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A novel antimicrobial peptide derived from human BPIFA1 protein protects against *Candida albicans* infection

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Abstract

Bactericidal/permeability-increasing fold containing family A, member 1 (BPIFA1) is an innate immunity defense protein. Our previous studies proved its antibacterial and antiviral effects, but its role in fungi remains unknown. The study aimed to identify antifungal peptides (AFP) derived from BPIFA1, and three antimicrobial peptides (AMP1–3) were designed. The antifungal effects were proved by growth inhibition assay. AMP3 activity was confirmed by germ tube growth experiment and XTT assay. Its effects on cell wall and membrane of *Candida albicans* were assessed by tannic acid and Annexin V-FITC/PI double staining, respectively. Additionally, scanning electron microscope (SEM) and transmission electron microscopy (TEM) were used for morphological and ultrastructural observation. The expression of ALS1, EAP1, and SUN41 was tested by qPCR. Ultimately, three AMPs could fight against *C. albicans in vitro*, and AMP3 was highly effective. It functioned by destroying the integrity of cell wall and normal structure of cell membrane. It also inhibited biofilm formation of *C. albicans*. In addition, AMP3 down-regulated the expression of ALS1, EAP1, and SUN41, those are known to be involved in virulence of *C. albicans*. Altogether, the study reported successful development of a novel AFP, which could be used as a new strategy for antifungal therapy.

Keywords

BPIFA1, innate immunity, anti-fungal peptide, *candida albicans*, biofilm

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Introduction

The mucosal surface of oral and upper respiratory tract of mammals is generally covered with a layer of mucus, which is known to act as an innate immune and protective barrier. In particular, this barrier prevents a direct contact of mucosal epithelium with microorganisms, harmful chemicals, and dust present in the external environment. This layer of mucus is characterized by the presence of several innate immune protective molecules, such as defensins and interleukins. Previous studies conducted in our laboratory identified an innate immune protective molecule, namely short palate, lung, and nasal epithelium clone 1 (SPLUNC1), in this layer of mucus. SPLUNC1 was found to be highly-expressed in the oral and respiratory tract. It was later renamed as BPI fold containing family member A1 (BPIFA1), based on the presence of bacterial permeability increasing (BPI) domain and function.[1] It has not only been shown to have antibacterial and antiviral activities, but also to inhibit the potential oncogenicity of EBV in respiratory epithelium.[2] Several other studies reported that SPLUNC1/BPIFA1 actively participated in protecting the host against bacterial infections, including *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and non-typeable *Haemophilus influenzae*, and viral infections, like influenza A and syncytial virus.[3,4] In a separate study, Jian et al. reported the development of BPIFA1-derived AMP (α4-short), which exhibited broad-spectrum bactericidal and antibiofilm activities.[5]

In addition to bacteria and viruses, the upper respiratory mucosa might also come in contact with fungi and other

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microorganisms. In fact, fungal infections have emerged as a serious threat to human health in the past few decades, causing a wide range of infections in humans worldwide. Systemic infections associated with fungi can even be life-threatening. It is estimated that fungal diseases affect more than one billion people globally. Among these, 150 million people suffer from severe infections.[6] *Candida albicans* is the most common pathogenic species that accounts for 21–68% of invasive candidiasis in different countries.[7] BPIFA1 is known to act as an innate immunity defense protein, which exhibits a broad spectrum of antimicrobial effects. However, it is not known whether BPIFA1 exhibits antifungal effects. Identification of BPIFA1 protein or its derived peptides as antifungal agents would provide a very important target/lead for the development of low-toxicity, broad-spectrum, and natural antimicrobial drugs.

Currently, only a small number of antifungal agents are available in the market. These are limited to three major classes, namely polyenes, azoles, and echinocandins. Traditional antifungals are generally expensive and confer severe toxicity. Additionally, widespread use of antifungal agents has resulted in an increase in drug resistance of *C. albicans*.[8] Thus, to address these issues, there is an urgent need to develop novel potent drugs that confer low toxicity and do not elicit resistance easily. Antimicrobial peptides (AMPs) are now recognized as naturally occurring cationic amphipathic small peptides (usually fewer than 50 amino acids) that can combat infections caused by microorganisms. These molecules are expressed across a diverse range of species, including microorganisms, plants and animals.[9] Despite the presence of anti-microbial and immunomodulatory activities, the clinical application of AMPs is limited owing to certain factors including toxicity, immune damage caused by heterogeneity of AMPs, high manufacturing cost, and low metabolic stability.[10] In past few years, studies have majorly focused on devising effective strategies to design synthetic peptides using sequences of these AMPs, to optimize antimicrobial functions while minimizing cytotoxicity. Some of these molecules are currently undergoing preclinical or clinical trials (Dramp Database).[11]

The present study reports successful designing, synthesis, and *in vitro* evaluation of three AMPs on the basis of SPLUNC1/ BPIFA1 protein. Among these, AMP3 exhibited strongest antifungal effect, and was selected for further explorations to unravel the possible antifungal mechanism. The findings of this study would provide compelling anti-*Candida* evidence of BPIFA1-derived AMP3, which could be further developed as a novel peptide-based therapy to combat invasive candidiasis.

Materials and methods

Chemicals and reagents

RPMI-1640 medium was obtained from Yuanju Biotechnology Co., Ltd (Shanghai, China). Yeast extract peptone dextrose (YPD) medium was composed of 1% yeast extract, 2% peptone (OXOID, Basingstoke, Britain), and 2% Glc (SINOPHARM, Shanghai, China). Fluconazole (FCZ) was sourced commercially (Sigma-Aldrich, MO, United States). Sabouraud dextrose broth (SDB) and Sabouraud dextrose agar (SDA) were purchased from Tianhe Microbial Reagent Co., Ltd (Hangzhou, China) and Barrett Biotechnology Co., Ltd (Zhengzhou, China), respectively.

Design and synthesis of AMPs

BLAST, CDD software, and EXPASY bioinformatics database were used to obtain functional domains and conserved motifs of human BPIFA1 protein. The PEPTIDE_MASS programme was utilized for enzyme digestion analysis to predict all the residues of BPIFA1 protein in the body. Following the sequence conservation principle and functional validity principle, several peptides were extracted from the full-length BPIFA1 protein. In full consideration of the amphiphilic, cationic, and hydrophobic properties of AMP and the optimal peptide length (usually 20–40 amino acid residues), peptides with the same physicochemical properties (analyzed by ProtParam) as AMP were selected. Then spiral wheel analysis and theoretical antibacterial activity prediction were conducted by HeliQuest database and AMP prediction model (APD, http://aps.unmc.edu/AP/), respectively. Ultimately, three of these peptides were screened out. Through amino acid substitution (glutamate replaced by histidine), the biological activity of the newly designed AMPs was greatly enhanced. The amino acid sequences are shown in Table 1. Then, AMP 1–3 were synthesized by Chinapeptides Co., Ltd (Suzhou, China) using solid-phase peptide synthesis method with purity over than 95%.

