Study on the inhibitory activity and possible mechanism of myriocin on clinically relevant drug-resistant *Candida albicans* and its biofilms

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Abstract: Objective: In order to prevent and control the infection of *Candida albicans*, the antifungal activity, possible mechanism of myriocin against *C. albicans* and its biofilm were studied. Methods: The antifungal activity of myriocin was investigated by microdilution method. The effect of myriocin on fungal cell wall or membrane was evaluated by adding sorbitol, ergosterol or phytosphingosine (PHS). The damage to the cell membrane was investigated with propidium iodide (PI) staining and visualized by Scanning electron microscope (SEM). The effects on biofilms and extracellular polysaccharides (EPS) were observed by crystal violet staining method and phenol-sulfuric acid method respectively. The adhesion of *C. albicans* cells to hydrocarbons was tested to evaluate cell surface hydrophobic (CSH). The combined effects of myriocin and antifungal drugs commonly used in clinical practice were investigated by using the checkerboard microdilution method. Results: MICs were found to be 0.125~4 µg/ml. Myriocin was found to affect both cell wall and cell membrane. After exposure to myriocin, biofilm and EPS were found to be inhibited and removed, and the CSH was decreased. The combined fungistasis of myriocin and voriconazole (VCZ) or amphotericin B (AMB) were additive. Conclusion: Myriocin had significant antifungal activity against *C. albicans*, and the antifungal mechanisms might be cell wall and membrane damage. Myriocin effectively inhibited and eliminated biofilms, and its mechanism may be related to the inhibition of EPS and CSH.

Keywords: myriocin; *Candida albicans*; antifungal activity; biofilm
1. Introduction

In recent years, the incidence of fungal infection has increased sharply, because of the widespread using of broad spectrum antibiotics, indwelling medical devices and more immuno-compromised patient population. Studies have shown that *C. albicans* is the most common fungi species of Vulvovaginal Candidiasis (VVC), Candidemia, and invasive Candida infections (IC). \(^1\)-\(^3\) The five major antifungal agents used in the treatment of *C. albicans* infection are azoles, allylamines, echinocandins, polyenes and nucleoside analogues. According to a Japanese study, the trend of antifungal drugs use for invasive mycoses increased between 2006 and 2015. \(^4\) With the long-term and extensive use, clinical antifungal agents show many limitations, such as high drug resistance rate, high hepatorenal toxicity and so on. The mechanisms of drug resistance of *C. albicans* include overexpression of efflux pump gene, mutation and overexpression of target protein and biofilm formation. \(^5\) Biofilm is a complex structure composed of associated microbial cells, attached to the surface of body's tissues or biological material and restricted in the extracellular polymeric substances which are mainly composed of polysaccharides. Meanwhile, it provides many benefits for microorganism, such as controlling the flow of nutrients, protecting self from host immune system and antimicrobial agents. \(^6\), \(^7\) Biofilms present great difficulties to clinicians. Once biofilms are formed, it may even be necessary to treat IC by removing implanted medical devices and using high doses of antifungal drugs. \(^8\) However, many severe patients cannot tolerate these treatments.

Myriocin is also called 2S,3R,4R,6E,2-amino-3,4-dihydroxyl, and Fig 1 shows its molecular formulas. Myriocin has dual activity, anti-inflammatory and antifungal activity. \(^9\) It is a compound isolated from the filtrate and mycelium of *Myriococcus albomyces* by Kluepfel et al., \(^10\) and is also present in the secondary metabolites of Cordyceps and Cordyceps cicadæ. \(^11\), \(^12\) The Fingolimod (FTY720), chemically modified by myriocin, has been listed as an immunosuppressant. In addition, myriocin can inhibit the proliferation of tumor cells. \(^13\), \(^14\) It is reported that the minimal inhibitory concentration (MIC) of myriocin against *Cryptococcus neoformans* and
Cryptococcus germicus is in the range of 0.48~1.95 μg/ml, and the MIC of Fusarium oxysporum is 1.25 μg/ml. Based on the antifungal activity of myriocin in vitro, which is comparable to the clinical antifungal drug, we urgently want to know the inhibitory activity against the clinically isolated strains.

