C16-Fengycin A affect the growth of *Candida albicans* by destroying its cell wall and accumulating reactive oxygen species

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Abstract

Candida albicans is the most common clinical pathogenic fungus, which is highly susceptible to immunodeficiency. Development of novel antifungal agents has become a growing trend in the treatment of *Candida* infections. C16-Fengycin A, a lipopeptide isolated from *Bacillus amyloliquefaciens* fmb60 showed significant fungicidal activity against *C. albicans*. In the study, we explored the possible antifungal mode of C16-Fengycin A. It was predicted that C16-Fengycin A had the ability to disrupt the cell wall due to its alterations of cell ultrastructure, and reduction of cell wall hydrophobicity. This was further confirmed by the changes in the exposure of the cell wall components and down-regulation of the genes related in the cell wall synthesis. Meanwhile, with the treatment of C16-Fengycin A, the levels of reactive oxygen species (ROS) increased, resulting in mitochondrial dysfunction in the cells. We hypothesized that the antifungal mechanism of C16-Fengycin A might be via the destruction of the cell wall and the accumulation of ROS, which could activate the High-Osmolarity Glycerol Mitogen-Activated Protein Kinase (HOG-MAPK) pathway. Our findings indicated that C16-Fengycin A could be a potential antifungal agent that could be used to treat candida infections.

Keywords Antifungal · *Candida albicans* · Cell wall · ROS

Introduction

One of the most prevalent pathogenic fungi in the clinical setting is *Candida albicans* (*C. albicans*). It is generally observed in the skin, mucous membranes, vagina, and other parts of the human body (Papon et al. 2013; Peleg et al. 2010; Pfaller and Diekema 2010; Turner and Butler 2014). In recent years, there has been a rise in fungal infections caused by *Candida* spp., which accounts for 73.4% of all fungal infections. This has become a serious issue in clinical settings (Calderone et al. 2014; Chin et al. 2016).

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The antifungal drugs available for clinical use, include azoles, polyenes, echinococcosis classes, and other antifungal agents, such as allylamines, flucytosine, and griseofulvin (Anderson 2005). However, clinically resistant strains are constantly being isolated, which greatly increases the risk of clinical treatment of fungal infections (Anderson 2005; Ford et al. 2015; Kullberg and Arendrup 2015). In addition, the majority of the antifungal drugs have poor targeting, and severe toxic and side effects such as nephrotoxicity, hepatotoxicity, hemolytic anemia, and even arrhythmia (Bayhan et al. 2016; Odds et al. 2003; Perlin 2015; Vincent et al. 2013). The rate of discovering new antifungal drugs is quite slow as compared to the increased cases of serious fungal infections recently. Therefore, searching for novel antifungal drugs which are efficient, safe, and non-toxic with less drug resistance is of top priority.

Natural products play an extremely important role in the development of new drugs, and they provide abundant resources for the discovery of new drugs (Newman 2008). Antimicrobial peptides are relatively small molecules, less than 50 amino acids, which have a broad spectrum of antimicrobial activity. Peptide was reported for the first time in 1982 (Hultmark et al. 1982). In the past few decades, more and more antifungal peptides have been discovered. Antimicrobial peptides are found in both

prokaryotes and eukaryotes. *Bacillus subtilis* (*B. subtilis*) is a Gram-positive bacterium that produces antimicrobial compounds with a variety of structures. They include both ribosome synthesized and post-translationally modified peptides such as lantibiotics and lantibiotic-like antimicrobial peptides, and also include non-ribosomal synthetic lipopeptides such as surfactin, iturin, and fengycin (Arima et al. 1968; Besson et al. 1976; Stein 2005). The lipopeptides can be produced by *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) (Sun et al. 2006), *Bacillus cereus* (*B. cereus*) (Nishikiori et al. 1986), *Bacillus licheniformis* (*B. licheniformis*) (Horowitz and Griffin 1991), etc., which are of the same genus as *B. subtilis*.

Fengycin is a decapeptide containing a β-hydroxy hydrocarbon chain that has shown distinct antifungal (Tang et al. 2014; Wei et al. 2010) and anti-tumor activity (Cheng et al. 2016; Yin et al. 2013). In comparison of traditional antifungal agents, Fengycin shows fewer side effects and less drug resistance (Vanittanakom et al. 1986). C16-Fengycin A is a monomeric lipopeptide isolated from *B. amyloliquefaciens* fmb60 (Liu et al. 2019; Lu et al. 2016). However, there are no relevant reports on the inhibitory activities of C16-Fengycin A against *C. albicans*. In the study, the antifungal effect and mode of C16-Fengycin A against *C. albicans* were investigated for the first time. First, we evaluated the antifungal properties of C16-Fengycin A, and then we further elucidated its effects on the cell wall polysaccharides, synthetase, and synthesis-related genes of *C. albicans*. In addition, the increase in intracellular ROS and mitochondrial membrane potential, as well as the signaling pathways involved in fungistatic behavior were illustrated. Therefore, our study was to explore the role of C16-Fengycin A as an antifungal agent, which would contribute to expand the theoretical research on the mechanisms of antifungal drugs and discover novel alternative antifungal agents.

Materials and methods

C16-Fengycin A, strains, and culture conditions

The microbial strains *C. albicans* (ATCC 10231) and *B. amyloliquefaciens* fmb60 (CGMCC 7.222) were provided by the Enzyme Engineering Laboratory, Nanjing Agriculture University (Nanjing, China). The *C. albicans* was stored in the Yeast-Peptone-Dextrose (YPD, Solarbio Co., Ltd., China) with 20% glycerol at − 80 °C and the *B. amyloliquefaciens* was stored in the Luria-Bertani broth (LB, Solarbio Co., Ltd., China) with 20% glycerol at − 80 °C. The strains were subcultured twice at 30 °C or 37 °C before every single usage of culture.

C16-Fengycin A was isolated and purified by our laboratory (Liu et al. 2019). The fermentation broth of *B. amyloliquefaciens* fmb60 was adjusted to pH = 2.0 with

6 M HCl, precipitated overnight, and then centrifuged to obtain the precipitate, that was re-dissolved with methanol. Sephadex LH-20 column chromatography, using 80% methanol, and RP-18 reverse column chromatography, using 60% acetonitrile were carried out. After high performance liquid chromatography (HPLC) (UltiMate 3000, Dionex, California, USA) detection, the C16-Fengycin A was collected. The purity of C16-Fengycin A reached 95%. The structure of C16-Fengycin A was shown in Fig. S1.

Determination of drug concentration with inhibition rate up to 80% (MIC$_{80}$) and minimum fungal concentration (MFC)

MIC$_{80}$ was determined by microdilution assay (Lu et al. 2014). In a 96-well flat-bottomed microtiter plate, YPD medium containing *C. albicans* was added. To this, varying concentrations of C16-Fengycin A (0, 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg/mL) were also added. After incubation at 30 °C for 48 h, the MIC$_{80}$ was determined in comparison to the control (organisms grown without C16-Fengycin A). The 96-well plate was further cultured at 30 °C for 48 h and applied to a YPD agar plate, and the concentration of the antimicrobial peptide corresponding to the growth of no colonies was MFC.

