In vitro antifungal activity of Shikonin against Candida albicans by inducing cellular apoptosis and necrosis

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Abstract

Our previous studies showed that Shikonin (SK) had a strong anti-*Candida albicans* (*C. albicans*) activity, especially against some fluconazole-resistant strains, which is probably due to the oxidative damage of SK to *C. albicans*. In this study, we further analyze the exact mechanism of SK against *C. albicans*. Through some experiments, such as detecting the apoptosis and necrosis rates of *C. albicans* SC5314 cells treated with different concentrations of SK by Annexin V-FITC / PI double staining, observing the ultrastructure of cells by transmission electron microscopy, testing mitochondrial cytochrome c oxidase activity, caspase activity, and caspase-related gene *CaMCA1* expression level. We found that SK could induce a series of apoptosis characteristics, including phosphatidylserine (PS) externalization, chromatin condensation and fragmentation, decreased cytochrome c oxidase activity as well as caspase activation. In summary, this study highlighted the antifungal activity and mechanism of SK against *C. albicans*, providing a potential therapeutic strategy for *C. albicans* infection.

Introduction

C. albicans is an endophytic fungus, which usually lives in the human gastrointestinal tract and skin. However, as an opportunistic pathogen, *C. albicans* would cause severe mucosal infections and fatal invasive infections in people with lowered immune function due to AIDS or cancer radio-or chemotherapy. Besides, *C. albicans* can provide suitable living and establishing conditions for other fungus, with such complicated infections leading to more severe infections and antimicrobial resistance [1–2]. Nowadays, with the mass application of broad-spectrum antifungal drugs, more and more multidrug-resistant strains are isolated from *C. albicans*-infected patients [3]. Therefore, it is necessary to explore new antifungal drugs with high efficiency and low drug resistance to deal with fatal oidiomycosis.

Many studies have shown that extracts from plants used in traditional medicine have broad antifungal activities, such as berberine, forsythiaside, baicalein, etc [4–6]. Shikonin (SK) is a kind of fat-soluble naphthoquinone pigment extracted from the rhizome of the traditional Chinese herbal medicine named Zicao [7]. It has been reported that SK can induce apoptosis of many kinds of tumor cells, thereby achieving antitumor effects [8]. Our previous study found that SK had a strong inhibitory effect on various kinds of fungi, including *Candida krusei*, *Saccharomyces cerevisiae* and *Candida glabrata*. Specially, the azole-resistant *C. albicans* strains were more susceptive to SK than fluconazole [9]. Nevertheless, the detailed mechanisms of SK against *C. albicans* remain unclear. Based on our original works, this study will further explore the mechanism of SK-induced apoptosis of *C. albicans*, in order to provide an important experimental basis for its antifungal infection.

Materials And Methods

1. Strains, Media and Compounds

C. albicans SC5314 was kindly provided by Prof. Cao (School of Pharmacy, Naval Medical University, Shanghai, China.). For all vitro experiments, 6.4 mg / mL SK (purity > 99%, Beijing Suolaibao Technology Co. Ltd, Beijing, China, dissolved in DMSO (Sigma)) was used as the stock solution stored at -20°C and added to the culture medium to obtain the required concentration. Media used in this study included Sabouraud dextrose agar plate (SDA, 1% peptone, 4% glucose, and 1.8% agar), yeast extract peptone dextrose (YPD, 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose), phosphate buffered saline (PBS) [10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride (pH 7.4)] and RPMI 1640 (Gibco, Bethesda, MD, U.S.A.) supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS). *C. albicans* strains were grown in SDA plates medium, and cultivated in a liquid complete medium YPD medium at 30°C with constant shaking (200 rpm).

2. Methods

2.1 Protoplast preparation

To study the effect of test SK on apoptotic markers, protoplasts were prepared as described previously [10], with modifications. Briefly, mid-exponential phase cells were harvested and exposed to various concentrations of SK shaken at 30°C for 2 h at 200 rpm. The cells were then washed twice with PBS. Next, the cells was resuspended at a concentration of 3×10^7 CFU / mL and digested with lyticase (100 U / mL) at 30°C for 30 min. The digested cells were centrifuged at 1500 rpm for 5 min again and the supernatant was discarded. Finally, the obtained protoplasts were washed twice with PBS and resuspended for further use.