Fungal strain

C. albicans ATCC 90028 was obtained and cultured in SDB for 24 h. Fresh cultures of the fungus were prepared by sub-culturing on SDA and used throughout the study. In order to prepare the cell suspension, one colony was picked from the SDA cultures and re-suspended in SDB to obtain a concentration of 1×10^6 cells/ml.

Erythrocyte lysis assay

For hemolytic assays, human erythrocytes were isolated from EDTA anticoagulated blood of healthy donors by centrifugation at 2500 *g* for 5 min, after four times washes, cells re-suspended to 4% (vol/vol) in PBS. To determine erythrocytes lysis, a volume of 100 µl of the cell suspension was mixed with 100 µl AMP solutions at final concentrations ranging from 1 to 1024 µg/ml in a 96-well plate. The reaction mixture was incubated with gentle shaking at 37°C for 60 min. After that, the mixture was spun at

Table 1. Primary sequences and physicochemical properties of AMPs.

Peptide	Length (no. of amino acids)	Primary sequence	Charge	μH	H	Theoretical molecular mass (Da)	Detected molecular mass (Da)
AMP1	32	NLPLLDILKPGGGTSGGLLGGLLGKVTSVIPG	+1	0.268	0.590	3014.6	3014.4
AMP2*	27	AVRDKQERIHLVLGDCTHSPGSLQISL	0	0.295	0.377	-	-
AMP2	27	AVRDKQ**H**RIHLVLGDCTHSPGSLQISL	+1	0.270	0.406	2981.4	2981.4
AMP3*	36	PIQGLLDSLTGILNKVLPELVQGNVCPLVNEVLRGL	−1	0.137	0.629	-	-
AMP3	36	PIQGLLDSLTGILNKVLP**H**LVQGNVCPLVN**H**VLRGL	+1	0.148	0.671	3841.6	3841.4

μH, Hydrophobic moment; H, hydrophobicity; *original sequence of peptide. The marked residues indicate that the original glutamate was replaced by histidine.

1000 *g* for 10 min, and 100 μl of the supernatant from each well was transferred to a 96-well plate for spectrophotometric analysis at 414 nm. To calculate % lysis, the average absorbance value of negative control (PBS and cells only) was determined. Triton X-100, a detergent which causes erythrocytes lysis, served as hemolysis control (cells suspended in 0.1% Triton X-100). Hemolysis percentage (%) = (mean absorbance value of supernatant in AMP group - absorbance value in PBS group)/(absorbance value in hemolysis control group - absorbance value in PBS group) × 100%. These experiments were verified by three independent trials.

Growth inhibition kinetics

Briefly, cells in exponential phase were adjusted to 2×10^3 CFU/ml in fresh RPMI-1640 medium, and 50 μl of the cell suspension was seeded in a 96-well plate. Next, 50 μl of the AMP solutions were added in appropriate final concentrations (8, 16, 32, 64, and 128 μg/ml) and incubated at 37°C. Then, the growth kinetic was monitored at an optical density at 620 nm (OD 620) by using the ELx800 Microplate Reader (BioTek, VT, USA) at the presumed time intervals (8, 12, 16, 20, 24, 32, 36, 40, and 48 h). FCZ was used as the positive control, and PBS was the negative control. The results were represented as the average of triplicate measurements from three independent assays.

C. albicans germ tube formation (GTF)

To determine the effect of AMP3 on the yeast-mycelium transition, *C. albicans* in the YPD media at a density of 1×10^6 CFU/ml were treated with 128 and 256 μg/ml of AMP3. After incubation at 37°C under constant shaking at 180 rpm for 2 h and 4 h, the cell suspensions were added to neubauer hemocytometer and observed for GTF by light microscopy (400×) (Olympus, Japan). The germ tubes were considered when the germinating protuberance was at least as long as the diameter of the blastopore. GTF (%) = (number of germ tubes/*C. albicans* cells) × 100%. The conventional antifungal drug FCZ (100 μg/ml) served as the positive control, while PBS treatment served as the negative control.

Anti-biofilm growth test

The biofilm was then quantified as per previously described metabolic assay based on the reduction of a tetrazolium salt (XTT). Briefly, *C. albicans* was first cultured in the YPD media to achieve exponential growth, followed by washing in normal saline (NS) and finally suspending at a final concentration of 3×10^6 CFU/ml in the RPMI-1640 medium. The cells were inoculated in a 96-well plate at the concentration of 100 μl/well, and the same volume of AMP3 was added at different concentrations in each well. The microplates were then incubated for 24 h at 37°C. The spent media and free-floating microorganisms were then removed and the wells were washed twice with PBS. Next, 50 μl of the XTT-menadione mixture were added to each well. Following incubation for 2 h at 37°C, the absorbance of XTT formazan was measured at 490 nm. PBS was used as the negative control and 100 μg/ml of FCZ as the drug control. The experiment was repeated three times. Biofilm inhibition rate (%) = (1-A treatment group /A negative control) × 100%.

Cell wall integrity test

C. albicans cells at the concentration of $1–5 \times 10^3$ CFU/ml were cultured in SDB containing 256 μg/ml of AMP3 at 37°C for 12 h. PBS was used as the negative control and FCZ (at the final concentration of 100 μg/ml) as the positive control. The cells were centrifuged for 5 min at 9391 *g* and washed thrice in PBS. The fungal precipitate was transferred to a slide, and the cells were treated with 10% tannic acid for 60 min, followed by staining with 1% crystal violet for 5 min. After washing, the cells were observed under a microscope (Olympus, Japan). Then, 100 cells were randomly counted to determine the cell wall integrity.

Flow cytometry analysis

FITC-Annexin V Apoptosis Detection Kit (Beyotime Biotechnology, Shanghai, China) was used to determine the apoptosis and necrosis of *C. albicans* after treatment with AMP3. *C. albicans* cells were grown and diluted to $1–5 \times 10^5$ CFU/ml concentration. Next, the cells were incubated with different concentrations of AMP3 (128 and 256 μg/ml), 100 μg/ml of fluconazole (as the positive control), and PBS (as the negative control) for 4 h at 30°C under constant shaking (180 rpm). The cultures were washed twice and re-suspended in PBS to the concentration of $1–5 \times 10^6$ CFU/ml. Subsequently, lyticase (15 U/ml) was added, and the solution was further incubated for 30 min at 37°C and then washed with PBS. The washed cells were incubated for 20 min in an Annexin binding buffer containing 5 μl of FITC-Annexin V and 10 μl of propidium iodide (PI). The cells were then analyzed by fluorescence-activated cell sorting (FACS) Calibur Flow Cytometer (BD Biosciences, San Jose, CA, United States).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analysis

C. albicans (at an initial density of $1–5 \times 10^3$ cells/ml) were treated with 256 μg/ml of AMP3 at 37°C for 24 h. The cells without treatment served as control. Then, the treated cells were collected by centrifugation at 9391 *g* for 5 min and washed thrice in PBS buffer, followed by fixing with 3% glutaraldehyde. Finally, the cells were sent to the Electron Microscope Laboratory of Central South University and observed under the Hitachi S-3400N TEM and the Hitachi HT-7700 SEM (Tokyo, Japan).