In this study, several common clinical pathogenic fungi were screened for the antifungal activity of myriocin, and compared with the clinical antibiotics. The fungistatic mechanism of myriocin on C. albicans CAU-01, isolated from the urine of patients with urinary tract infection, was discussed systematically. The dynamic process of cell membrane damage by myriocin was revealed. On the other hand, crystal violet staining method was used to evaluate the inhibition and clearance effect of myriocin on biofilm, and its mechanism was investigated by investigating the effect of myriocin on extracellular polysaccharides (EPS) and cell surface hydrophobic (CSH).

2. Materials and Methods

2.1. Drugs and reagents

Myriocin (≥98%), voriconazole (VCZ, ≥98%), amphotericin B (AMB, 80%), sorbitol (98%) and ergosterol (≥95%) were purchased from the Aladdin biochemical technology Co., Ltd. (Shanghai, China). Phytosphingosine (PHS, 95%) was acquired from Jiangsu Aikon Biopharmaceutical R&D Co., Ltd. (Nanjing, China). Sodium chloride injection (NS, 10 ml:90 mg) was acquired from Hebei tiancheng pharmaceutical Co., Ltd. (Hebei, China). All other chemicals (methanol, dimethylsulfoxide, glutaraldehyde) were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd.. Potato dextrose agar (PDA) medium and Potato dextrose broth (PDB) medium were purchased from Hope biotechnology Co., Ltd. (Qingdao, China).

2.2. Fungal strains and culture condition

C. albicans (CAU-01, CAU-02, CAB-03, CAB-04, CAS-05, CAS-06, CAV-07, CAV-08), Candida tropicalis (CTS-01, CTS-02, CTV-03, CTV-04), Candida krusei (CKW-01, CKW-02), Candida glabrata (CGU-01, CGU-02), Candida parapsilosis
(CPU-01, CPU-02), Cryptococcus neoformans (CNB-01, CNB-02) were clinical isolated strains provided by the clinical laboratory of Wuxi No. 2 People's Hospital. Strains were isolated from the patients' blood, urine, sputum, vaginal secretions or wound secretions (serial description: the first two letters were fungal initials; the third letter was the strain source, B represented blood, U represented urine, S represented sputum, V represented vaginal secretion, W represented wound secretion), among which CAU-01, CAB-04, CTS-01 were VCZ-resistant strains, CAU-02 and CAB-03 were VCZ-intermediate strains. All strains were identified by automatic microbial mass spectrometry detection system (bioMerieux, VITEX MS, France). In addition, there were standard strains purchased from Beina Chuanglian Biotechnology Co., Ltd.: C. albicans BNCC186382 and C. parapsilosis BNCC336515. In this study, CAU-01 was selected to investigate the mechanism of myriocin, aiming the clinically relevant drug-resistant C. albicans.

These strains were stored at –80°C in PDB containing 50% glycerol. Cells were defrosted on ice and then inoculated and activated in PDA plate and cultured at 35°C for 24h (Cryptococcus neoformans for 48h). Single colony of uniform size was taken in 50 ml PDB medium and incubated at 35°C in 150 rpm shaker for 24h (Cryptococcus neoformans cultured for 48h).

2.3. MIC and minimal fungicidal concentration (MFC)

As previously reported with minor modifications\textsuperscript{17,18}, the logarithmic fungi were diluted with PDB to 10\textsuperscript{1-4} cfu/ml. Myriocin solution and AMB solution were two-fold diluted from 32 µg/ml to 1/64 µg/ml, and VCZ solution was from 128 µg/ml to 1/16 µg/ml. Then, 100 µl fungal solution were added in each well and incubated with myriocin at 35°C for 24h (Cryptococcus neoformans for 48h). 100 µl PDB instead of drug solution was used as the blank control and 200 µl PDB without fungal solution as culture medium control. The absorbance value of each hole was read at 625 nm on the enzyme meter. The calculation method of inhibition rate is shown in formula (1).

\[
\text{Inhibition rate } \% = 1 - \frac{OD_{\text{dosing well}} - OD_{\text{culture medium}}}{OD_{\text{blank control}} - OD_{\text{culture medium}}} \times 100\%
\] (1)
MIC was defined as the lowest drug concentration capable of inhibiting 80% of the growth of strain. The evaluation criteria for antimicrobial activity was: MIC < 100 µg/ml was considered as good/significant antimicrobial activity, 100 µg/ml ≤ MIC < 500 µg/ml was medium activity, 500 µg/ml ≤ MIC < 1000 µg/ml meant weak activity, and MIC ≥ 1000 µg/ml was inactive. 