2.2 Annexin V-FITC/PI-staining

This assay was used to identify necrosis (cellular integrity) and apoptosis (externalization of phosphatidylserine). Annexin-V-FLUOS kit (Roche Applied Science, Germany) was used following the manufacturer's instructions [11]. Briefly, protoplasts of *C. albicans* cells were treated with different concentrations of SK, as mentioned in above section, and 10 mM H_2O_2 was used as a positive control. Then these preparation protoplasts were resuspended and incubated at 25°C for 15 minutes by 5 uL propidium iodide (PI) and FITC-labeled annexin-V. After incubation, 100 µL binding buffer was added to resuspend each sample, then which was analyzed using the flow cytometry (FACSCalibur, USA). The detection conditions are as follows: Annexin V-FITC: excitation wavelength 488 nm, emission wavelength 518 nm; PI: excitation wavelength 488-540nm, emission wavelength 617 nm.

2.3 Transmission Electron Microscope (TEM) [12]

The *C. albicans* SC5314 (3×10^7 CFU / mL) were firstly administrated by 4 μg / mL SK for 2 h at 37°C, and then harvested by centrifugation (3000 g, 5 min). The collected cells were promptly placed in 3% glutaraldehyde at 4°C overnight, fixed with 1% (w/v) osmium acid for 1 h. After gradient dehydration with ethanol and acetone, it was immersed, embedded, polymerized, and sliced. Finally, thin sections were prepared and stained by uranyl acetate for 2 h, being observed under Hitachi H-800 transmission electron microscope.

2.4 Cytochrome c oxidase (COX) activity

Firstly, *C. albicans* mitochondria of the cells were separated by Cell Mitochondria Isolation Kit (Beyotime). Then, COX detection kit (GENMED) was used to detect mitochondrial COX activity [13]. All experiments were performed according to the manufacturer's instructions. Briefly, protoplasts of *C. albicans* cells were treated with 1, 2, 4 μg / mL SK as mentioned in above section, and 10 mM H_2O_2 was used as a positive control. Then these preparation protoplasts were homogenized in order to release the mitochondria. After centrifugation at a speed of 10 000 × g for 10 min at 4°C, the supernatant was discarded, and the precipitation particles (mitochondrial precipitate) were resuspended in 200 uL of storage solution. Protein concentration was determined by the TCA Lowry method [14]. After that, the mitochondrial COX activity need be determined. Firstly, these prepared mitochondrial samples were melted in ice. Secondly, set the spectrophotometer parameters: temperature 25°C, wavelength 550 nm, reading at 0s and 60 s each time. Thirdly, 850 μL buffer solution and 50 μL reaction working solution were added into the colorimetric cup, then which was detected for the background reading (0 s reading – 60 s reading) by spectrophotometer. Fourthly, added another 100μL sample (complete mitochondria containing 2 μg mitochondrial protein) into the foregoing colorimetric cup for detecting the sample reading (0 s reading – 60 s reading). Finally, calculation of sample activity: (sample reading - background reading) / [0.1 (sample volume, mL) × 21.84 (molar absorptivity)] = Unit / mL or $\mu mol \cdot min^{-1} \cdot mL^{-1}$.

2.5 Real time RT-PCR

RNA isolation and real-time RT-PCR were performed as described previously [9]. Total RNA was extracted according to the instructions of *C. albicans* RNA extraction kit (TIANZ, Beijing, China). Isolated RNA was resuspended in diethyl pyrocarbonate-treated water. The OD260 and OD280 were measured. First-strand cDNA was obtained using the cDNA synthesis kit (TaKaRa Biotechnology, Dalian, China) for RT-PCR according to the manufacturer's instructions. Real-time PCR was performed using the 7500 Applied Biosystems. SYBR green I (TaKaRa Biotechnology, Dalian, China) is used for real-time monitoring of amplification products. *CaMCA1* was amplified with the forward primer 5-TATAATAGACCTTCTGGAC-3 and the reverse primer 5-TTGGTGGACGAGAATAATG-3.

