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ARTICLE

Bafilomycin C1 exert antifungal effect through disturbing sterol biosynthesis in *Candida albicans*

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Abstract

In a previous study on discovering new antimicrobial agents from microbial sources, nine bafilomycins were isolated from the fermentation broth of *Streptomyces albolongus*. Among them, bafilomycin C1 (Baf C1) showed strong antifungal activity against *Candida albicans*, with MIC value of 1.56 μg/mL. The aim of this study was to evaluate the action mechanism of Baf C1 against *C. albicans*. Quantitative PCR analysis revealed that ergosterol biosynthesis-related genes of *C. albicans ACS1*, *HMG1*, *IDI1*, *ERG1*, *ERG2*, *ERG6*, *ERG7*, *ERG8*, *ERG9*, *ERG12*, *ERG13*, *ERG20*, *ERG24*, *ERG251*, *ERG252*, *ERG26*, *ERG27*, and *ERG28* were all down-regulated (Log_2fold change < -1) after Baf C1(4 μg/mL) exposure. Moreover, the expression of *MET6* gene, encoded methionine synthase, was also down-regulated (2.7-fold). It is corresponding with the quantitative PCR result, the content of ergosterol has dropped about 41% compared with the control. Transmission electron microscope examination also revealed that the Baf C1 strongly destroyed the cell membrane of *C. albicans*. In addition, the content of farnesol was significantly increased, about 2.1-fold compared with the control. The results indicated Baf C1 caused aberrations in sterol biosynthesis, leaded to the lack of ergosterol of the fungal membrane.

Introduction

In recent decades, the number of opportunistic fungal infections has greatly increased in patients with severe immunocompromised [1–3]. *Candida albicans* is the most commonly encountered human fungal pathogen, causing skin and mucosal infections in healthy individuals and immunocompromised patients [4]. Systemic candidiasis, caused by *C. albicans*, has become one of the main causes of death in deep fungal infection patients [5, 6]. Unfortunately, there are still major weaknesses of currently available antifungal agents for the treatment of candidemia in their spectra, potencies, safety, and pharmacokinetic properties [7, 8]. Besides, with the long-term and large scale application of broad-spectrum antifungal agents, there has been a notable increase in drug resistance [2, 3]. Therefore, the search for new antifungal drugs and the exploitation of the molecular mechanism of antifungal reagents are becoming a focus of researchers' attention.

In the progress of discovering new antimicrobial agents from microbial sources, our research group had isolated nine bafilomycins from a fermentation broth of *Streptomyces albolongus* [9]. Among them, bafilomycin C1 (Baf C1) showed strong inhibition activity against *C. albicans*, with MIC value of 1.56 μg/mL [9]. Bafilomycins, a group of 16-membered macrolide antibiotics, were first isolated from *Streptomyces griseus* [10]. Earlier studies showed that bafilomycins possessed a broad spectrum of biological activities, including antibacterial, antifungal, insecticidal, and cytotoxic [9, 11]. However, the underlying molecular mechanisms responsible for the antifungal effect of bafilomycins remained unknown, although some investigators have recorded its bioactivity [10, 11].

Ergosterol belongs to the most important fungal sterols [12], is present in the phospholipid bilayer of the fungal cell membrane, maintaining both fungal membrane structural integrity and fluidity [13], and can affect the activity of membrane-bound enzymes [14]. It has gained in popularity as a quantitative chemical index for fungal mass [15]. The lack of ergosterol and nonplanar polyol precursor accumulation will lead to rupture of the fungal membrane. The ergosterol biosynthesis pathway (Fig. 1) is the important

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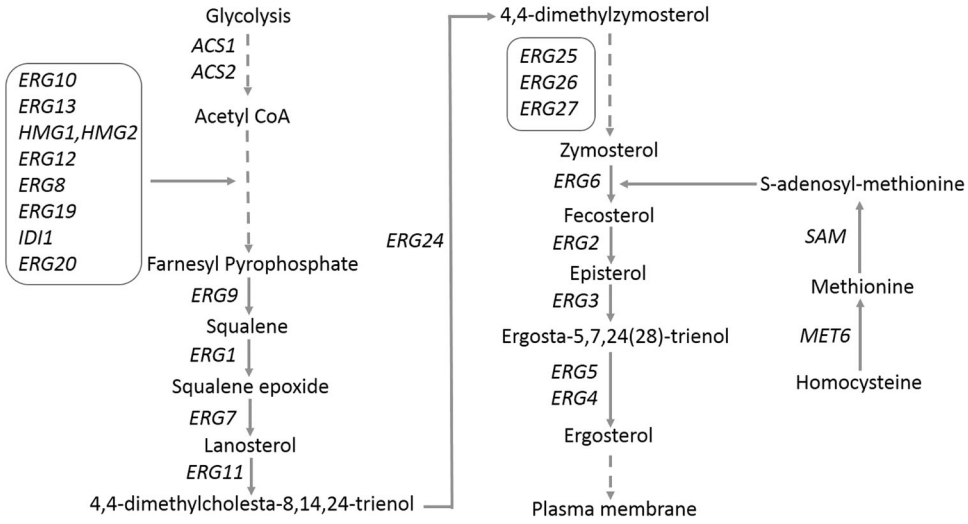

Fig. 1 Ergosterol biosynthesis pathway. Only selected substrates or products are shown. Genes (in italics) that encode enzymes in the pathway are shown to the left. CoA, coenzyme A

target for many antifungal agents [6, 16–20]. This pathway involves a variety of enzymes, and their encoding genes in *C. albicans* had been clearly illuminated (Table 1) [4]. When cells exposed to a drug, measurement of changes in gene expression can help us to understand the mechanism of how drugs work in cells and organisms [21]. In current study, we examined the relative expression of the ergosterol biosynthesis-linked genes after exposure to Baf C1 using RT-qPCR to reveal the effect of Baf C1 on these genes in *C. albicans*.