To determine MFC, 100 µl from the wells with no fungal growth and the blank control group were seeded in PDA plate, which was then incubated at 35°C for 48h. MFC was defined as the minimum concentration with no more than 3 cfu in the plate.

2.4. Sorbitol assay

As an osmotic protective agent, sorbitol can stabilize fungal protoplasts. In a medium containing sorbitol, the antifungal activity of compounds that specifically inhibit fungal cell walls can be reversed. As previously reported with appropriate modifications, the microdilution method described above was used to determine MIC in two groups: one group was added sorbitol (the final concentration was 0.8 mol/L) to the medium, and the other group was not added. AMB was used as the negative control. The plates were sealed under aseptic conditions, cultured at 35°C, and read 48h later to compare whether there was any difference in MIC.

2.5. Ergosterol and PHS assay

Ergosterol is a major sterol component in fungal plasma membrane, which plays the same role in fungal plasma membrane as cholesterol in mammalian membrane. Referring to the experimental method of LeiteMCA et al. with appropriate modification, ergosterol was ground and dissolved in dimethyl sulfoxide (DMSO) and Tween 80, and the mixed emulsion was poured into PDB and then sterilized under high pressure. The ergosterol group and the non-ergosterol group were set up by microdilution method. The final concentration of ergosterol was 200 µg/ml, and AMB was used as the positive control. The plates were sealed under aseptic condition, cultured at 35°C, and read 48h later to compare whether there was any difference in MIC.
Sphingolipids (SPLs) are also important components of the membrane of fungal cells and have important signal transduction functions, playing a key role in regulating the formation of membrane. Although the disruption of SPLs biosynthesis can cause fatal damage to fungal cells, exogenous addition of PHS or dihydrosphingosine (DHS) can restore fungal growth. Referring to the ergosterol assay, the experiments were repeated by replacing ergosterol with PHS at 5 μmol/L.

2.6. Flow cytometry (FCM) analysis

Referring to the method of Jiang Q et al. with appropriate modifications, fungal cells were stained with PI (BBI Life Science, Shanghai, China) to evaluate membrane permeability. The logarithmic fungi (10^5-6 cfu/ml) were incubated with myriocin at 35°C. NS was the blank control. Cells were collected by centrifugation and then suspended with PBS. The cells were incubated with 5 μl PI in dark for 30min at room temperature. Then the cells were centrifuged and excess stains were removed with PBS. The samples were analyzed by FACSAtia II (BD Biosciences, USA) within 1h. Argon laser (488 nm) was used to excite the PI, and the red fluorescence emitted by PI was collected at 585 ± 21 nm. At least 10,000 cells were detected for each sample.

2.7. Scanning electron microscope (SEM) analysis

As previously reported but minor exception, fungi suspension was inoculated in PDB with myriocin (2 μg/ml) at 35°C for 3h, 6h and 12h. NS was substituted for myriocin as the control. Then the cells were centrifuged at 4,000 rpm for 10min and washed with NS twice. After washing, the cells were suspended in 2.5% glutaraldehyde and fixed overnight at 4°C. Then samples were washed twice with NS for 5min and centrifuged at 3000 rpm for 5min to collect thallus. We dehydrated them by different concentrations of ethanol (50%, 80%, 100%) gradient, each time for 10min, and then soaked samples in 100% ethanol at 4°C for 10min. Samples were then dried in a vacuum freeze dryer for 1h. Finally, the sample was attached to the conductive adhesive and fixed on the copper sample table, and then, gold was
sputtered under vacuum. SEM (su1510, Hitachi, Japan) was used to observe the morphology of *C. albicans* at an acceleration voltage of 5 kV.

2.8. Effects on biofilm

Through two groups of experiments, the inhibitory and eradicating effects of myriocin on *C. albicans* biofilm were evaluated. Fungi suspension ($10^{4-5}$ cfu/ml) at logarithmic stage and myriocin with different final concentrations (0.1 µg/ml, 0.05 µg/ml) were cultured in a 96-well plate at 37°C for 24h. NS was the blank control. The solution was discarded, and each well was lightly washed with PBS (pH=6.5) twice. After drying at 60°C, 200 µl of 0.1% crystal violet solution was added to each hole for staining at room temperature for 5min. Excess stain was removed by PBS, and the plate was dried at 37°C. Each well was soaked with 200 µl 33% glacial acetic acid for 10min. Then the biofilms were quantified with value of OD$_{595nm}$.