The PCR protocol consisted of denaturation program (95°C for 10 s); 40 cycles of amplification and quantification program: 95°C for 10 s (denaturation), 60°C for 20 s (annealing), 72°C for 30 s (extension);

melting curve program: 60–95°C with a heating rate of 0.1°C per second; finally, a cooling step to 40°C. The changes in SYBR Green I fluorescence in every cycle were monitored by the LightCycler system software, and the threshold cycle (*C*T) above background for each reaction was calculated. The *C*T value of 18S ribosomal RNA (amplified with the forward primer 5-TCTTTCTTGATTTTGTGGGTGG-3 and the reverse primer 5-TCGATAGTCCCTCTAAGAAGTG-3) was subtracted from that of the tested gene to obtain a Δ*C*T value. The Δ*C*T value of an arbitrary calibrator (*e.g.*, an untreated group) was subtracted from the Δ*C*T value for each sample to obtain a ΔΔ*C*T value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$. Triplicate experiments were conducted to generate a mean value.

2.6 Assessment of Caspase Activity

Caspase activity was detected by the CaspSCREEN Flow Cytometric Apoptosis Detection Kit (BioVision, USA) [15]. The kit used dye D_2R (rhodamine 110 containing two aspartic acid residues). The experimental procedures are as follows: protoplasts of *C. albicans* cells were treated with 1, 2, 4 μg / mL SK as mentioned in above section, then were resuspended in D_2R staining solution (296 μL incubation buffer, 3μL 1M DTT and 1μL D_2R reagent) at 30°C for 45 min before viewing and counting under a fluorescence microscope.

2.7 Statistical analysis

Data were analyzed using SPSS 19.0 (IBM Corp.) and presented as the mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

1. SK concentration-dependently induces *C. albicans* cells death

C. albicans SC5314 cells treated with SK at different concentrations were analyzed by double staining with annexin V-FITC and propidium iodide (PI). Annexin V-FITC was used to detect the apoptotic cells with externalized PS, whereas PI staining was used to detect necrotic cells.

As shown in Fig. 1, after being treated with 2 μg/ml SK, the percentage of death cells was 30.51% (17.42% as apoptosis and 13.09% as necrosis). When the concentration of SK increased to 4 μg / mL, the proportion of death cells was about 60.82% (34.12% as apoptosis and 26.70% as necrosis). When the concentration of SK reached 8 μg / mL, the proportion of death cells further increased (most cells were necrosis). The negative control (unstimulated cells) resulted in apoptosis (5.50%) and necrosis (4.73%), whereas positive control (10 mM H_2O_2) showed apoptosis (51.43%) and necrosis (19.75%). The results

also show that cell death was dose-dependent with a significant increase in the percentage of apoptosis and necrosis compared to unstimulated control.

2. Effect of SK on the ultrastructure changes of *C. albicans*

The effect of SK on the ultrastructure changes of *C. albicans* SC5314 cells was observed by transmission electron microscopy (TEM). As shown in Fig. 2, compared with the untreated control, the cells exposed to 4 μg/ml SK showed extensive chromatin condensation and fragmentation along the nuclear envelope, a leading mark of cellular apoptosis. Cells treated at high fungicidal concentration of SK (8 μg/ml) showed extensive granular cytoplasm with little evidence of an organized internal structure, and nuclear bodies were only rarely observed, a leading mark of cellular necrosis.

3. SK concentration-dependently reduced *C. albicans* mitochondrial COX activity

Mitochondrial COX activity is an important indicator for the evaluation of cell function. In this study, the mitochondrial COX activity of *C. albicans* cells was determined by the transformation of reduced cytochrome c into oxidized cytochrome c.