Determination of growth curve

The growth curve was drawn by measuring the OD at different time points. The specific experimental scheme was as follows. 100 μL of different concentrations of C16-Fengycin A solution were added to a 96-well plate by using a checkerboard microdilution method. Then, 100 μL of *C. albicans* suspension (10^6 CFU/mL) was added to each well and cultured at 30 °C. The absorbance OD$_{600}$ value was measured every 4 h from 0 h. The results were expressed as Mean ± S.D. on the growth curve.

Determination of the permeability of cell membrane

The *C. albicans* was adjusted to OD$_{600}$ = 0.2 with PBS buffer, and six treatment groups were established, in which the concentration of C16-FengycinA were 0, 8, 16, 32, 64, and 128 μg/mL, and the cells were incubated at 30 for 4 h. Then the Prodium Iodide (PI) dye was added at a final concentration of 30 μg/mL and incubated for 30 min in the dark. After washing in PBS buffer, the ratio of stained cells was measured by flow cytometry.

Transmission electron microscopy (TEM)

Candida albicans cells were suspended in 0.01 M PBS. C16-Fengycin A was added to a final concentration of 32 μg/mL,

along with an equal volume of dd H_2O as a control. Cultures were incubated at 30 °C for 3 h and then collected the cells. The cells were fixed with 2.5% glutaraldehyde. The morphological changes of *C. albicans* were performed by TEM (JEM-1011, Japan).

Hydrophobicity determination

The concentration of *C. albicans* was adjusted with YPD medium to $OD_{600} = 0.1$. Five concentrations of C16-Fengycin A (8, 16, 32, 64, and 128 μg/mL) were used and negative control (ddH_2O) was maintained. Cultures were incubated at 30 °C for 16 h. The fungal concentration was adjusted with YPD medium to $OD_{600} = 1.0$, and then 0.75 mL cyclohexane was added to 3 mL of the solution and vortexed vigorously for 3 min. 200 μL of the aqueous solution was taken to determine the OD_{600} value. The formula for calculating the hydrophobicity was: (control group OD_{600} − OD_{600} after stratification) / control group OD_{600}.

Determination of the exposure of glucan and chitin in *C. albicans*

The concentration of *C. albicans* was adjusted with PBS buffer to $OD_{600} = 1.0$. A series of concentrations of C16-Fengycin A (8, 16, and 32 μg/mL) was used and negative control (ddH_2O) was maintained. The cultures were incubated at 30 °C for 4 h, and then were adjusted to $OD_{600} = 0.8$ with PBS. To determine the exposure of glucan, 0.1% aniline blue solution was added. The reaction was incubated at 80 °C for 15 min in the dark. Fluorescence was measured with a microplate reader (excitation - 398 nm, emission - 508 nm). To determine the exposure of chitin, the cells were stained with 3.5 μg/mL of Calcofluor white (CFW) for 30 min. The fluorescence intensity was measured by a multi-function microplate reader (excitation - 365 nm, emission - 435 nm).

(Lee and Kim 2017; Sherrington et al. 2017).

Molecular docking of C16-Fengycin A and the synthetase of glucan and chitin

According to previous reports (Rajasekharan et al. 2018), the structure of C16-Fengycin A was elucidated by ChemBiodraw 11.0, and the glucan synthase and chitin synthase structures were obtained by homology modeling. AutoDock4.0 software was used to treat them by hydrogenation, charge calculation, and non-polar hydrogen treatment. Then, a 37.5 × 37.5 × 37.5 box was constructed centering on the active site of the protein, and the structure of the small molecular was handled in a flexible manner. The AutoDock 4.0 program performs automated molecular docking. Molecular docking simulation was performed by Vina algorithm, and 100 hybrid Vina runs were

carried out per docking job. The simulation results were analyzed with Pymol.

Measurement of the content of ROS in *C. albicans*

The cells of *C. albicans* were adjusted to a concentration of 10^6 cells/mL by YPD, and varying concentrations of C16-FengycinA (0, 8, 16, and 32 μg/mL) were added in the gradient. After incubation at 30 °C for 3 h, the cells were collected by centrifugation and then stained with 40 μg/mL of DCFH-DA. After incubation for 30 min in the dark, the samples were detected by flow cytometry. The experimental data were analyzed by flow analysis software WinMDI2.9. Meanwhile, green fluorescence observation was performed by fluorescence microscopy.

Effects of vitamin C (VC), glutathione (GSH), and *N*-acetyl-cysteine (NAC) on the activity of C16-Fengycin A

Candida albicans was diluted to 10^6 with YPD medium, and 100 μL of the bacterial solution was added to each well in a 96-well plate. The experiment was divided into five groups. In each group, only C16-Fengycin A was

Table 1 PCR primer sequence of the target gene

| Gene	Sequence (5'-3')
Gls1-F	CCAGATGTTTGGCGGTAACT
Gls1-R	ACCGTCGATGAGAGCAGAGT
Fks1-F	GCACAAAAGGGTTTGCATTT
Fks1-R	TTTGTTCACCCATACCAGCA
Chs1-F	CAGCACACCAAGAGCACAAT
Chs1-R	TTGTGGAGGAGGCAAAAATC
Chs2-F	TCCCCACTGAACGTGTCATA
Chs2-R	GGCTCGAGTGAGTTTCTTGG
Chs3-F	GATGAAAGCCATCCACGATT
Chs3-R	GGGATCCCTTCGTCTTCTTC
Chs8-F	AGCTGCTACGTGTGGACCTT
Chs8-R	TTCGAAAACCCCTTTCAATG
Sho1-F	GGAGGACATCCTGAATCACC
Sho1-R	GACACCACTAGCAGCAAATC
Ssk1-F	CCAGTAACAACTCAAGAGCC
Ssk1-R	CCTCCAAGTGATACTTTTCGG
Pbs2-F	AGCAGGGTAAGGATAATGGC
Pbs2-R	CACCCAGGGAAAAGTAAAGTTG
Hog1-F	GTCTGTGGGTTGTATCTTAG
Hog1-R	TCACTAAATGGGATAGGGTC
18Sr RNA-F	AATTACCCAATCCCGACAC
18Sr RNA-R	TGCAACAACTTTAATATACGC