Reverse transcription-quantitative PCR

C. albicans cells were diluted to a concentration of 3×10^6 CFU/ml in the RPMI-1640 medium. After incubation with AMP3 at a final concentration of 128 and 256 μg/ml at 37°C for 24 h, PBS was used as the negative control and FCZ (100 μg/ml) as the positive control. The cells were collected and washed, and the total RNA was extracted by using the Yeast RNAiso Kit (TaKaRa, Japan), while cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Takara Bio, Japan) as per the respective manufacturer's instructions. qPCR was performed using the SYBR Premix Ex Taq™ II (Takara Bio). The reaction mixtures were incubated for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The expression of ALS1, EAP1, and SUN41 were detected, those are known to be involved in biofilm formation, adhesion, and virulence of *C. albicans*. The primer sequences used in this study are listed in Table 2. β-Actin served as the internal reference gene, and the relative gene expression was calculated based on the formula $2^{-\Delta\Delta CT}$.

Table 2. Gene-specific primers used for relative quantification of gene expression by RT-PCR.

Gene	Primer sequence	
ALS1	FORWARD	GACTAGTGAACCAACAAATACCAG
	REVERSE	ACCAGAAGAAACAGCAGGTG
EAP1	FORWARD	CTGCTCACTCAACTTCAATTGTCG
	REVERSE	GAACACATCCACCTTCGGGA
SUN41	FORWARD	AACCCTTTCCCTTCCATCTG
	REVERSE	ACCAGAACCAGAACCACCAG
ACTIN	FORWARD	CTTCTCAATCTTCTGCCATTGA
	REVERSE	TTCTGGACTCTGAATCTTTCG

Statistical analysis

All experiments were performed in at least three biological replicates with experimental triplicates. Data were expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined using one-way analysis of variance (ANOVA). The statistical analysis was performed using the Prism 5.0 Software (GraphPad Software). Differences were considered significant at $P < 0.05$.

Results

Successful designing and synthesis of low toxicity AMPs derived from human BPIFA1

On the basis of comprehensive bioinformatics analysis of BPIFA1 (including its conserved domain, enzyme-digested peptides, α-helix regions, and physicochemical properties) and cationic and amphiphilic characteristics of AMPs, sequences located at 70–101, 165–191, and 199–234 sites of BPIFA1 protein were used to design three representative peptides: AMP1, AMP2, and AMP3, respectively. In order to make AMPs more electropositive and hydrophobic, glutamate was replaced with histamine in the original sequence of AMP2 and AMP3. The primary sequences and physicochemical properties (charge, hydrophobic moment, hydrophobicity, and theoretical molecular mass) of three AMPs are described in Table 1, and the helical wheel models are represented in Figure 1A. As shown in Table 1, these three peptides were electropositive, with net charge of + 1. AMP1, AMP2, and AMP3 exhibited good hydrophobicity of 0.590, 0.406, and 0.671, respectively. At the same time, these three AMPs exhibited certain amphiphilicity.

Three AMPs were synthesized by Chinapeptides Co., Ltd (Suzhou, China). HPLC analysis and mass spectrometry analysis of the synthesized AMPs are presented in Figure 1B and 1C. AMP1 exhibited molecular mass of 3014.4 Da, which was close to its theoretical molecular mass (3014.6 Da). HPLC analysis showed that the peptide