The logarithmic fungi were diluted with PDB to $10^{3-4}$ cfu/ml and then cultured in a 96-well plate at 37°C for 24h. After removing the planktonic fungi with PBS, different concentrations of myriocin (1 µg/ml, 0.5 µg/ml, 0.25 µg/ml) were added to the plate and incubated at 37°C for 4h. NS was the blank control. The next steps were the same as the inhibition experiment.

2.9. Effects on EPS

The effects of myriocin on EPS were also studied through inhibition and clearance experiments. *C. albicans* ($10^{5-6}$ cfu/ml) were inoculated into PDB and co-cultured with different concentrations of myriocin (0.1 µg/ml, 0.05 µg/ml, 0) at 37°C for 24h. The supernatant was removed by centrifugation at 4,000×g for 10min. To remove precipitation and plankton, the tubes were washed gently with PBS, and then dried naturally. The supernatant were poured back into these tubes. The EPS in the supernatant was precipitated with ethanol (80%) overnight at 4°C, and then collected by centrifugation at 8,000×g for 20min. In the eradication experiment, *C. albicans* was incubated under the same conditions without myriocin. After the removal of planktonic fungi, different concentrations of
myriocin (1 µg/ml, 0.5 µg/ml, 0.25 µg/ml, 0) were added to co-culture at 37°C for 4h. Then the EPS precipitation was obtained by the previous method. Referring to previous reports, EPS was quantified by phenol-sulfuric acid method. Each tube containing precipitation was added with water to 2 ml. Then 1 ml of 5% phenol solution was added to each tube and thoroughly shaken. After that, 5 ml sulfuric acid was slowly added to the test tube, which was immediately mixed thoroughly and placed in a water bath at 40°C for 15min. Finally, the tubes were removed and quickly cooled to room temperature. The eradication rate could be calculated by the value of OD\(_{490}\)nm.

2.10. Effects on CSH

CSH plays an important role in the pathogenesis of opportunistic fungal pathogen \(C.\ \textit{albicans}\). Compared with hydrophilic cells, hydrophobic cells show stronger adhesion to epithelial, endothelial, and extracellular matrix proteins, appear to be more resistant to phagocytic cell killing, and are more virulent in mice. In this experiment, the adhesion of \(C.\ \textit{albicans}\) cells to hydrocarbons was tested to evaluate CSH. According to the method of Souza et al. but appropriate modifications, the logarithmic phase fungi suspension was co-cultured with different concentrations of myriocin (0.1 µg/ml, 0.05 µg/ml) at 37°C and 200 rpm for 24h. PDB instead of myriocin was used as the blank control. The culture medium was discarded, and the precipitate was washed with NS. The cells were suspended with NS until the absorbance at 520 nm was about 0.5. This absorbance value was considered C\(_{0}\). In each group, the fungi suspension 4 ml was taken into the test tube, and then 1 ml toluene was added. The test tube was bathed in water at 37 °C for 10min, then shaken for 30sec, and then bathed 30min, finally shaken thoroughly. After 15min of rest, the two phases were separated, the aqueous phase was transferred to a clean test tube and measured at 520 nm. This absorbance value was considered C\(_{1}\). The value of CSH was calculated as formula (2).

\[
\text{CSH} = \frac{C_{0} - C_{1}}{C_{0}}
\]

2.11. Combined fungistasis
According to the MIC determined in the above experiments, this test was carried out by checkerboard microdilution method. Fungi solution (100 µl, 10\(^{3-4}\) cfu/ml) and different concentrations (half diluted) of two antifungal agents were added to the wells of the 96-well plates. The final concentration of myriocin in rows was from 1 to 1/64 µg/ml, and the final concentration of VCZ in columns was from 64 to 1/32 µg/ml. 100 µl PDB instead of drug solution was used as the blank control and 200 µl PDB without fungal solution as culture medium control. The plates were incubated at 35°C for 24h. The absorbance value of each hole was read at 625nm on the enzyme meter.

The experiments were repeated by replacing VCZ with AMB (the final concentration was from 8 to 1/256 µg/ml).