Note: F, forward; R, reverse
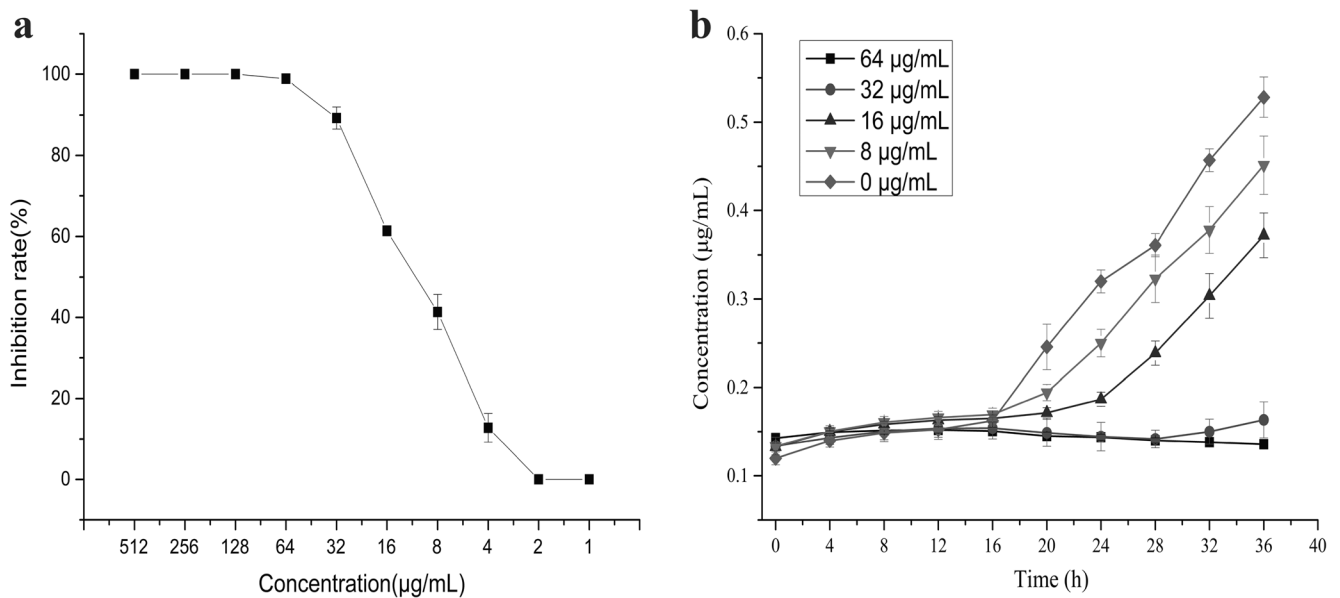
Fig. 1 The effect of C16-Fengycin A on the growth of *C. albicans*. **a** antifungal activity of C16-Fengycin A against *C. albicans*. **b** the effect of C16-Fengycin A on the growth curve of *C. albicans*

added as a control, and 5 mM VC, GSH, and NAC were added to the experimental group. Ten gradients were set for each group, and the MIC_{80} values of each group were determined.

Detection of mitochondrial membrane potential

The *C. albicans* was adjusted to a concentration of 10^6 cells/mL in PBS, and different concentrations of C16-Fengycin A
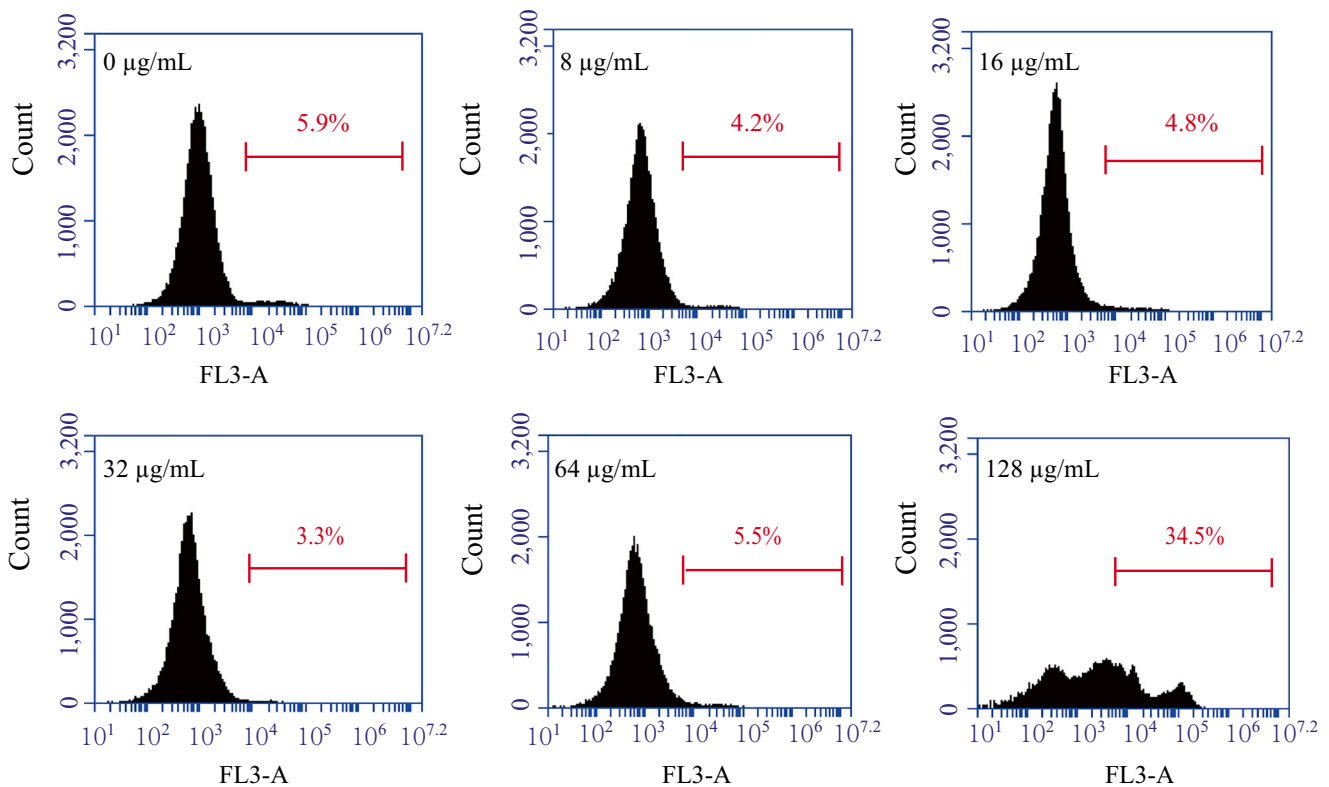

Fig. 2 The effect of C16-Fengycin A on the permeability of cell membrane The cells were treated with various concentrations of C16-Fengycin A. M, events of dead cells; 50,000 cells were calculated

(0, 8, 16, 32 μg/mL) were added and then cultured at 30 °C for 3 h. The cells were stained by 5 μM Rh123 for 30 min in the dark. The fluorescence intensity was measured by flow cytometry FL1 channel. The experimental data were analyzed by the flow analysis software WinMDI2.9.

Determination of the content of glycerol

The total amount of intracellular glycerol was measured using the Glycerol Assay Kit (Nanjing Jiancheng Bioengineering Institute, China). The total amount of protein in the sample was determined using the protein detection kit (Beyotime, China). The content of glycerol was calculated based on the total amount of glycerol and the total amount of protein: the content of glycerin = Total glycerol / Total protein.

Quantitative real-time PCR assay of the target genes

Quantitative RT-PCR was performed based on previously described protocols (Lu et al. 2014). The cells were treated with C16-Fengycin A (0, 8, 16, and 32 μg/mL) and grown in YPD at 30 °C for 3 h. Then the cells were harvested. Reverse transcription was performed using HiScript®II 1st Strand cDNA Synthesis Kit (Vazyme Co., Ltd., China). RT-PCR was performed with TaKaRa SYBR Premix ExTaqTMII (TaKaRa

Inc., Japan) using a Step One System (Applied Biosystems, Singapore). $2^{-\Delta\Delta Ct}$ method was used to compute the gene expression. The primers used were listed in Table 1, and the gene of 18S was used as an internal reference gene.