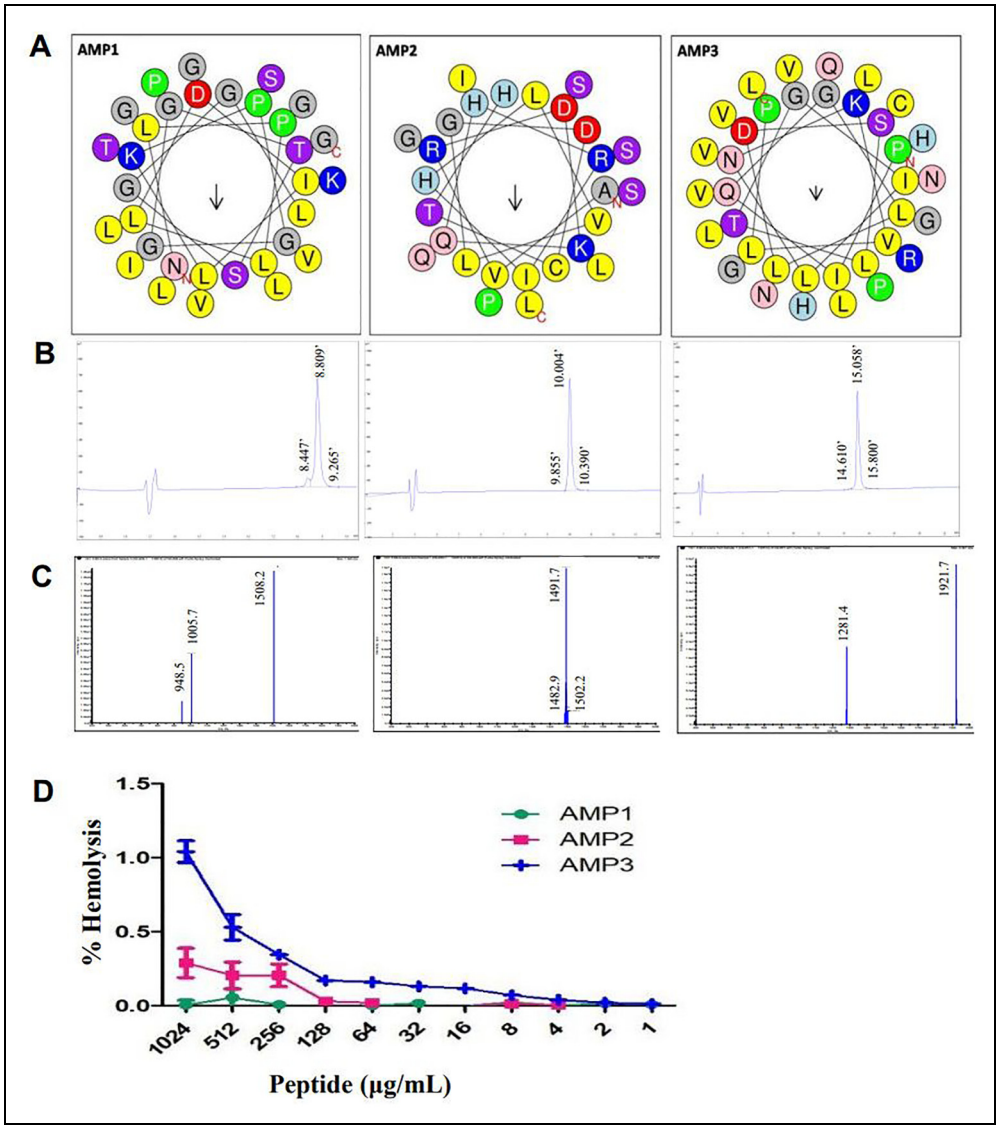

Figure 1. Biological characteristics and host toxicity of AMPs. (A) Helical wheel diagrams of AMP1, AMP2, and AMP3, the black arrows indicated the direction of the hydrophobic moment, different kinds of residues were presented in different colours, hydrophobic residues were shown in yellow and blue circles represented cationic residues. (B) HPLC chromatographic fingerprints and (C) mass spectrograms of AMPs. (D) Erythrocyte suspension was mixed with AMP solutions at final concentrations ranging from 1 to 1024 μg/ml in a 96-well plate. Followed by 1-h incubation, and then centrifuge at 1000 g for 10 min, the supernatant was used for spectrophotometric analysis at 414 nm. Percentage of hemolysis was determined.

was pure, as indicated by the presence of a single sharp peak with retention time (RT) of 8.809 min. Similarly, molecular masses for AMP2 and AMP3 were recorded to be 2981.4 and 3841.4 Da, and the purity of these two peptides were found to be 98.28% and 97.90%, respectively. For primary characterization of cytotoxic property, hemolytic activity was assessed and compared for these peptides. The results showed that AMP1 exhibited no hemolytic toxicity, even at very high concentration (> 1000 μg/ml). Comparatively, both AMP2 and AMP3 showed negligible hemolytic activity at concentrations up to 128 μg/ml (Figure 1D).

AMPs exhibit potent antifungal activity against C. albicans

To assess antifungal activity, *C. albicans* was treated with 128 μg/ml of AMPs, and colony morphology was observed in SDA medium. The colony numbers of each group were 26 (AMP1 group), 32 (AMP2 group), 11 (AMP3 group), and 52 (negative control group), respectively. The results suggested that all three peptides could fight against *C. albicans*, and AMP3 exhibited most significant anti-candida effect (Figure 2A). Further, the growth inhibition kinetics of three AMPs against *C. albicans* was assessed using

different concentrations of the peptides (8, 16, 32, 64, and 128 μg/ml), at various time intervals (Figure 2B-2D). The data demonstrated that the growth of *C. albicans* cells was inhibited by peptides at aforementioned concentrations, as indicated by a decrease in optical density of *C. albicans* in AMP treated samples. Among these three AMPs, AMP3 exhibited strongest antifungal activity.

AMP3 inhibits C. albicans *GTF and possesses anti-biofilm growth activity*

Based on aforementioned results, the study further focused on exploring the antifungal properties of AMP3. Since yeast-to-hypha transition is believed to be a key determinant for the pathogenesis of *C. albicans*, the effect of AMP3 on GTF in *C. albicans* was assessed. As shown in Figure 2E, treatment with 128 μg/ml of AMP3 for 4 h resulted in a reduction in GTF as compared to negative control group. Furthermore, AMP3 at a concentration of 256 μg/ml resulted in sustained GTF inhibition as compared to FCZ, and GTF in *C. albicans* was recorded to be 20.33% and 20.67% after 2 h and 4 h of intervention, respectively.

Next, the effect of AMP3 on *C. albicans* biofilms was evaluated, and it was found that AMP3 inhibited biofilm formation in a dose dependent manner (Figure 2F). Biofilm formation was inhibited by 25% ($P < 0.05$) in the presence of 32 μg/ml of AMP3. Increase in the concentration of AMP3 resulted in an enhancement in its anti-biofilm effect. In fact, the inhibition rate of AMP3 at the concentration of 256 μg/ml was found to be comparable to FCZ. Altogether, AMP3 exhibited a remarkable anti-biofilm effect.

AMP3 alters cell wall properties

The study further explored the effect of AMP3 on the cell wall of *C. albicans*. First, the integrity of cell wall was tested by tannic acid staining. Interestingly, AMP3 treatment destroyed the cell wall of *C. albicans*. Consequently, the dye entered the cells, resulting in dark purple appearance. As shown in Figure 3A, majority of untreated *C. albicans* (about 86.67%) appeared ovoid, with dark purple cell wall and light purple cytoplasm. After treatment with 256 μg/ml of AMP3 for 12 h, the number of intact cells decreased, which accounted for only 55% of the cells. In comparison to these, FCZ treated group exhibited minimum proportion (38.30%) of normal cells. Statistical analysis for cell wall integrity rates in three groups are shown in Figure 3B.

AMP3 increases apoptosis and necrosis of C. albicans

Annexin V is a phospholipid-binding protein, which exhibits high affinity towards phosphatidylserine. Phosphatidylserine is generally located on the inner leaflet of the cytoplasmic membrane lipid bilayer. It has been previously shown that phosphatidylserine translocate to the outer leaflet during cell apoptosis and necrosis, resulting in binding to annexin V. PI is a membrane-impermeant fluorescent dye, which can only pass through damaged membrane to bind nucleic acids. Thus, PI positive cells represent cell necrosis. As shown in Figure 4, treatment with 128 and 256 μg/ml of AMP3 significantly increased the proportion of apoptotic cells in annexin V^+/PI^- quadrant (14.33% and 12.13%), as compared to negative control (1.58%). In fact, the proportion of apoptotic cells in AMP3 group was found to be even higher than FCZ group (6.3%). For PI^+ necrotic cells, the population increased to 19.53% and 22.43% after treatment with 128 and 256 μg/ml of AMP3, respectively. These results indicated that treatment with AMP3 resulted in induction of both apoptosis and necrosis, which was mediated via externalization of phosphatidylserine and membrane disruption.