The combined fungistasis of two drugs was elucidated by the fractional inhibitory concentration index (FICI). The formula is as follows:

\[
FICI = \frac{MIC_{drugA in combination}}{MIC_{drugA alone}} + \frac{MIC_{drugB in combination}}{MIC_{drugB alone}}
\]  

(3)

FICI ≤ 0.5 was considered as synergistic, 0.5 < FICI ≤ 1 meant additive, 1 < FICI ≤ 2 was defined as irrelevant, and FICI > 2 was classified as antagonistic.

2.12. Statistical analysis

All experiments were repeated at least 3 times. The SPSS 23 was used to perform statistical analyses by means of independent one-way ANOVA tests. The Dunnett's test was employed to compare between groups. The p value less than 0.05 meant the difference statistically significant.

3. Results

3.1. Antifungal activity of myriocin

The antifungal activity against 22 strains of 6 kinds of fungi were determined, and the MIC values of myriocin, VCZ and AMB are listed in Table 1. The results showed that the MICs of myriocin against these strains were 0.125~4 µg/ml. Among C. albicans strains, the MIC of myriocin against CAU-01 was significantly lower than that...
of CAB-03, CAS-06 and CAV-07 (*p < 0.05). At low concentration, myriocin could significantly inhibit the growth of all experimental strains, and its fungistatic activity was close to that of AMB, a classic polyenes antifungal drug, and its antifungal activity against drug-resistant and intermediate strains was better than that of VCZ, which is commonly used in clinic.

MFC was determined by plate culture, and CAU-01 and BNCC 186382 were the test strains. No colony growth was observed when the concentration was 4 μg/ml, so the MFC of myriocin against *C. albicans* CAU-01 and BNCC 186382 was 4 μg/ml.

3.2. Effects on cell wall and cell membranes

3.2.1. Changes in MIC after addition

As shown in Table 2, the MIC value of myriocin in the presence of sorbitol increased twice, indicating that myriocin could affect the synthesis of cell wall of *C. albicans* to some extent. The MIC values of myriocin in the presence and absence of ergosterol were 0.5 μg/ml and 8 μg/ml, respectively, against CAU-01. And the positive control (AMB) used in this assay showed MIC values of 0.5 μg/ml and 64 μg/ml. These results indicated that myriocin has affinity for ergosterol, but the affinity was weaker than that of AMB. Besides, the MIC value of myriocin in the presence of PHS significantly increased by more than 128 times, which was much higher than that of ergosterol. It can be seen that myriocin might exhibit antifungal effect mainly by blocking the biosynthesis of SPLs.

3.2.2. FCM analysis

In order to illustrate the dynamic changes of membrane more intuitively, we drew Fig 2 according to the ratio of fluorescent cells (Supplementary Material: Fig S1 and Fig S2). As shown in Fig 2a, the percentage of fluorescence cells were 4.6% at 0.25 μg/ml during the test time (3h), while the proportion of fluorescent cells increased to 25.70% and 32.27% at 0.5 μg/ml and 1 μg/ml respectively. Compared with the untreated group, fluorescent cells increased significantly after exposure to 0.5 μg/ml or 1 μg/ml myriocin for 3 hours (*p < 0.05). As shown in Fig 2b, the proportion of
stained cells gradually increased with increasing exposure time at 0.5 µg/ml. It takes 120 min and 180 min to increase the ratio of fluorescent cells to 14.10% and 28.57%, respectively. The above results indicated that the damage of the membrane by myriocin leads to entering of PI to cells. The degree of damage was positively correlated with the concentration and exposure time. The destruction of cell membrane is one of the mechanisms of myriocin for inhibiting *C. albicans*.

3.2.3. SEM analysis

The effects of myriocin at 2 µg/ml on the cell surface of *C. albicans* CAU-01 were observed via SEM. The results are shown in Fig 3. The untreated control group cells presented smooth surface and complete, uniform near-spherical structures (Fig 3A and Fig 3a). After exposure for 3 h, the cell surface became rough (Fig 3B and Fig 3b). Six hours later, some cells appeared uneven and irregular spherical structures with obvious bulges and collapses (Fig 3C and Fig 3c). The extent of cell shrinkage was further increased after exposure for 12 h, with saclike protuberant and exudation of contents, and some cells could no longer maintain the spherical shape (Fig 3D and Fig 3d). It can be seen that the surface roughness increased as the increase of treatment time.