Statistical analysis

All experiments were repeated three times, and the results were represented by mean ± S.D. t test was performed to determine the significance levels using SPSS 22.0 (IBM, USA).

Results

Effect of C16-Fengycin A on the growth of *C. albicans*

The MIC_{80} of C16-Fengycin A against *C. albicans* was determined by microdilution method. C16-Fengycin A inhibited the growth of *C. albicans* at 32 μg/mL, indicating its MIC_{80}, and the MFC was 256 μg/mL (Fig. 1a). The growth curve was also measured as shown in Fig. 1b. In the control group, it was found that *C. albicans* entered the logarithmic growth phase after 16 h of incubation. When incubated with C16-Fengycin A, the growth was retarded, and then entry into the log phase was delayed. Logarithmic growth phase-delayed by nearly 8 h

Fig. 3 The images of TEM of *C. albicans* exposed to C16-Fengycin A. **a** the cells treated without C16-Fengycin A. **b** the cells treated with 32 μg/mL of C16-Fengycin A
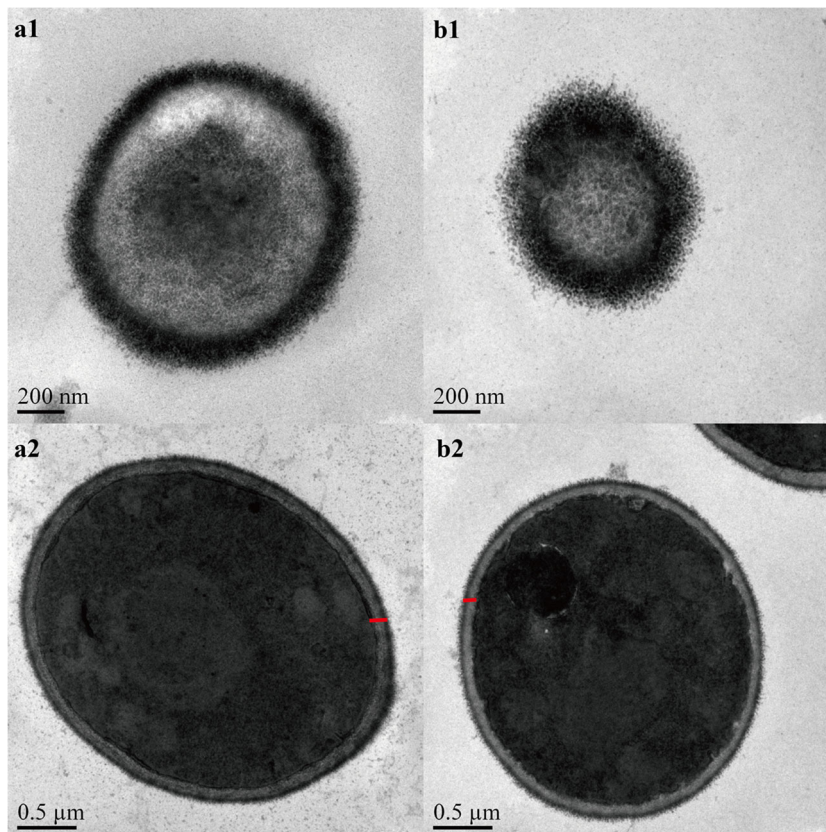
in the presence of 16 μg/mL of C16-Fengycin A, indicating the growth of *C. albicans* was inhibited. When the cells were exposed to 32 μg/mL of C16-Fengycin A, the growth of the cells was more severely inhibited.

Effect of C16-Fengycin A on the integrity of cell membrane

PI is a membrane-selective permeable dye that can only pass through cell membranes that are damaged and have increased permeability (Bauer 1993). Flow cytometry analysis showed that the addition of low concentration of C16-Fengycin A could not increase the amount of cells stained by PI. When the cells were exposed to 128 μg/mL of C16-Fengycin A, the cell membrane permeability increased from 5.9 to 34.5% (Fig. 2). This result indicated C16-Fengycin A could only increase the permeability of cell membrane when it reached a high enough concentration. At low concentrations, it could not affect the cell membrane permeability.

Effect of C16-Fengycin A on the cell ultrastructure of *C. albicans*

The control group indicated that *C. albicans* had a clear and complete cell wall structure and normal cell wall thickness. When the cells were exposed to 32 μg/mL of C16-Fengycin A, the cell wall structure was loosely dispersed, similar to the disintegration, and the cell wall was damaged. At the same time, the internal electron density was not uniform that can cause lesions. Meanwhile, the cell wall was significantly thinner after treatment with 32 μg/mL of C16-Fengycin A (Fig. 3). The results of TEM showed that C16-Fengycin A mainly caused cell wall damage.

Reduce the hydrophobicity of cell wall

Cell surface hydrophobicity is an important factor in the non-specific adhesion of bacteria and fungi and also plays a role in the adhesion strength. Studies have shown that the stronger the hydrophobicity of the cell surface, the greater the adhesion to the host (Naito et al. 1993). We observed that the hydrophobicity of *C. albicans* decreased with increasing the concentration of C16-FengycinA (Fig. 4).

Effect of C16-Fengycin A on the exposure of gluan and chitin and the expression levels of their synthesis-related genes

To determine whether C16-Fengycin A had the ability to interfere with the cell wall, aniline blue and CFW were used to detect the exposure content of glucan and chitin (Fig. 5a). When exposed to aniline blue, the glucan exhibited fluorescence. The fluorescence intensity was found to increase in the
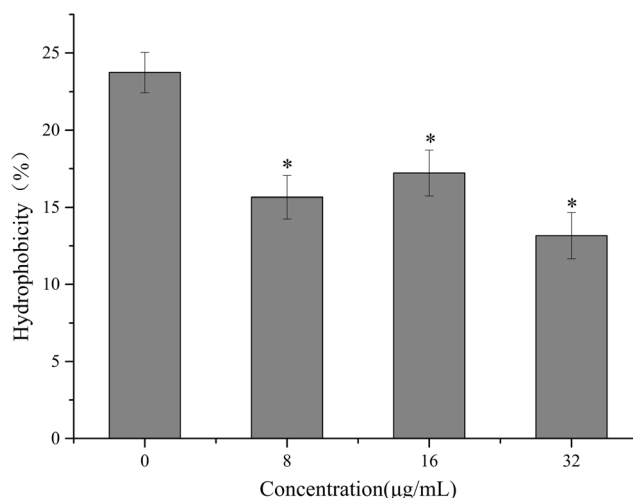

Fig. 4 The effect of C16-Fengycin A on the hydrophobicity of *C. albicans*

presence of C16-Fengycin A. CFW has a high affinity to chitin and exhibits fluorescence. When exposed to varying concentrations of C16-Fengycin A, the value of fluorescence intensity was also observed to rise. The results indicated that C16-Fengycin A could increase the exposure of glucan and chitin, resulting in the disruption of the structure of the cell wall, thereby inhibiting cell growth.