AMP3 destroys cellular microstructure of C. albicans

Treatment of *C. albicans* with AMP3 (256 μg/ml) for 24 h resulted in morphological changes, as observed by optical microscopy (400×). When compared with control group, AMP3 group was characterized by the occurrence of significantly reduced budding cells and filamentous pseudohyphae (Figure 5A and B). Besides this, the morphology and ultrastructure of fungal cells were analyzed by SEM and TEM. SEM images for untreated *C. albicans* showed normal shape with plump appearance, smooth surface, and clear cell boundaries (Figure 5C and E). Similarly, intact cell walls, cell membranes, and organelles were observed in TEM analysis (Figure 5G and H). In comparison to this, the cells exposed to AMP3 showed the presence of lysed cells and some cell debris (Figure 5D and F). Additionally, significant changes in ultrastructure were also observed. These included loss of structural integrity of the cell membrane, uneven thickness of the cell wall, occurrence of vague organelles accompanied by electron dense area in the cytoplasm (green arrows), and oozing out of intracellular content (red arrows) (Figure 5I–K).

AMP3 decreases the expression of ALS1, EAP1, and SUN41 in C. albicans

The expression of cell wall associated genes of *C. albicans* was detected by qRT-PCR. The results indicated that treatment with 128 μg/ml of AMP3 apparently decreased the expression of *ALS1*. Additionally, it also down-regulated *EAP1* and *SUN41*. Importantly, treatment with 256 μg/ml of AMP3 significantly down-regulated the expression of *ALS1*, *EAP1*, and *SUN41* by 93.9%, 96.1%, and 84.8%, respectively, which was similar to that of FCZ (Figure 6).

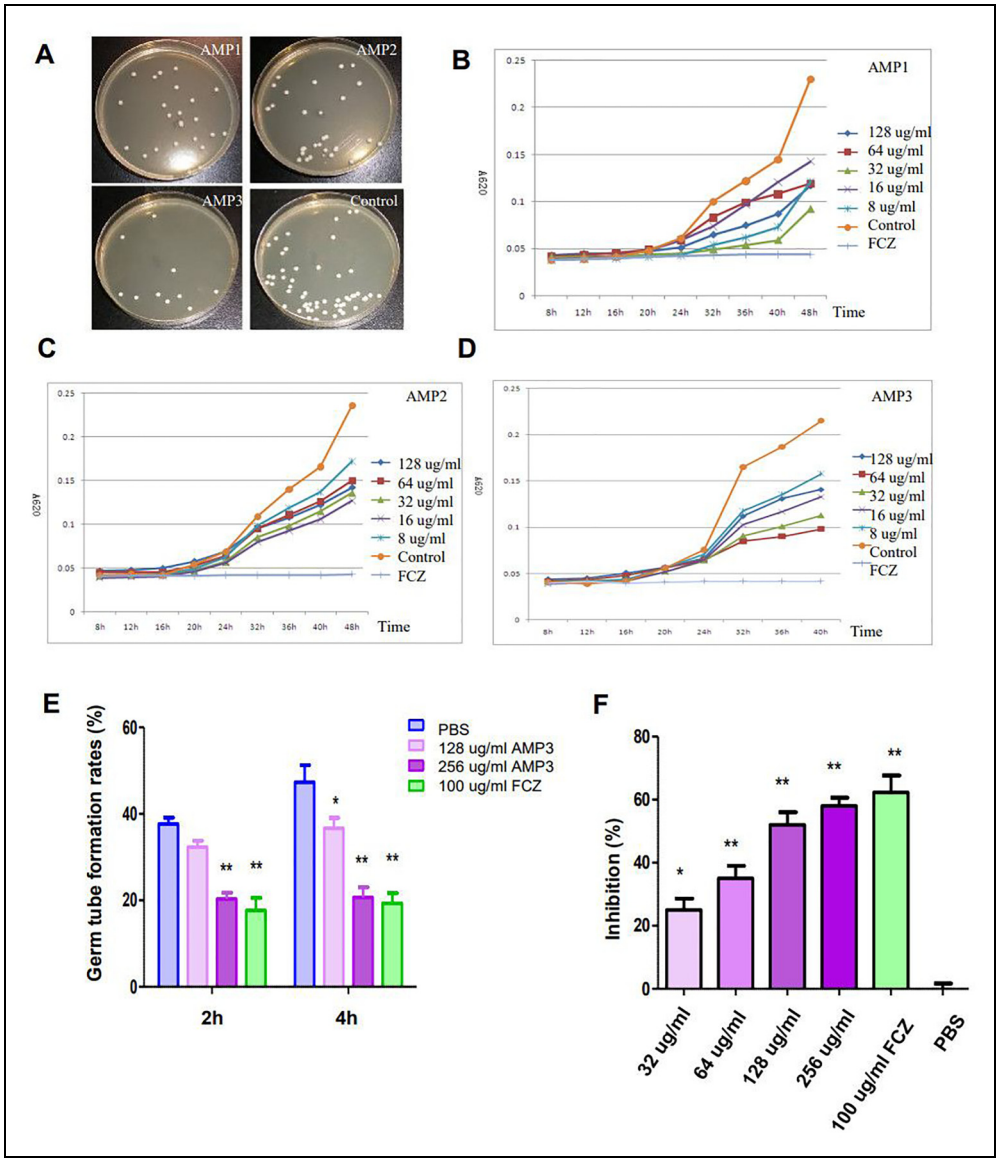

Figure 2. The activity of AMPs against *C. albicans*. (A) Colony morphology of *C. albicans* grown in Sabouraud's dextrose agar (SDA) medium for 48 h after 24 h incubation with 128 μg/ml AMPs at 37°C. (B-D) Inhibition kinetics of AMPs against *C. albicans*. Optical density measurements at 620 nm of *C. albicans* treated with peptides at 8–128 μg/ml concentrations. (E) Germ tube formation (GTF) assays were conducted in the YPD medium containing variable levels of AMP3. After 2 h and 4 h of culture, GTF of *C. albicans* was observed. Sterile PBS served as the negative control and FCZ served as the drug control group. (F) The anti-biofilm growth activity of AMP3 was detected by using the XTT assay. Briefly, *C. albicans* were inoculated in a 96-well plate, and treated with different concentrations of AMP3, FCZ, or PBS. After incubation for 24 h at 37°C, XTT-menadione mixture were added. 2 h later, the absorbance of XTT formazan was measured at 490 nm. All the experiments were performed in triplicate. *$P < 0.05$; **$P < 0.01$.

Discussion

BPIFA1/SPLUNC1 is a protein that is known to be highly expressed in the upper respiratory tract and oral cavity. It was discovered using yeast two-hybrid system.[12] And was identified to be an innate immune protein. Interestingly, it has been shown to exhibit anti-bacterial and anti-viral activities against a variety of organisms, including *P. aeruginosa* and EB virus.[1,2] The present study showed that the polypeptides derived from BPIFA1 protein exhibited well anti-fungal effect, and provided new evidence for the broad-spectrum antibacterial activity of BPIFA1.