Hyphae are an important factor of fungal virulence. *C. albicans* is a dimorphic yeast. Both yeast cells and hyphae are present in the host during infection [1]. Hyphae formation is considered as an important virulence factor, and close relationship with the infection ability of pathogenic fungi [22]. Its ability to switch from yeast cells to hyphae is considered to be important for the interactions of *C. albicans* with its host [1]. Farnesol phosphate derivatives were the precursors of steroids in fungi. It could influence the yeast-to-hypha transition and the virulence of *C. albicans* [23].

In the present work, we reported the influence of Baf C1 on the production of ergosterol and farnesol in *C. albicans*. The effects of Baf C1 on ergosterol biosynthesis-related (*ERG*) genes expression were also assayed to explore the mechanism of action.

Materials and methods

General experimental procedures

Baf C1 was isolated from a fermentation broth of *Streptomyces albolongus* by one of the authors (N. Ding) using our previously established method [9]. The fermentation broth of *S. albolongus* was collected and treated with the polymeric resin Amberlite XAD-16 to get bound compounds. The crude extracts were then isolated by sequential chromatography over Sephadex LH-20, silica gel, and ODS to yield pure Baf C1 with a purity of 98.0% determined by high-pressure liquid chromatography (HPLC). Baf C1 were accurately weighed, dissolved in DMSO to prepare 8.0 mg/mL of stock solution and stored at −20 °C. A series of working solutions of Baf C1 were prepared over the concentration of 0.5, 1.0, 2.0, 4.0, and 8.0 mg/mL by diluting the stock solution with DMSO before test. *C. albicans* SC5314 (ATCC MYA-2876) was maintained on two complete media consisting of a YPD liquid medium (1% yeast extract, 2% peptone, 2% dextrose), and a solid medium prepared by adding 2% agar (Sangon). Methanol, ethanol and potassium hydroxide (pellet form) were all of analytical grade (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). Ergosterol and farnesol (Sigma-Aldrich, Germany) were accurately weighed, dissolved in methanol to prepare the stock solutions of ergosterol (2.3 mg/mL) and farnesol (1.0 mg/mL) and stored at 4 °C. Go Taq® qPCR Master Mix and GoScript™ Reverse Transcription System were purchased from Promega (Madison, WI, USA).

Growth curve study

The effect of Baf C1 exposure in relation to time and concentration on *C. albicans* was determined in YPD liquid medium. *C. albicans* were cultured for 12 h in 5 mL YPD liquid medium and then 5 µL of this cell suspension was inoculated to 100 mL of fresh YPD liquid medium and cultured overnight at 37 °C in a shaking incubator. Following, adjust the cell suspension concentration to form an optical density (OD) of 0.1 (measured at a wavelength of 600 nm, 2×10^6 cfu/mL). Then, 10 µL of various concentration of Baf C1 working solutions (in DMSO) were added to 10 mL cells suspension, and the final