To further confirm this, gene expression studies were carried out. The *C. albicans* glucan synthase-related genes Fks1 and Gls1 and the chitin synthase-related genes Chs1, 2, 3, and 8 were targeted (Fernandes et al. 2014). RT-PCR assay showed that after C16-Fengycin A intervention, a down-regulation of these genes was noted with a statistical difference of $P < 0.05$ (Fig. 5b). This indicated that C16-Fengycin A could play a role in inhibiting the biosynthesis of glucan and chitin of *C. albicans*.

Docking of C16-Fengycin A and the synthetase of glucan and chitin

To illustrate the interactions of the C16-Fengycin A with glucan synthase and chitin synthase, molecular docking simulations were investigated. The ligand (C16-Fengycin A) was docked with the synthase of glucan and chitin (Fig. 5c).

For glucan synthase, C16-Fengycin A was found to bind to the large hydrophobic groove by hydrophobic interactions and

Fig. 5 The effect of C16-Fengycin A on the cell wall components, their ▶ synthetase, and synthesis-related genes. **a1** Fluorescence intensity of aniline blue binding to glucan. **a2** Fluorescence intensity of CFW combining with chitin. **b** The effects of C16-Fengycin A on the expression levels of biosynthesis-related genes of glucan and chitin. **c** Docking of C16-Fengycin A and the synthetase of cell wall components. **c1** and **c4** 3D structure diagram of the synthase of glucan and chitin by PyMOL. **c2** and **c3** An active pocket of C16-Fengycin A docked with glucan synthase. **c5** and **c6** Active pocket of C16-Fengycin A docked with chitin synthase
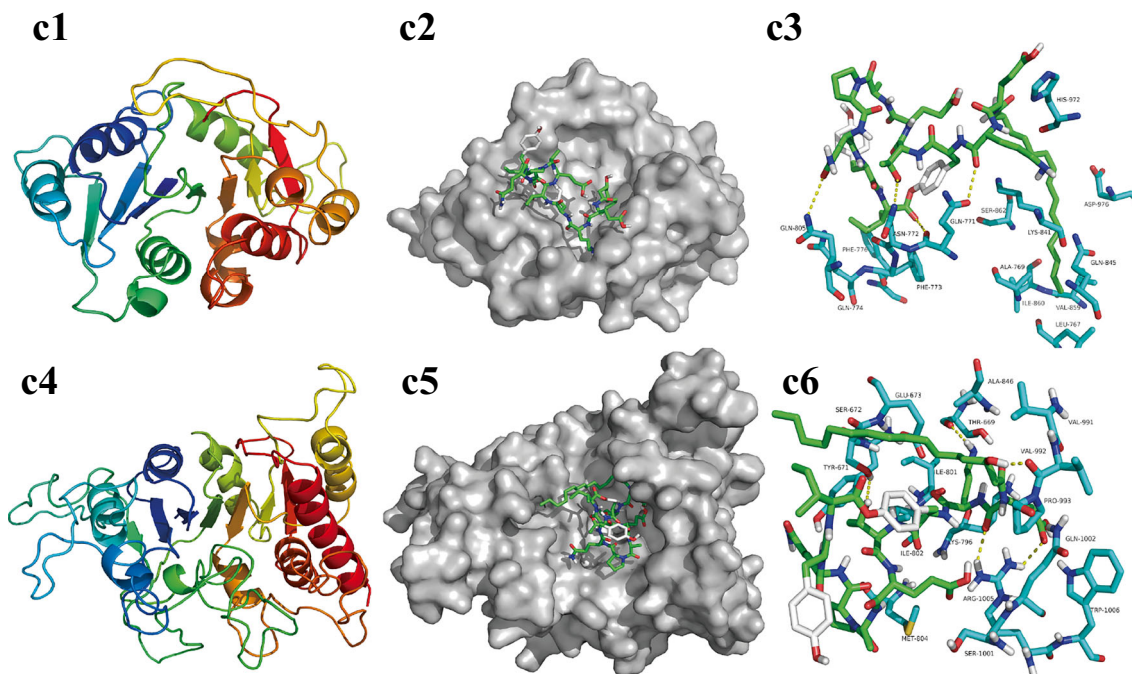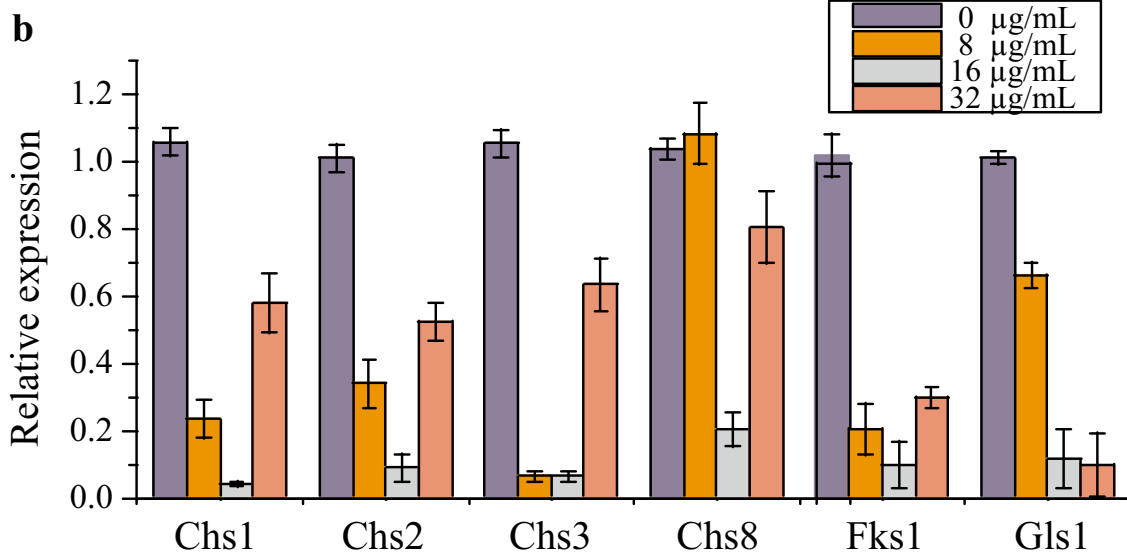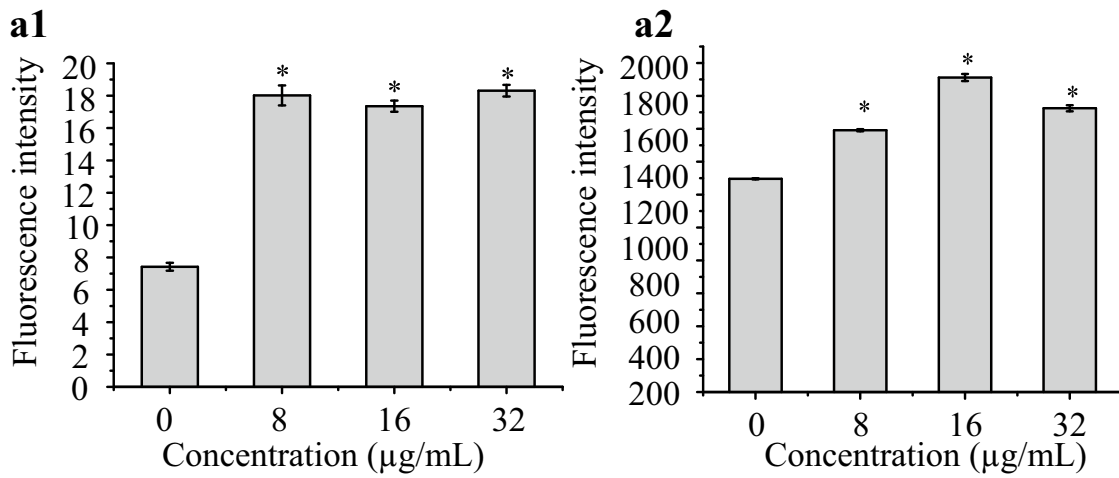
Van der Waals forces. The binding energy was −8.6 kcal/M. C16-Fengycin A interacted with the glucan synthase residues Leu767, Ala769, Asn772, Phe773, Gln774, Gln805, Lys841, Gln845, Val859, Ile860, His972, and Asp976. Hydrogen bonds were formed between C16-Fengycin A and the residues Gln771, Asn772, and Gln805 of the glucan synthase.