The study utilized the APD database, and identified three sequences from human BPIFA1 protein that exhibited AMP properties. APD database is a comprehensive database that is used for peptide information search, calculations, prediction, and design of AMPs.[13] Following

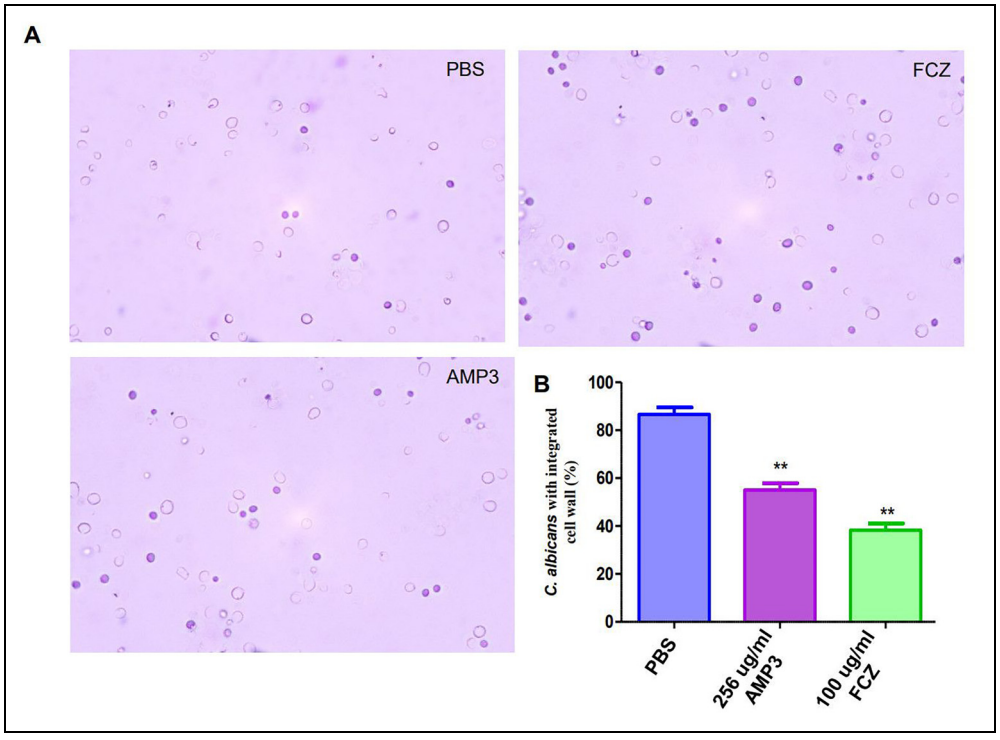

Figure 3. AMP3 altered cell wall properties and inhibited biofilm formation of *C. albicans*. (A and B) Cells were grown overnight in the presence or absence of AMP3. Cell wall integrity was tested by tannic acid staining, and intact *C. albicans* were observed as a dark purple cell wall enclosing a light purple cytoplasm. Cell wall integrity rates in three groups were calculated. *$P < 0.05$; **$P < 0.01$.

amino acid substitution, AMPs became more electropositive and hydrophobic. These two properties are known to influence the interaction of AMP with bacterial membranes, which are negatively charged and rich in lipids. Although these AMPs exhibited lower charge (+1) as compared to most of other AMPs, such as LL-37, lactoferrin, lysozyme, and defensin, which exhibit a positive charge in the range of 3–11.[14] However, no optimal structure function profiles have been determined for these molecules.[10] Amphiphilicity is another key factor that is known to affect antimicrobial action of AMPs. In the present study, AMP1, AMP2, and AMP3 were predicted to have certain amphiphilicity, with hydrophobic moment of 0.268, 0.270, and 0.148, respectively. Besides this, helical wheel models showed that the designed AMPs exhibited helical structure, which is generally present in most of the AMPs. Thus, the present study reported successful designing and synthesis of cationic amphipathic helical AMPs derived from human BPIFA1 protein. Interestingly, these three newly synthesized AMPs exhibited anti-*C. albicans* activity, which has never been mentioned while discussing BPIFA1 protein and its associated peptides. Among these three peptides, AMP3 demonstrated highest effect.

For the successful clinical application of polypeptides, the safety, represented by the cytotoxicity of the peptides, should be considered first. A considerable number of naturally occurring peptides derived from toxins and other types of natural host defense systems have been previously shown to be cytotoxic.[10] Interestingly, the newly designed and synthesized AMPs, reported in the present study, exhibited negligible toxicity towards mammalian cells. Only slightly higher hemolytic activity (about 1%) was reported for AMP3, at a high concentration of 1024 μg/ml. This might be attributed to an off-target effect of AMP3 after optimization of its antibacterial activity.[5] Therefore, comprehensive consideration should be taken while designing AMPs.

After confirming the antifungal effect of AMPs, the study further explored the mechanism of action. The germ tube of *C. albicans* is an outgrowth that is produced by the fungi during germination, which will grow, differentiate, and develop into hyphae. Assessing this feature is of great importance as yeast-to-hypha transition represents the main virulence factor that is associated with candidiasis. Additionally, filamentation is pivotal for the development of robust biofilms. A biofilm mode of growth is another major virulence factor involved in the pathogenesis of *C. albicans*, which is difficult to eradicate, and inherently enhances the resistance to treatment.[15,16] The results of the present study indicated that AMP3, at a concentration of 256 μg/ml, could significantly inhibit GTF of *C. albicans*, which was comparable to the activity of classic agent fluconazole. Importantly, AMP3 inhibited biofilm