The results showed that the enzyme activity was significantly decreased in a concentration-dependent manner after exposure of *C. albicans* SC5314 cells to various concentrations of SK compared to the unstimulated control. As shown in Fig. 3, the enzyme activity was reduced to 74.54%, 50.48%, and 24.91% at concentration of 1, 2 and 4 μg/mL SK, respectively. As a positive control, the COX enzyme activity of the 10 mM H_2O_2 treatment group was 11.09% of the unstimulated control.

4. SK concentration-dependently improved caspase enzyme activity and *CaMCA1* gene transcription level in *C. albicans*

Since *C. albicans* caspase and its encoding gene *CaMCA1* has been found to play an important role in apoptosis, here we investigated the caspase activity by staining the cells with D_2R and the staining rate can reflect the activation of caspase in cells. As shown in Fig. 4A, after exposure of *C. albicans* SC5314 strains to various concentrations of SK for 2 h, an increase in caspase activity was observed in a dose dependent manner. Briefly, the cells staining rates in the 1 μg / mL, 2 μg / mL and 4 μg / mL SK treatment groups were 11.3%, 19.57% and 33.80% respectively, significantly higher than the unstimulated control (5.2%). As the positive control, the cell staining rates in the 10 mM H_2O_2 treatment group were 37.50%.

In addition, the transcription of caspase activity-related gene *CaMCA1* in *C. albicans* was studied by real time RT-PCR. As shown in Fig. 4B, the expression levels of *CaMCA1* in different concentrations of SK-treated groups were increased in a concentration-dependent manner.

Discussion

Apoptosis refers to one of main pathways of programmed cell death under certain physical and pathological conditions, following the activation of extrinsic or intrinsic death signals regulated by genes. It is a common process in eukaryotes [16]. Inducing or accelerating tumor cell apoptosis has become an important strategy in anti-tumor therapy [17]. In recent years, it has been reported that some natural products of traditional Chinese medicine, such as resveratrol, baicalein and pterostilbene, can induce the apoptosis of *C. albicans* at a suitable concentration, suggesting that inducing apoptosis of pathogenic fungi is expected to become a potential mean in clinical therapy of fungal infection [18].

In this study, the effect of SK on *C. albicans* apoptosis was investigated. The results suggested that *C. albicans* SC5314 strains after being exposed to different concentrations of SK exhibited concentration-dependent effect on the classical traits of yeast apoptosis such as PS externalization, chromatin condensation, decrease in cytochrome c oxidase activity as well as increase in caspase activity and mRNA expression of the gene encoding *C. albicans* caspase, *CaMCA1*.

We first analyzed the proportion of apoptotic cells, necrotic cells, and living cells by Annexin V-FITC / PI double staining, which is the most direct method to detect apoptosis. The results showed that apoptosis occurred in *C. albicans* cells treated with a low concentration of SK, and the proportion of apoptotic and necrotic cells gradually increased with the increase of SK concentration. In addition, compared to the unstimulated control, the chromatin of *C. albicans* cells with 4 μg / mL SK treatment showed obvious condensation and fragmentation along the nuclear envelope, further suggesting that SK could be an inducer of apoptosis in *C. albicans*. However, to our surprise, at higher concentration of SK (8 μg / mL), most cells were in necrosis. It suggested high concentration of SK exert direct cytotoxic effect leading to necrotic rather than apoptotic cell death, which could be the reason of rapid and irreversible antifungal activity of SK and can be correlated to the ROS accumulation-induced internal structural and functional damage of many organelles [11, 19].