For chitin synthase, it was found that C16-Fengycin A could bound to large hydrophobic groove through hydrophobic interactions and Van der Waals forces with a binding energy of −8.4 kcal/M. C16-Fengycin A was found to mainly interact with Thr669, Tyr671, Ser672, Glu673, Lys796, Ile801, Ile802, Met804, Ala846, Vla991, Val992, Pro993, Ser1001, Gln1002, Arg1005, and Thr1006. Hydrogen bonds were formed between the chitin synthase residues Thr669, Ser672, Val992, Arg1005, and C16-Fengycin A.

The formation of hydrogen bonds enhances the ability of the ligand to target the proteins. This result indicated that the synthetase on the cell wall may be the target of C16-Fengycin

A. We predicted that C16-Fengycin A may inhibit cell wall synthesis by binding to the target, thereby affecting the growth of *C. albicans*.

Increase in the content of intracellular ROS

DCFH-DA was used to indicate the content of ROS in *C. albicans* cells. Fluorescence microscopy and flow cytometry were used for visualization. It was found that when exposed to C16-Fengycin A for a duration of 3 h, a cumulative increase of ROS in the cells were noted. Increased drug concentrations resulted in increased content of ROS. When compared to the blank control group, 32 μg/mL C16-Fengycin A caused the fluorescence signal value to increase by seven times (Fig. 6a). Fluorescence microscopy revealed that more *C. albicans* cells were stained with green fluorescence after C16-Fengycin A treatment than the non-dosed negative
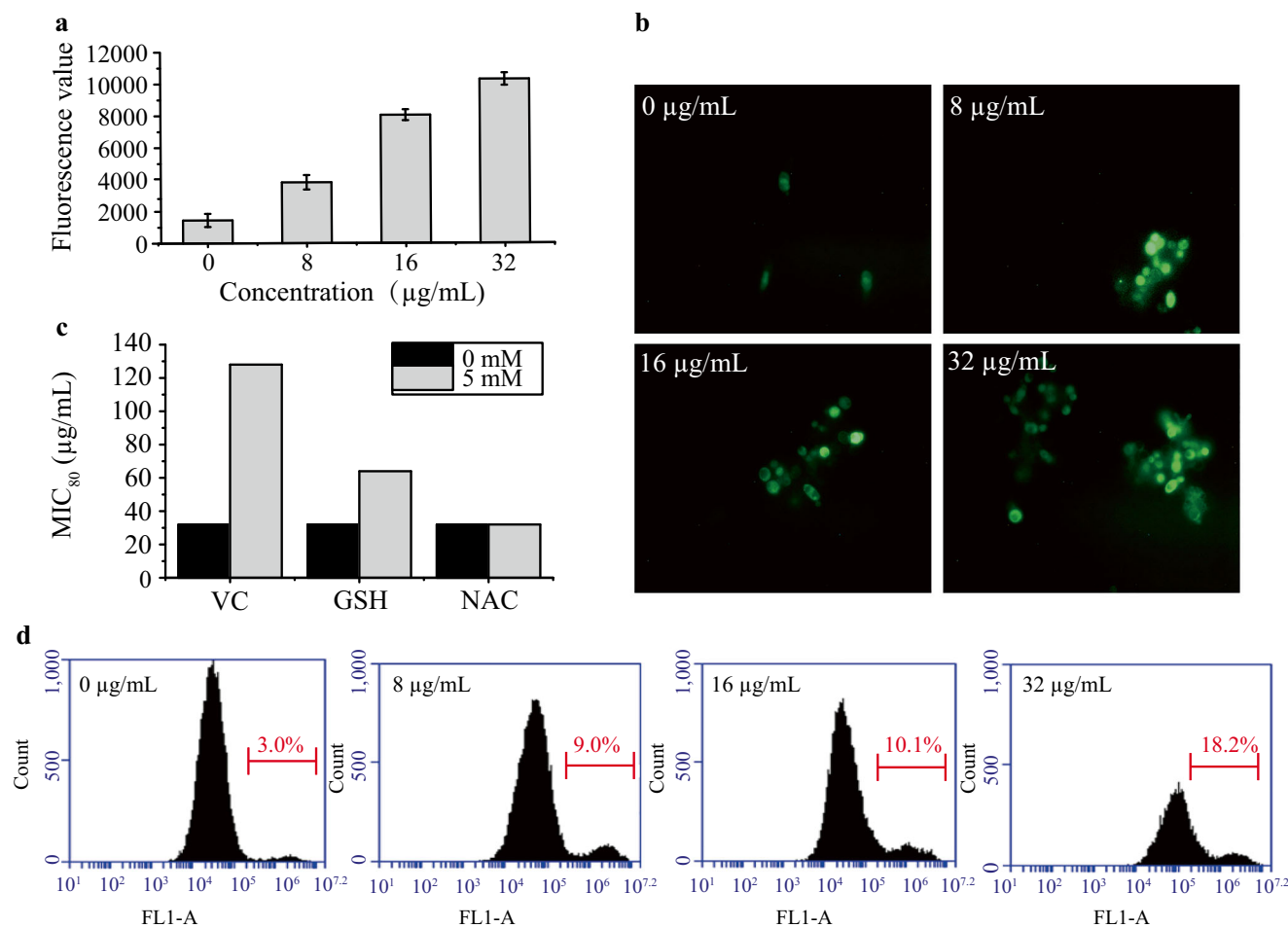

Fig. 6 The effect of C16-Fengycin A on the content of ROS and mitochondrial membrane potential and ROS scavenger reduced the activity of C16-Fengycin A. **a** and **b** The effect of C16-Fengycin A on intracellular ROS production. After staining with 40 mg/L of DCFH-DA, the samples were detected by flow cytometry and visualized by fluorescence microscopy with 488 nm of excitation and 525 nm of emission. **a** Showed the results of flow cytometry and **b** showed the observation of fluorescence microscopy. **c** ROS scavengers effected on the MIC_{80} of C16-Fengycin A against *C. albicans*. **d** The effect of C16-Fengycin A on the mitochondrial membrane potential in *C. albicans*

control group, which was consistent with the flow cytometry results (Fig. 6b).