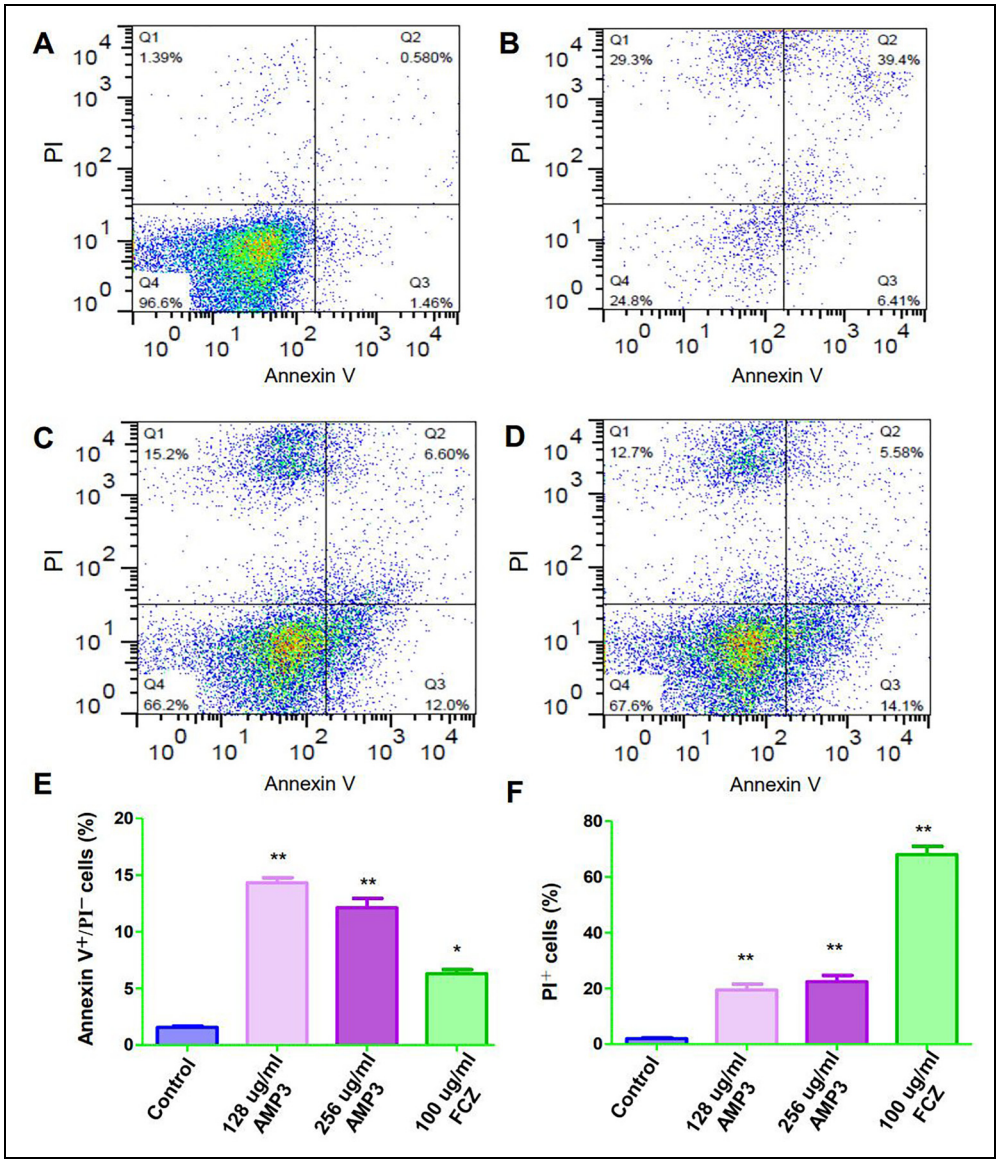

Figure 4. The apoptotic and necrotic cells were distinguished by double staining with annexin V-FITC and PI, and further analysed by flow cytometry. (A) Non-treated cells; (B) 100 μg/ml FCZ-treated cells; (C) 256 μg/ml AMP3-treated cells; (D) 128 μg/ml AM3-treated cells. (E) The percentages of annexin V^+PI^- cells in these groups were presented. (F) And PI^+ cells of different gruops were evaluated. $^{**}P < 0.01$.

growth of *C. albicans* in a dose-dependent manner. This might be closely related to the strong hydrophobicity of AMP3, which was contributed by the enrichment of leucine residues.[17] AMP3 could bind to the cell surface lipids and interfere with cell attachment to solid surfaces, which is an early step in biofilm formation. Besides this, AMP3 treatment also inhibited the following proliferation phase, which is usually characterized by the initiation of GTF/filamentation. All these factors contributed to anti-biofilm activity of AMP3 against *C. albicans*.

The cell wall of fungi is known to act as a protective barrier, which limits the access of molecules to the plasma membrane. Additionally, it also takes important part in cell adhesion, cell signaling, and pathogenesis. Thus, it acts as an important target for the action of AMPs.[18] In the present study, AMP3 acted on *C. albicans*, and destroyed cell wall integrity, as indicated by tannic acid staining, wherein significantly increased dark purple cells were observed in AMP3 treated group.

The cell membrane acts as the second barrier in fungal cells, and is known to be rich in anionic phospholipids and ergosterol. Fungal cell membranes are significantly different from mammalian cells that consist of zwitterionic phospholipids and cholesterol. This difference in lipid content has been widely exploited to develop antifungal drugs, such as amphotericin B and azoles, and AMPs.[18]