It is well known that apoptosis can be activated by two main signaling pathways: the death receptor-mediated extrinsic apoptosis pathway and the mitochondrial-mediated (cytochrome c, caspase) intrinsic apoptosis pathway [20]. Because SK is a lipophilic quinone natural compound [21]. it is very important to pay attention to the intrinsic pathway rather than extrinsic pathway, which is the classical pathway of apoptosis. Previously, our study found that SK could significantly reduce mitochondrial membrane potential and ATP level, as well as increase production of ROS in *C. albicans* [9]. In this study, mitochondrial COX and caspase activity were further measured to understand the effect of SK on the mitochondrial pathway. Mitochondria has been reported an important cell organelle and play a key role in the physiological of cell death [22]. When apoptotic signaling molecules act on mitochondria, mitochondrial permeability changes, membrane potential being decreased, resulting in a large amount of ROS, then cytochrome c being released into the cytoplasm [23–24]. The release of cytochrome c from mitochondria to cytoplasm is directly related to the decrease of cytochrome c oxidase activity [25]. After cytochrome c being released from the mitochondria to cytoplasm, yeast metacaspase Yca1p (it is the

only caspase that plays an important role in yeast apoptosis, and it is also an ortholog of mammalian caspases) is activated, thereby in turn resulting in the activation of caspase cascade and inducing apoptosis [26]. Our results demonstrated significant decrease in COX activity in *C. albicans* after treatment with different concentrations of SK. In addition, the caspase activity and the expression of related gene *CaMCA1* increased. Previous studies have found that *CaMCA1* is a homologous of metacaspase *YCA1* in *Saccharomyces cerevisiae*. As the only gene responsible for caspase activity in *C. albicans*, *CaMCA1* is involved in H_2O_2-induced apoptosis [27].

In addition to the mitochondrial pathway, the Ras-cAMP-PKA signaling pathway can also mediate the yeast apoptosis [28]. Our study found that SK could affect *C. albicans* biofilm formation by inhibiting the Ras-cAMP-PKA pathway (data not shown). Therefore, whether the apoptosis of *C. albicans* induced by SK is also related to the Ras-cAMP-PKA pathway remains to be further studied.

In summary, this study reported for the first time that SK could induce apoptosis of *C. albicans*, showing some typical apoptosis characteristics including of PS externalization and chromatin condensation. It is related to the accumulation of intracellular ROS, the decrease of mitochondrial membrane potential, the release of cytochrome c, and the increase of caspase activity caused by mitochondrial injury. This study provides an effective laboratory basis for SK in the clinical treatment of *C. albicans* infection and further enriches the theory of Chinese traditional medicine antifungals.

Declarations

Acknowledgement

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Chong Pang, Jianshuang Chen, Shuangyan Liu and Hao Miao. The first draft

of the manuscript was written by Hao Miao and Chong Pang, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Ethics approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent: Not Applicable.

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Figures

Figure 1

Effect of various concentrations of SK on cell apoptosis in *C. albicans* SC5314. Untreated cells and cells exposed to H_2O_2 (10 mM) were used as negative and positive controls respectively. (A) The fluorescence was determined by flow cytometer using annexin V-FITC and PI double staining. Fluorescence showing healthy (lower left) annexin (-) PI (-) protoplasts, apoptotic (up right + lower right) annexin (+) PI (-/+) protoplasts, and necrotic (up left) annexin (-) PI (+) protoplasts. (B) Percentage of cells showing apoptosis and necrosis. The experiment was repeated at least 3 times. Data represent the mean ± SD.

Figure 2

Electron microscopy images of *C. albicans* SC5314 cells treated with different concentrations of SK. Black arrow marks chromatin condensation and fragmentation representing apoptosis; White arrow marks organellar swelling and membrane disintegration representing necrosis. (Scale bar:1 μm)

Figure 3

Effect of various concentrations of SK on mitochondria COX activity in *C. albicans* SC5314. Untreated cells and cells exposed to H_2O_2 (10 mM) were used as negative and positive controls respectively. The sample of untreated control was regarded as 100%. These data were mean ± SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control.

Figure 4

Effect of various concentrations of SK on caspase activity in *C. albicans* SC5314. Untreated cells and cells exposed to H_2O_2 (10 mM) were used as negative and positive controls respectively. (A) Percentage of cells with activated caspases staining with D_2R. (B) Transcription levels of *CaMCA1* were determined by real-time RT-PCR. The mRNA levels were normalized on the basis of their 18S rRNA levels. Gene expression was calculated as the fold increase relative to the control group. These data were mean ± SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$ compared with the control.