ROS scavenger reduced the activity of C16-Fengycin A

To verify whether the antifungal activity of C16-Fengycin A on *C. albicans* is related to the accumulation of ROS in the cell, three ROS scavengers: VC, NAC, and GSH were used. 5 mM VC and GSH could significantly increase the MIC_{80} of C16-Fengycin A by four and twofold, respectively, while NAC had no effect on the MIC_{80} of C16-Fengycin A (Fig. 6c). Therefore, the inhibitory activity of C16-Fengycin A against *C. albicans* was positively correlated with the accumulation of intracellular ROS.

Mitochondrial membrane potential

Rh123 is a potential-dependent distribution of fluorescent probes that can be used as an indicator to detect mitochondrial membrane potential (Zoratti and Szabo 1995). It was found that C16-Fengycin A could significantly enhance the fluorescence intensity of Rh123 in *C. albicans* cells. Specifically, C16-Fengycin A at 8, 16, and 32 μg/mL enhanced Rh123 fluorescence intensity from 3.0% (negative control) to 9.1%, 10.1%, and 18.2%, respectively (Fig. 6d). The results indicated that C16-Fengycin A could significantly enhance the mitochondrial membrane potential of *C. albicans* and induce hyperpolarization.

Osmotic pressure and HOG-MAPK pathway

Cell wall destruction could lead to osmotic stress in *C. albicans*, and the accumulation of ROS could result in the cells in the oxidative stress. As a defense measure, they could activate the HOG-MAPK pathway to adjust those pressures (Brewster et al. 1993; Gregori et al. 2007).

To determine if the intracellular osmotic pressure increased, the content of intracellular glycerol was estimated. As seen in Fig. 7a, C16-Fengycin A had the ability to induce a significant increase in the content of intracellular glycerol. As the dose of C16-Fengycin A increased (8, 16, and 32 μg/mL), the content of intracellular glycerol increased from 2.92 to 7.699 nmol/mg, 12.3 nmol/mg, and 6.99 nmol/mg, respectively. It proved that C16-Fengycin A could cause an increase in intracellular osmotic pressure of *C. albicans*. We also examined the expression levels of Sho1, Ssk1, Pbs2, and Hog1 genes in the HOG-MAPK pathway. As seen in Fig. 7b, when the cells exposed the C16-Fengycin A, the expression levels of Sho1, Ssk1, Pbs2, and Hog1 genes decreased.

Discussion

C. albicans is a fungal pathogen that could cause mucosal and systemic infections in the host. Currently, the major drugs for the treatment of diseases caused by *Candida* spp. are azoles and polyene antibiotics, which could easily lead to the emergence of clinically resistant strains. Therefore, searching for new antifungal drugs is a top priority in fungal treatment. C16-Fengycin A is a lipopeptide isolated from *B. amyloliquefaciens* fmb60, and it has good fungicidal effects against various fungi. The discovery prompted us to further explore the antifungal mechanism of C16-Fengycin A on *C. albicans*.

Most antifungal peptides target the cell wall and membrane of fungi (Hwang et al. 2010; Mandal et al. 2013; Qi et al.
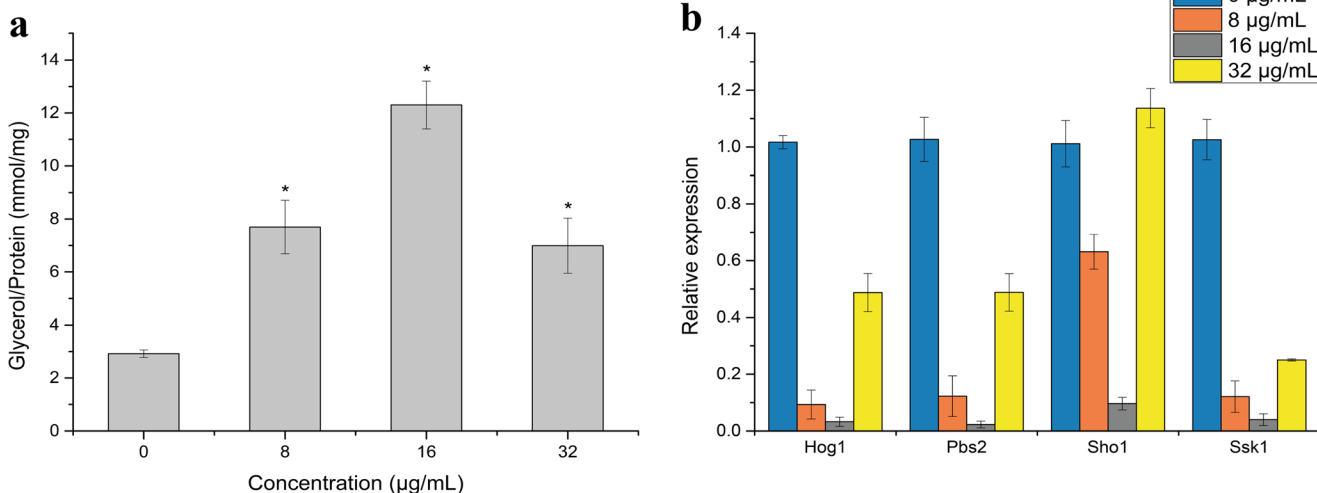
Fig. 7 The effect of C16-Fengycin A on osmotic pressure and the expression levels of genes related to HOG-MAPK signaling pathway. **a** The contents of intracellular glycerol were measured. The bars indicate standard deviations. **b** The expressions levels of Sho1, Ssk1, Pbs2, and Hog1 genes were measured by RT-PCR. Bars indicate standard deviations

2010; Zhu et al. 2015). In our previous study, we also found that C16-Fengycin A mainly acted on the fungal cell membrane (Liu et al. 2019). Therefore, we first investigated whether the presence of C16-Fengycin A could cause the change in the permeability of cell membrane of *C. albicans*. PI staining results showed that when the cells were treated with $1 \times MIC_{80}$ of C16-Fengycin A, the permeability of cell membrane was not changed, only if the concentration of C16-Fengycin A reached $4 \times MIC_{80}$, the permeability of cell membrane increased. In fact, any antimicrobial peptides at a sufficiently high dose could cause the damage and perforation of cell membrane (Shai 1995). Therefore, we believed that the destruction of cell membrane was not the main factor in the inhibition of C16-Fengycin A against the growth of *C. albicans*. In order to explore its antifungal mechanism, we further investigated the effect of C16-Fengycin A on the cell wall of *C. albicans*.

The cell wall is the outermost structure of *C. albicans*. It plays an important role in maintaining the strength, shape, and integrity of the fungal cells (Ene et al. 2015; Mouyna et al. 2000). The observation on the ultrastructure of *C. albicans* showed that C16-Fengycin A made the cell wall became loose and thinner. Submicroscopic observation provided direct evidence for the damage of C16-Fengycin A against the cell wall. In addition, C16-Fengycin A could also reduce the hydrophobicity of cell surface of *C. albicans*. At the same time, we used the value of fluorescence intensity of CFW and aniline blue to

detect the exposure of polysaccharides on the cell wall of *C. albicans*. The results showed that C16-Fengycin A could increase the exposure of chitin and β-glucan on the cell wall. Furthermore, RT-PCR results showed that the synthase-related genes of glucan and chitin were down-regulated in the presence of C16-Fengycin A. We hypothesized that C16-Fengycin A might bind to these synthetases, thereby reducing the activity of synthetase and inhibiting the synthesis of the cell wall components. The molecular docking simulations confirmed that C16-Fengycin A could bind to synthetases by means of hydrophobic interactions and hydrogen bonding. The above results indicated that C16-Fengycin A could effectively inhibit the growth of fungi by inhibiting or interfering with the biosynthesis of cell wall components.