Table 1 Primers used in this study

Genes	Description	GenBank accession no.	Primer	Sequence (5′ to 3′)
ACS1	Acetyl-coenzyme A synthetase	XM709254	Forward	GATGTTGAAGGGGTTCTTGC
			Reverse	TCTCTAGCAGCACCATCACC
ACS2	Acetyl-coenzyme A synthetase	XM708575	Forward	TGCCACCAGTTTCAGTCAAC
			Reverse	CAACCAACATCACCAGCAGT
ERG1	Squalene epoxidase	XM706801	Forward	TTGGTGCAGGGGTTATTGGT
			Reverse	CCCAGTACAATCAACAGCCC
ERG2	Sterol C8-C7 isomerase	XM713793	Forward	CAGCAATTGGGACTGAAGGT
			Reverse	TTCGGGAATCAATGCACCAG
ERG3	Sterol C5 desaturase	XM708484	Forward	GCTTCTCATGCTTTCCATCC
			Reverse	CAAGCGGTACCATTGACAAC
ERG4	Sterol C-24 (28) reductase	XM712569	Forward	CTTCGGAAGGTCAATCTTGG
			Reverse	GTCCAAACACCGGGTAAAGT
ERG5	Sterol C22 desaturase	XM711840	Forward	GAAGAGCAATTGCGTGTGAG
			Reverse	TGGTGGACGGTATCTCAAAG
ERG6	Sterol 24-C-methyltransferase	XM716495	Forward	AGATGCTGCTTCTGTTGCTG
			Reverse	GGAATGAAGAACCCCAACC
ERG7	Squalene cyclase	XM717519	Forward	GGTATCTTCCCCTGCCAGTA
			Reverse	AGAATGAAGTCCCCAACCAC
ERG8	Phosphomevalonate kinase	XM717731	Forward	GTTGAGCCTTTGACTGTTGC
			Reverse	CAACTGGGTTTGGACATCAG
ERG9	Squalene synthase	XM709367	Forward	TGGCCTCGAGAAATTTGG
			Reverse	CAGTGACATGACCCAATGC
ERG10	Acetoacetyl-CoA thiolase A	XM705032	Forward	GTTGTTGGTGGTGCTGAATC
			Reverse	TCTGGTGAATCCATGGTCAG
ERG11	Lanosterol 14α-demethylase	XM711668	Forward	CCATTTGGTGGTGGTAGACA
			Reverse	CAGGGTCAGGCACTTTATAACC
ERG12	Mevalonate kinase	XM718212	Forward	GGGCGACTAAATTGACAGGT
			Reverse	CTGAGAACTTTGTGGCATCG
ERG13	3-Hydroxy-3-methylglutaryl coenzyme A synthase	XM711353	Forward	CAGCTTTGCAAGTCCCTACC
			Reverse	GCCAAACCAGAACCATAGGA
ERG20	Farnesyl diphosphate synthetase	XM707792	Forward	TATGCCTCAAGAAGCCATTG
			Reverse	ATGGCCCAACCTAATAATGC
ERG24	Sterol C-14 reductase	XM705578	Forward	TCTTGGTGTTTGCCTACTGG
			Reverse	GCTCTGCACTTCATTTCGTC
ERG251	C-4 sterol methyl oxidase	XM708363	Forward	TAGGATTGGGTACGGTTGGT
			Reverse	GCATCAACGGCTTGGAAT
ERG252	C-4 sterol methyl oxidase	XM717610	Forward	CCATTTGGTTTAGCAGCAGA
			Reverse	GAGAATCAACGGCTTGGAAT
ERG26	C-3 Sterol dehydrogenase/C-4 decarboxylase	XM710527	Forward	AACTTGGCCATACCCTGAAG
			Reverse	CAATTGACGATCACCAGGTC
ERG27	Sterol C-3 ketoreductase	XM712838	Forward	GAAGTTTTGCAAAGCCCAAT
			Reverse	CCACCATTTTCCAACAAGTG

Table 1 (continued)

Genes	Description	GenBank accession no.	Primer	Sequence (5′ to 3′)
ERG28	Involved in synthesis of ergosterol	XM714372	Forward	CAGCAAGAACTTTTGGAACTTG
			Reverse	AAAATGCCATGCAGCAATAG
MET6	Cobalamin-independent methionine synthase	XM713126	Forward	TCCACTCTCCAAGAATTCCA
			Reverse	TCTGGCCAGCCTCTAGTTTT
HMG1	Hydroxymethylglutary-CoA reductase	XM708543	Forward	TCACCAGAAGATGTCCCTCA
			Reverse	AACCCGGTTAAATGATGAGC
IDI1	Isopentenyl diphosphate isomerase	XM715202	Forward	CCAGAAGTTACCCCCTTGAA
			Reverse	GCACCAACAGGCTTGTCAT
ACT1	Actin	XM717232	Forward	TGGAAGCTGCTGGTATTGAC
			Reverse	TCCTTTTGCATACGTTCAGC

concentrations of Baf C1 were 0, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/mL, respectively. The flasks were cultured for 48 h at 37 °C on a rotary shaker at 180 rpm. The growth was monitored by measuring the optical density (600 nm) of the cultures during the subsequent 48 h. There were three independent experiments for each concentration.

Ultrastructure analysis by transmission electron microscopy

Transmission electron microscopy was performed to observe the effect of Baf C1 on cell ultrastructure. *C. albicans* cells were collected after being treated with Baf C1 at 2 μg/mL or 4 μg/mL for 6 h. The cells were collected through centrifuging at 6000 × g for 5 min and washed twice with PBS (phosphate buffered saline) solution. After that, cells were collected, fixed in 2% glutaraldehyde at 4 °C for 72 h, and then placed in 1% phosphotungstic acid. The cells were dehydrate using graded ethanol, and embedded with EPON-812. Ultrathin sections were prepared and observed after double staining with uranium and plumbum under a transmission electron microscope (HITACHI H-7650, Japan) with 4×10^4 magnification. At the same time, the vehicle treated cells were used as control, and amphotericin B (AMB, 2 μg/mL) and fluconazole (FLC, 2 μg/mL) were served as the positive controls.

RNA isolation

C. albicans were cultured for 12 h in 5 mL YPD liquid medium and then 5 μL of this cell suspension was inoculated to 100 mL of fresh YPD liquid medium and cultured overnight at 37 °C in a shaking incubator. Following, adjust cultures to a final cell density of 2×10^6 cells/mL. Then, 10 μL of Baf C1 solutions (in DMSO) were added to 10 mL cells suspension, the final concentrations of which were 0, 2, 4 μg/mL (0, 2.8, 5.6 μM). The cells were cultured at 37 °C with shaking at 180 rpm and continued for 6 h. At the indicated times, 1×10^7 cells from each culture were transferred to microcentrifuge tubes, centrifuged for 10 min at 6000 × g, and the supernatant was discarded. The pellet was resuspended in 50 μL of ice-cold PBS buffer and transferred to a precooling mortar, following the addition of liquid nitrogen into a mortar and grounded into a powder. After that, RNA isolation using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen, China) according to the manufacturer's protocol. cDNAs were synthesized from total RNA using the GoScript™ reverse transcription system in accordance with manufacturer instructions (Promega, USA).