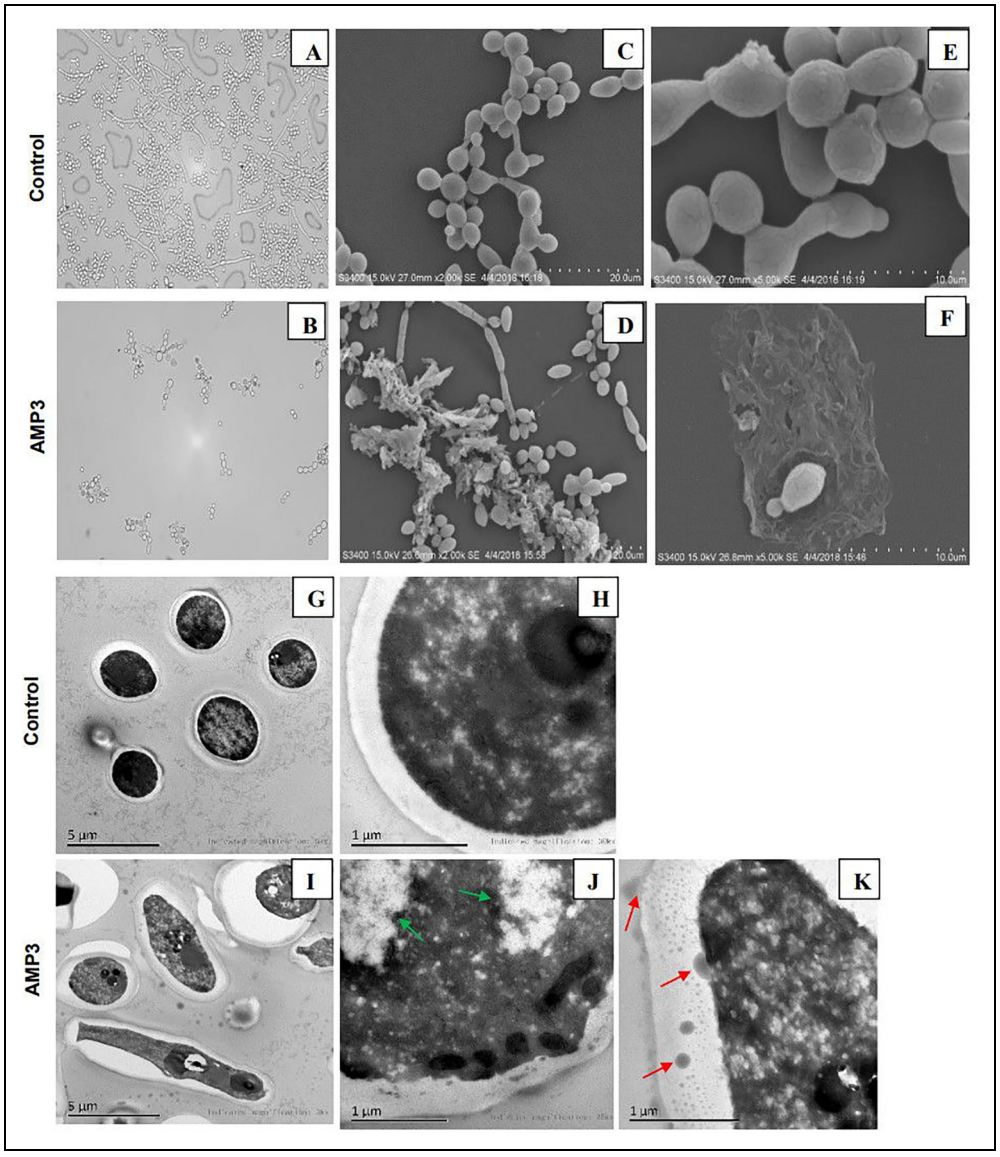

Figure 5. *C. albicans* treated with AMP3 (256 μg/ml) for 24 h, the morphological changes were observed by (A and B) optical microscopy (400×), (C-F) SEM, and (G-K) TEM. The Green arrows indicate high electron density areas, while red arrows indicate cytoplasmic extravasation. The bars indicate 20, 10, 5, and 1 μm, respectively.

In the present study, damage to cell membrane was confirmed by Annexin V-FITC and PI double staining, wherein the proportions of V-FITC + /PI − and PI + cells were found to be higher in AMP3 treated group as compared to untreated control group, which indicated that AMP3 not only caused cell membrane damage, but also initiated apoptosis and necrosis in the cells.

To gain better insights into the mechanism by which AMP3 affected *C. albicans*, optical microscopy, SEM, and TEM were used to observe changes in cell morphology. Light microscopy results showed that the number of cells, blastospores, and filamentous pseudohyphae were significantly reduced after treatment with AMP3. SEM showed that AMP3 treatment resulted in abnormal and irregular cell structure and cells lysis of *C. albicans*. Additionally, cell debris and substances that could be cell contents were observed around the cells. TEM micrographs showed that exposure to AMP3 resulted in morphological changes, including loss of structural integrity of the cell membrane, uneven thickness of the cell wall, presence of vague organelles accompanied by electron dense area in the cytoplasm, and oozing out of intracellular content. All these changes confirmed that the cell wall and cell membrane acted as targets for AMP3 action. Additionally, cell organelles might also be affected by AMP3 treatment.

Among various groups of genes that are involved in biofilm formation in *C. albicans*, it was reported that the family ALS (agglutinin – like sequence), encoding surface

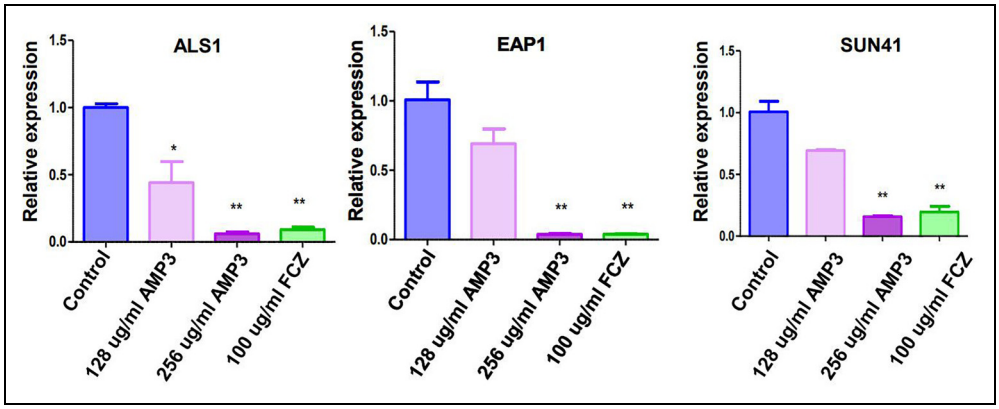

Figure 6. Effects of AMP3 on *C. albicans* cell wall-associated genes expression. *C. albicans* cells were incubated with AMP3 at a final concentration of 128 and 256 μg/ml at 37°C for 24 h, PBS was used as the negative control and FCZ (100 μg/ml) as the positive control. RT-PCR was used to detect the expression of ALS1, EAP1, and SUN41. *$P < 0.05$; **$P < 0.01$.

glycoproteins, plays a key role in this process.[19] The results of the present study showed that the expression of ALS1 was significantly down-regulated after application of AMP3, which might reduce the production of adhesion glycoproteins, thereby inhibiting the formation of biofilm. Another gene that is closely related to adhesion of *C. albicans* is EAP1 (Enhanced Adherence to Polystyrene), which is expressed in both yeast and hyphal form. EAP1 generally encodes glycosylphosphatidylinositol cell wall protein, and mediates cell adhesion and biofilm formation.[20] In the present study, the level of EAP1 in negative control was recorded to be 25.6 times higher than AMP3 group. These results were consistent with those of biofilm experiment. SUN41 protein, a putative cell wall glycoprotein specific to fungi, is required for biofilm formation, cell wall integrity, and virulence.[21] Real-time PCR showed that AMP3 inhibited SUN41 expression in *C. albicans*, providing evidence for the antifungal activity of AMP3. The decreased levels of these three proteins indicated that AMP3 could inhibit *C. albicans* by influencing biofilm formation and cell wall integrity.

Altogether, the present study reported the development of three AMPs based on BPIFA1 protein sequence. Among these, AMP3 exhibited strongest antifungal activity. In particular, AMP3 acted via inhibition of GTF, hyphal elongation, and biofilm formation. Additionally, it also destroyed the integrity of cell wall and membrane of *C. albicans*, resulting in the induction of cell apoptosis and necrosis. Thus, a polypeptide with antifungal properties was successfully designed and synthesized, based on BPIFA1 protein. The results of the study indirectly confirmed broad-spectrum antimicrobial activity of BPIFA1.

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Supplemental material

Supplemental material for this article is available online.

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