The production of ROS has been reported as a drug antifungal mechanism more and more in recent years (Miao et al. 2012; Helmerhorst et al. 2001; Phillips et al. 2003). It plays an important role in physiological processes such as cell cycle and proliferation. When the levels of intracellular ROS exceed their normal metabolic range, they could result in the destruction of the normal metabolism of cellular macromolecules, leading to cell death (Costa and Moradas-Ferreira 2001). In this study, in addition to affecting the synthesis of cell wall components, C16-Fengycin A could also increase the content of intracellular ROS in a dose-dependent manner. VC and GSH acted as antioxidants to neutralize ROS produced by the cells. It was found that VC and GSH could effectively
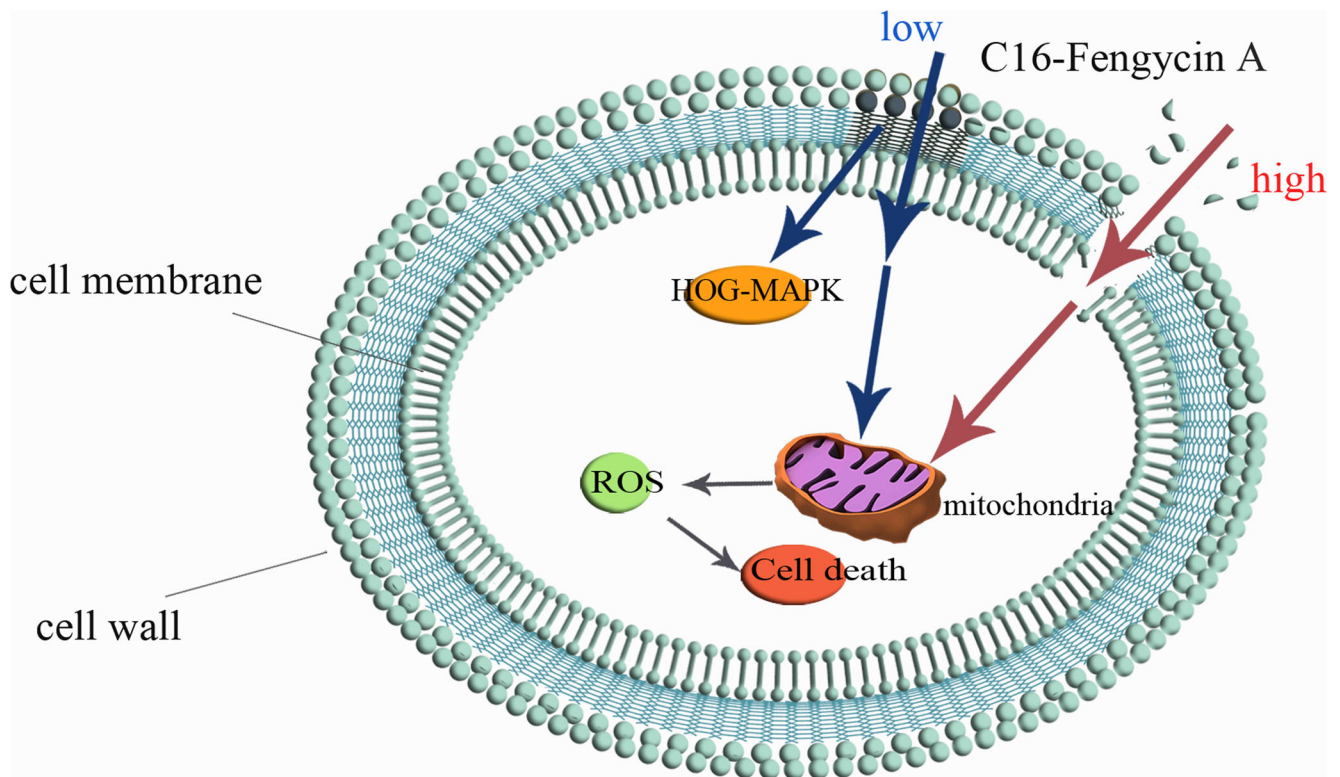

Fig. 8 The mode of action of C16-Fengycin A against *C. albicans*

inhibit the fungicidal activity of C16-Fengycin A. The protective effect of VC and GSH on *C. albicans* suggested that the accumulation of ROS aggravated the damage of C16-Fengycin A against *C. albicans*. Moreover, the TEM results showed that when the cells were subjected to 32 μg/mL of C16-Fengycin A, the organelles were shrunk and the cells had necrosis. The damage caused by C16-Fengycin A to *C. albicans* may be associated with the accumulation of ROS. These results proved that the accumulation of ROS was an important factor in the fungistatic effect of C16-Fengycin A on *C. albicans*.

At the same time, C16-Fengycin A could cause the rise of mitochondrial membrane potential and the disturbance of mitochondrial function. Mitochondria are the main site of ROS production. The increase in mitochondrial membrane potential may be related to the increase of intracellular ROS. High concentrations of ROS could cause oxidative damage in cells. Therefore, from the above results, we could infer that C16-Fengycin A stimulated mitochondria to produce excessive ROS. When the content of ROS exceeded the normal metabolic range of *C. albicans*, it would further damage mitochondrial function, lead to an increase in mitochondrial membrane potential, inhibit the synthesis of the cell wall components, and ultimately cause cell death. This was consistent with the antifungal mechanisms reported in previous studies, which played a fungicidal role by promoting the increase of ROS (An et al. 2007; Andres et al. 2008).

The HOG-MAPK pathway plays an important role in coping with cellular oxidative stress and maintaining the structure of the cell wall (Cheetham et al. 2011). Glycerol, as an osmotic stress regulator, can balance intracellular and extracellular osmotic pressure (Gregori et al. 2007). We have found that the addition of C16-Fengycin A could down-regulate the related genes in the HOG-MAPK pathway, and increase the amount of intracellular glycerol. It was inferred that C16-Fengycin A at low concentrations could cause the damage to the cell wall of *C. albicans*, and induce the accumulation of ROS, which resulted in the cells to be in oxidative stress and under high osmotic pressure, thereby activating the intracellular HOG-MAPK pathway and increasing the amount of glycerol.

In conclusion, we hypothesized that C16-Fengycin A inhibited the growth of *C. albicans* mainly by destroying the structure of cell wall, reducing the hydrophobicity of the cell wall and accumulating the amount of ROS. Whereas C16-Fengycin A reached a sufficiently high concentration, it could lead to damage the cell membrane and aggravate cell death. Based on the above findings, we proposed the following model: C16-Fengycin A could damage the cell wall and cell membrane, promote the increase of content of ROS, and affect mitochondrial function, increasing in the content of intracellular glycerol and activating the HOG-MAPK pathway (Fig. 8). The study confirms that C16-Fengycin A is an effective antifungal agent which has the excellent application prospect.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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