Quantitative real-time PCR

Quantitative PCR (polymerase chain reaction) was conducted with 1 μL reverse transcribed product in a CFX Connect™ real-time PCR system (BIO-RAD, USA) using GoTaq® qPCR master mix (Promega, USA). Each reaction set three parallel reaction. PCR was performed with the primer sets listed in Table 1. Cycling conditions for all genes were 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s and 40 cycles to ensure that amplification during the logarithmic phase was obtained. *ACT1* gene was used as the internal control. Fold changes were calculated using the formula $2^{-(\Delta\Delta Ct)}$, where ΔΔCt is ΔCt (treatment)-ΔCt (control), ΔCt is Ct (target gene)-Ct (*ACT1*), and Ct is the

threshold cycle (user's manual for CFX Connect™ real-time PCR system).

Extraction and quantitation of ergosterol

The extraction and quantitation of ergosterol followed the methods as described by Munayyer et al [24]. *C. albicans* were cultured for 12 h in 5 mL YPD liquid medium and then 5 µL of this cell suspension was inoculated to 100 mL of fresh YPD liquid medium and grown overnight at 37 °C in a shaking incubator. Following, adjust the cultures to a final cell density of 2×10^6 cells/mL. Then, 100 µL of Baf C1 solutions (in DMSO) were added to 100 mL cells suspension at the final concentrations of 0, 2, 4 µg/mL, respectively and cultured at 37 °C with shaking at 180 rpm for 6 h. The numbers of cells was counted using a hemacytometer. The cells were harvested, washed twice with PBS solution, and then added 10 mL of 15% KOH in 90% ethanol. The mixture was saponified using a water bath for 2 h at 80 °C (Shaked once every 30 min), and cooled to room temperature. The mixture was extracted three times with 6 mL petroleum ether. The combined organic extracts were washed with saturated brine, and evaporated to dryness under reduced pressure in a rotary evaporator at 40 °C. The dried residue was redissolved in methanol, made up to 6 mL and filtered through a 0.2 µm PTFE filter (Agilent technologies, USA) for quantitative analysis.

A series of standard working solutions of ergosterol were prepared over the concentration of 230.0, 115.0, 57.5, 46.0, 23.0, and 4.6 µg/mL by diluting the stock solution with methanol. HPLC analysis were conducted using an Agilent 1290-6420 Triple Quadrupole LC/MS system with an Agilent SB-C18 column (2.1 × 50 mm, 1.8 µm). The mobile-phase solvent composition was a methanol–water mixture (95:5, v/v) with the flow rate of 0.4 mL/min and injection volume of 2 µL. The retention time of ergosterol is 2.5 min. Quantification was performed by the multiple reaction monitoring (MRM) method. The ion transitions of the precursor to the product ion were principally ions $[(M\text{-}H_2O) + H]^+$ at m/z $379 \rightarrow 69$ ($[C_5H_8 + H]^+$) (fragmentor voltage, 135 V; collision energy, 30 eV) for ergosterol. Each transition was monitored with a 200 ms dwell-time. Optimum values for the ESI parameters were: 330 °C of drying gas temperature, 10 L/min of drying gas flow and 35 psi of nebulizer pressure. Each analysis was conducted in triplicate.

Extraction and content determination of farnesol

The extraction and determination of farnesol followed the methods as described by Hornby et al. [25]. *C. albicans* were treated with Baf C1 as the same method above. The numbers of cells was counted using a hemacytometer. The culture supernatant fluid was collected after centrifuging for 10 min at 6000 × g. The supernatant fluid was extracted three times with 6 mL ethyl acetate. The combined organic extracts were washed with saturated brine, and evaporated to dryness under reduced pressure in a rotary evaporator. The dried residue was redissolved in methanol, made up to 6 mL and filtered through a 0.2 µm PTFE filter (Agilent technologies, USA) for quantitative analysis.

HPLC analyses were performed using the same column as above with a flow rate of 0.4 mL/min and injection volume of 2 µL. A gradient solvent system consisting of solvent A (water) and solvent B (acetonitrile) was used as following: 0–9 min, 20–80% B; 9–10 min, 100% B. The equilibration time for each injection was set at 12 min. A series of standard working solutions of farnesol were prepared over the concentration of 50.0, 25.0, 20.0, 10.0, 5.0, and 2.5 µg/mL by diluting the stock solution with methanol. The retention time of farnesol is 7.3 min. A positive SIM (selected ion monitoring) mode was used to analyze farnesol (*m/z* 205 $[(M\text{-}H_2O) + H]^+$). The TQ (triplequadrupole)-MS conditions utilized were a gas temp. of 330 °C gas flow of 10 L/min, nebulizer pressure of 35 psi, cap. voltage of 4000 V, and cell accelerator voltage of 1 V. Each analysis was conducted in triplicate.

Data analysis

The data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. The statistical significance of the differences between the means was determined by using Student's *t*-test. The *p*-values < 0.01 (* or #) was considered significant. The SPSS 17.0 statistical software package was used for data analysis.

Results

Growth curve

C. albicans cells were treated with Baf C1 at various concentrations (0-8 µg/mL) for 48 h. Baf C1 showed significant inhibition of proliferation for *C. albicans* cells in a time-dependent and dose-dependent manner ($p < 0.01$) (Fig. 2). By 6 h post-incubation, significant inhibition was observed at the concentrations as low as 4 µg/mL compared with the control. The data suggested a role of Baf C1 in suppressing *C. albicans* growth.

Ultrastructure analysis

The cell membrane and cell wall of *C. albicans* were observed through transmission electron microscope after treated with Baf C1 or vehicle. Baf C1 treated cells presented notable alteration in the morphology compared with

the control cells (Fig. 3). *C. albicans* cells displayed a normal cellular morphology with typical cell membrane and a distinct cell wall in vehicle treated group (Fig. 3a). However, after exposure to Baf C1, AMB or FLC, the cell wall was irregular, and more importantly, the cell membrane was extensively damaged (Fig. 3b-e).

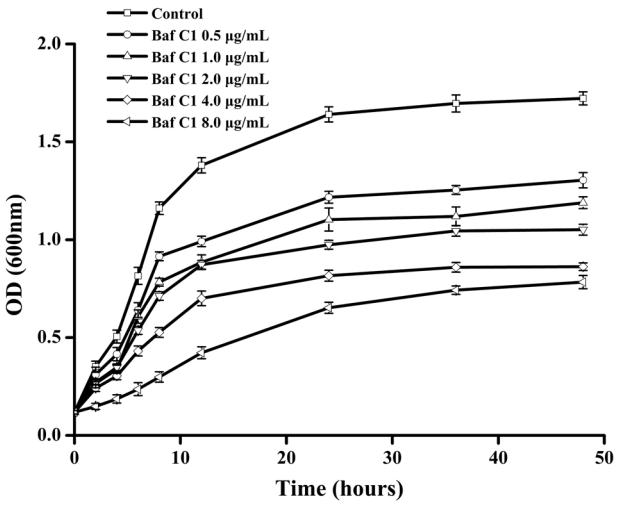

Fig. 2 The effect of Baf C1 on the growth of *Candida albicans* in liquid YPD medium. *C. albicans* cells were treated with Baf C1 (0, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/mL, respectively) for 48 h. The OD (measured at a wavelength of 600 nm) is plotted versus time. Data represent the mean ± SD of three independent experiments. Baf C1 showed significant inhibition of proliferation for *C. albicans* cells in a dose-dependent manner ($p < 0.01$)

Quantitative PCR analysis

Quantitative PCR (qPCR) analyses revealed that the expressions of ergosterol biosynthesis-related genes of *C. albicans* were significantly down-regulated in a dose-dependent manner after treated with Baf C1 compared with the control ($p < 0.01$) (Fig. 4). Among them, *ACS1*, *HMG1*, *IDI1*, *ERG1*, *ERG2*, *ERG6*, *ERG7*, *ERG8*, *ERG9*, *ERG12*, *ERG13*, *ERG20*, *ERG24*, *ERG251*, *ERG252*, *ERG26*, *ERG27*, and *ERG28* were all down-regulated (Log_2fold change < -1, $p < 0.01$). Moreover, the expression of *MET6* gene, encoded methionine synthase, was also down-regulated (2.7-fold).

Quantitative analysis of ergosterol

In order to determine the change of ergosterol content in *C. albicans* cells, the calibration curve of ergosterol was constructed (Fig. 5a). The peak areas showed a good linear relationship with ergosterol concentration in 4.6–230 μg/mL, regression equation: $y = 3.4E\text{-}03\ x - 1.2798$ ($R^2 = 0.9987$). Followed the methods as described by Ding et al. [26] the limit of detection (LOD) and limit of quantification (LOQ) for ergosterol were estimated by a signal to noise ratio of 3:1 and 10:1 respectively. The LOD and LOQ values of ergosterol were 0.36 μg/mL and 1.15 μg/mL respectively. The content of ergosterol in test samples was divided by the number of cells to yield the relative content of ergosterol in single cell. The quantification analyses based on % the cells counts revealed that the content of ergosterol was

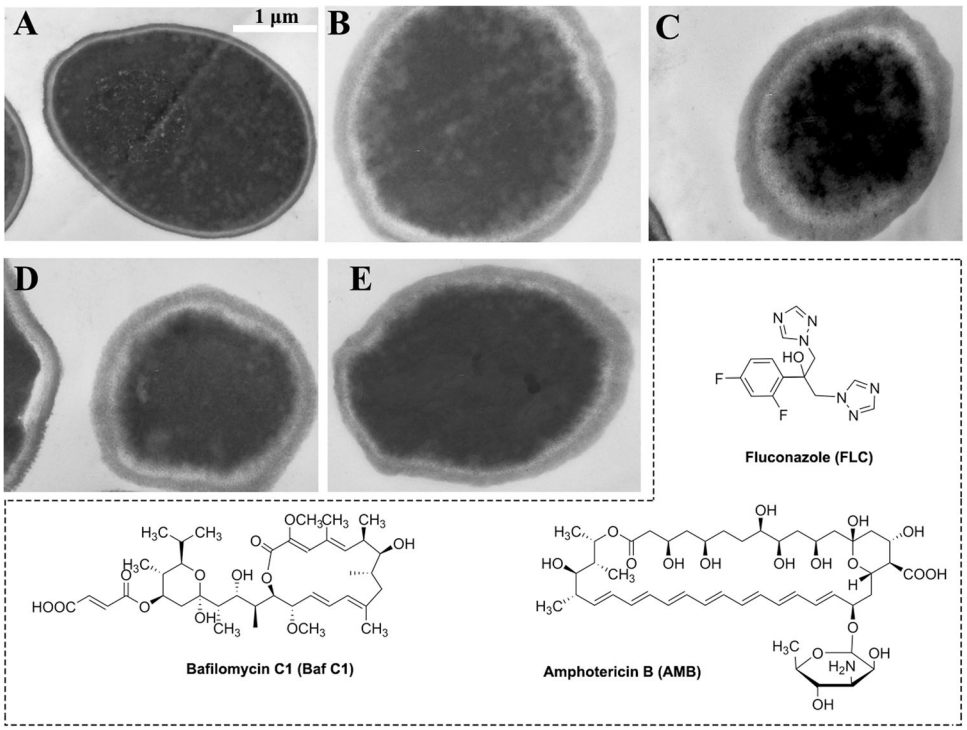

Fig. 3 Ultrastructure of *C. albicans* cell. *C. albicans* were treated with Baf C1 or vehicle and were observed by transmission electron microscopy. **a** vehicle treatment; **b** treated with 2 μg/mL of Baf C1; **c** treated with 4 μg/mL of Baf C1; **d** treated with AMB (2 μg/mL); **e** treated with FLC (2 μg/mL). The cell membrane of *C. albicans* was seriously destroyed by Baf C1, AMB or FLC. The white bar represents a length of 1 μm

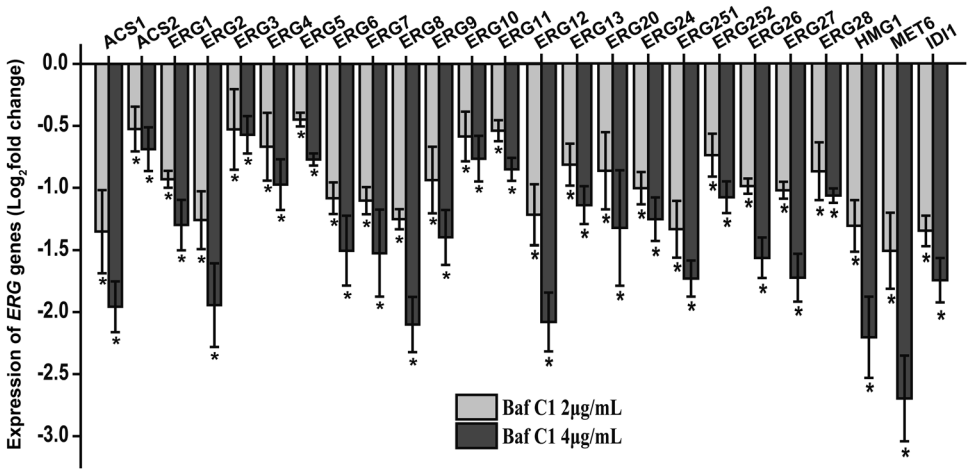

Fig. 4 Quantitative real-time PCR analysis of ergosterol biosynthesis-related genes. Real-time qPCR of *C. albicans* treated with 2 μg/mL of Baf C1 (light grey bars) or 4 μg/mL (dark grey bars) for 6 h. Cells treated with DMSO (0.1%) were used as control; *ACT1* gene was used as the internal control. Data represent the mean ± SD of three independent determinations. Significant differences from the control are indicated by $*p < 0.01$. The y-axis scale is $\log_2$ fold change

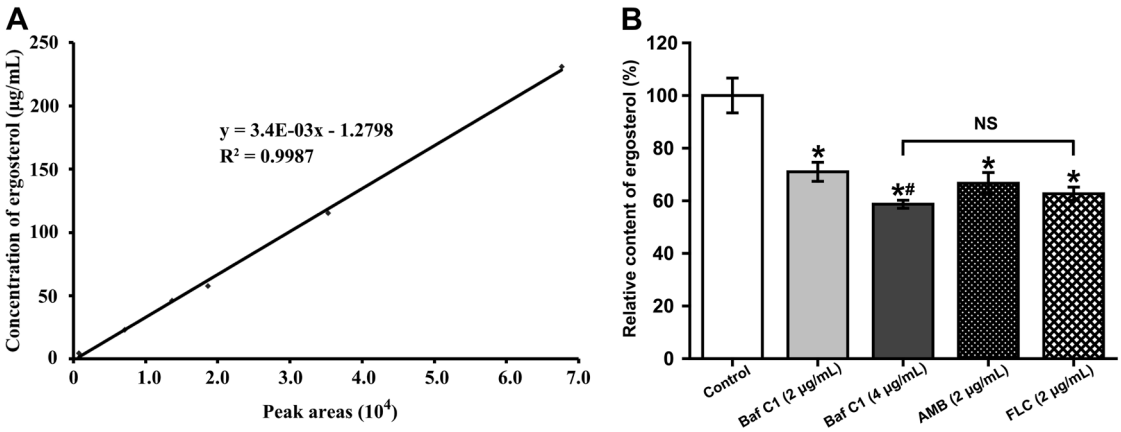

Fig. 5 The quantitative analysis of ergosterol. **a** The calibration curve of ergosterol. The regression analysis of the calibration curve was carried out by plotting the peak areas against the concentration of ergosterol. The concentrations of ergosterol were 230.0, 115.0, 57.5, 46.0, 23.0, and 4.6 μg/mL; **b** The quantitative analysis of ergosterol of *C. albicans* after treated with 2 μg/mL (light grey bars) or 4 μg/mL (dark grey bars) of Baf C1 for 6 h. AMB (2 μg/mL) or FLC (2 μg/mL) treated groups were served as the positive controls, and control group was treated with vehicle. Data represent the mean ± SD of three independent experiments. $*p < 0.01$ as compared with control group; $^{\#}p < 0.01$ with respect to AMB treatment group; NS, not significant

significantly decreased after treated with Baf C1, AMB or FLC compared with the control ($p < 0.01$). The content of ergosterol decreased about 29, 33, and 37%, respectively, when the *C. albicans* were exposed to 2 μg/mL Baf C1, AMB or FLC. Especially, when the final concentration of Baf C1 at 4 μg/mL, it dropped about 41% (Fig. 5b).

Quantitative analysis of farnesol

The calibration curve of farnesol was established by HPLC (Fig. 6a). The peak areas showed a good linear relationship with farnesol concentration between 2.5–50 μg/mL, regression equation: $y = 2E\text{-}07x - 10.595$ ($R^2 = 0.999$). The LOD and LOQ values of farnesol were 0.25 and 0.83 μg/mL respectively. The results showed that the content of farnesol were significantly increased in the culture liquid after treated with Baf C1, AMB or FLC ($p < 0.01$). The content of farnesol increased about 34%, 46% and 65%, respectively, when the *C. albicans* were exposed to 2 μg/mL Baf C1, AMB or FLC. Specifically, it presented about 2.1-fold higher when the final concentration of Baf C1 at 4 μg/mL compared with the control (Fig. 6b).

Discussion

In the present study, the molecular mechanisms of Baf C1 against *C. albicans* were evaluated; the influence of Baf C1 on ergosterol biosynthesis-related genes of *C. albicans* were investigated. Our results indicated various molecular mechanisms, including decreasing the ergosterol content, increasing the farnesol product and reducing the expression of *ERG* genes are involved in Baf C1 against *C. albicans*.

In TEM photographs, after Baf C1 treatment, the cell wall was irregular, and more importantly, the cell membrane of *C. albicans* appeared obvious shrinkage and breakage.

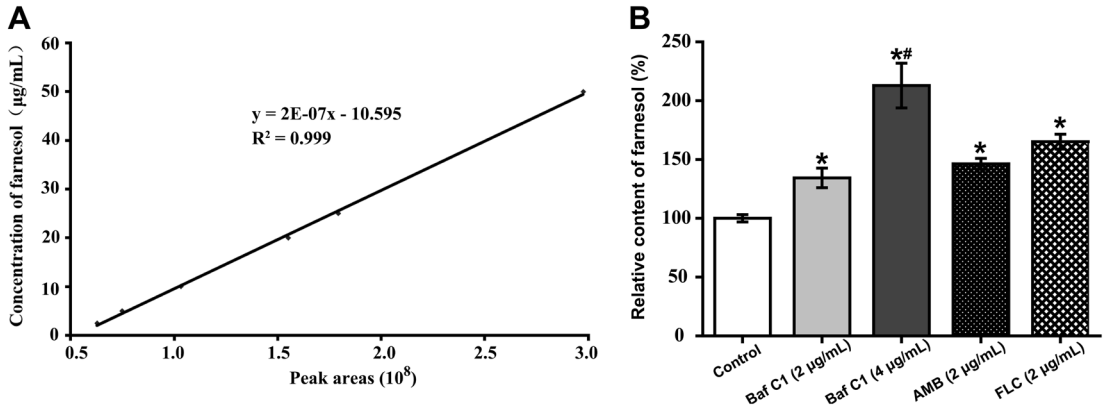

Fig. 6 The quantitative analysis of farnesol. **a** The calibration curve of farnesol. The concentrations of farnesol were 50.0, 25.0, 20.0, 10.0, 5.0, and 2.5 μg/mL; **b** The quantitative analysis of farnesol of *C. albicans* after treated with 2 μg/mL (light grey bars) or 4 μg/mL (dark grey bars) of Baf C1 for 6 h. AMB (2 μg/mL) or FLC (2 μg/mL) treated groups were served as the positive controls, and control group was treated with vehicle. Data represent the mean ± SD of three independent experiments. $^{*}p < 0.01$ as compared with control group; $^{\#}p < 0.01$ with respect to AMB or FLC treatment group

Furthermore, the cell membrane of *C. albicans* was also seriously destroyed in the AMB treated group. According to the literature, AMB could bind with ergosterol in the plasma membrane, disruption of the normal structure of cell membrane, lead to the cell membrane breakage [27–29]. The structure characterization of Baf C1 was obviously different with AMB (Fig. 3) and there is no macrocyclic polyene moiety in the structure of Baf C1. Therefore, we conclude that they would present different mechanisms on against *C. albicans*. The cell membrane breakage possibly caused by Baf C1 influencing the ergosterol product in *C. albicans*.

Ergosterol is the major sterol component in fungal membranes and therefore, thought to be necessary for fungal development and survival. Therefore, key enzymes involved in the ergosterol pathway had been attractive targets for antifungal agents [30]. In order to determine the effect of Baf C1 on sterol biosynthetic pathway in *C. albicans*, we examined the relative expression of the ergosterol biosynthesis-linked genes after exposure to Baf C1 using RT-qPCR. Quantitative PCR results showed that some key *ERG* genes [6, 20] including *ACS1*, *HMG1*, *IDI1*, *ERG1*, *ERG2*, *ERG6*, *ERG7*, *ERG8*, *ERG9*, *ERG12*, *ERG13*, *ERG20*, *ERG24*, *ERG251*, *ERG252*, *ERG26*, *ERG27*, and *ERG28* in *C. albicans* were significantly down-regulated after Baf C1 treatment (Log_2fold change < -1, $p < 0.01$). According to the literature, early intermediates prior to squalene in the ergosterol pathway are important for pathway regulation [30]. About ten enzymes, encoded by *ACS1, ACS2, HMG1, IDI1, ERG8, ERG9, ERG10, ERG12, ERG13*, and *ERG20* genes, are involved in the early steps of sterol biosynthesis. It was notable that these genes were significantly down-regulated after Baf C1 treatment ($p < 0.01$). The effect of Baf C1 on the production of ergosterol and the growth of *C. albicans* could be related with the down-regulation of these genes. Among them, ACS1 proteins, namely acetyl-coenzyme A synthetase, whose activity is central to the metabolism of prokaryotic and eukaryotic cells. The physiological role of this enzyme is to activate acetate to acetylcoenzyme A, provides the cell the two-carbon metabolite used in many anabolic and energy generation processes [31]. Thus, besides being involved in the biosynthesis of sterol, the acetyl-coenzyme A synthetase also play a major role in fatty acid biosynthesis and energy generation processes. Therefore, down-regulation of this gene possibly inhibits cell proliferation through lack of ergosterols or phospholipids in membranes. Moreover, *ERG1* (allylamines), *ERG2* (morpholines), *ERG24* (allylamine) were known as antifungal drugs' target enzyme genes [20]. And these genes were significantly down-regulated after Baf C1 treatment ($p < 0.01$). Squalene cyclase, a key enzymes in ergosterol biosynthetic pathway, was encoded by *ERG7* and considered as a potential antifungal target [30, 32]. Our current study showed that *ERG7* gene was significantly down-regulated after Baf C1 treatment ($p < 0.01$). The inhibition of these genes expression leaded to the decrease ergosterol production in the *C. albicans*. Interestingly, the azoles target enzyme gene *ERG11* [6] was not obviously regulated in our experiment. These facts indicated the action mechanisms of Baf C1 was different from those known drugs and possibly possessed potential to resist the azole-resistant strains of *C. albicans*. Notably, the expression of *MET6* gene, encoded methionine synthase, was also down-regulated. *MET6* is an essential gene in *C. albicans*, as methionine synthase converts homocysteine to methionine [33]. *MET6* was considered as an attractive antifungal drug target owing to its dual effect on not only causing methionine auxotropy but also homocysteine accumulation, which further inhibit sterol biosynthesis [16].

In addition, it was corresponding with the quantitative PCR results, the content of ergosterol in *C. albicans* was

obviously reduced after treatment with Baf C1 compared with the control. Thus we suggested that Baf C1 could decrease ergosterol production in the *C. albicans* through down-regulating the expressions of *ERG* genes. According to the literature, bafilomycins shows a high-affinity inhibitory function on vacuolar-type ATPase (V-ATPase) [34]. The V-ATPase is critical for generation of a pH gradient that drives secondary transporters to maintain cellular ion homeostasis. Thus, V-ATPase plays essential roles in diverse cellular processes, and is required for fungal virulence [35]. It is generally accepted that azoles exert their antifungal effect by inhibiting ergosterol biosynthesis, specifically targeting lanosterol demethylase (Erg11p) [35]. In addition, the studies of Zhang et al. [35] suggested that the requirement of ergosterol for V-ATPase function is conserved in fungi, and ergosterol could directly modulates the activity of V-ATPase. And their findings showed that FLC, an azoles antifungal drug, probably inhibited V-ATPase through depletion of ergosterol and then caused fungistatic effects. Given the facts above, we predicted that ergosterol depletion by Baf C1 treatment would impair V-ATPase function, thereby disruption of cellular ion homeostasis, and disruption of V-ATPase function is sufficient to repress growth and attenuate fungal virulence, but its theoretical basis needs further verification.

C. albicans is a dimorphic yeast. The ability to switch from yeast cells to hyphae was considered to be important for virulence of *C. albicans* [1, 36]. Farnesol had been proved to inhibit the yeast-to-hypha transition in *C. albicans* [23]. Increasing expression of farnesol in *C. albicans* was thought to be a novel route for the development of biofilm (formed by mycelium) formation inhibitor [37]. In the present study, we found that the content of farnesol in the culture liquid of *C. albicans* was significant increase after treated with Baf C1. Based on our results and the literature, we proposed that Baf C1 could increase the content of farnesol in *C. albicans* environment to inhibit the yeast-to-hypha transition, and therefore weakened the virulence of *C. albicans* [23, 36–38].

In conclusion, the action mechanism of Baf C1 against *C. albicans* may be that Baf C1 caused aberration in sterol biosynthesis, leaded to the lack of ergosterol of the fungal membrane, thereby killed fungi through destroying the cell membrane. In addition, Baf C1 could inhibit the yeast-to-hypha transition of *C. albicans* to weaken the virulence.

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Compliance with ethical standards

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

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