3.3. Inhibition and clearance effects on biofilm

Crystal violet staining was used to quantify biofilm and to evaluate the inhibition and clearance effects of myriocin on biofilm of *C. albicans*. From the data of growth curve (Supplementary material: Fig S3), it can be seen that myriocin at 1/5 MIC and 1/10 MIC hardly affect the growth of *C. albicans* CAU-01. Therefore, in order to ensure that myriocin inhibited biofilm formation is not related to its growth inhibition effect, we selected these two concentrations to do the experiments. As shown in Fig 4, the inhibition rate and clearance rate increased with the increase of the concentration. The inhibition rates reached 47.00% and 14.01% at 0.1 µg/ml and 0.05 µg/ml respectively, suggesting that myriocin at 0.1 µg/ml could effectively inhibit the formation of biofilm (Fig 4a). The clearance rates on mature biofilms at 0.25 µg/ml, 0.5
µg/ml and 1 µg/ml were 30.38%, 41.50% and 55.25%, respectively, indicating that myriocin played a certain role in the clearance of mature biofilm (Fig 4).

3.4. Inhibition and clearance effects on EPS

Phenol-sulfuric acid method was used to measure EPS and to evaluate the inhibition and eradication effects of myriocin on EPS of biofilm. At 0.05 µg/ml and 0.1 µg/ml, the inhibitory rates reached 21.37% and 40.94%, respectively, indicating that myriocin at 1/5MIC could effectively inhibit the secretion of EPS (Fig 5a). The eradication rate on EPS secreted by mature biofilms reached 54.75% at 1 µg/ml (Fig 5b), suggesting that myriocin had certain eradication effect on EPS.

3.5. Reduce CSH

As shown in Fig 6, the CSH of the blank control group was 0.2479, and it decreased rapidly to 0.1092 after exposure to myriocin at 0.1 µg/ml. The CSH of *C. albicans* treated with myriocin at 1/5MIC was significantly lower than that of the blank control group (*p < 0.05*). The above results indicated that myriocin could reduce the adhesion of *C. albicans*.

3.6. Combined fungistasis

The results of myriocin combined with VCZ or AMB against *C. albicans* CAU-01, CAB-04 and BNCC186382 are shown in Table 3. The FICI values of the two combinations were between 0.5 and 1, indicating that the two combinations had additive combined fungistatic effect on *C. albicans*. Similar results were observed in *C. tropicalis* CTS-01 and *C. glabrata* CGU-01 (Supplementary Material: Tab S1).

4. Discussion

*C. albicans* is one of the most common clinical conditional pathogenic fungus. There are few antifungal drugs available clinically, and with the long-term and extensive use of these drugs, the detection rate of clinical drug-resistant strains...
increases gradually. Therefore, it is particularly urgent to develop new products, especially against clinical drug-resistant strains.

The MICs of myriocin against all experimental strains were 0.125–4 μg/ml. At low concentrations, it could significantly inhibit the growth of all experimental strains, including clinical drug-resistant strains. In recent years, scientists have been looking for new antifungal substances in response to the problem of fungal drug resistance. More and more phytoconstituents have been proved their antifungal activity, such as carvacrol, citral, geraniol, citronellal, etc. \(^{19, 20, 23, 34}\) Compared with them, the MIC value of myriocin was much lower. Xu et al. \(^{16}\) found that the cell-free supernatant from *Bacillus amyloliquefaciens* LZN01 had strong antifungal activity, and its main antifungal component was myriocin. Combined with our results, it is suggested that myriocin is a promising antifungal substance with significant antifungal activity.

Although the infection rate of Non-*Candida albicans* (NCA), especially *C. glabrata* and *C. tropicalis*, has a significant upward trend, *C. albicans* still plays an important role in the fungi causing nosocomial urinary tract infection. \(^{35, 36}\) Only drugs that are metabolized to produce active drugs and reach sufficient concentrations in urine can be used to treat urinary tract infections, such as fluconazole. So other azoles (VCZ, itraconazole, etc.) and echinocandins are not recommended. \(^{37}\) Once azole resistance occurs, the treatment of urinary tract fungal infection will become tricky. Therefore, in order to cope with the current situation of fewer drugs available for urinary tract fungal infection, *C. albicans* CAU-01 strain was selected to further explore the mechanism of myriocin.

As an osmotic protective agent, sorbitol plays a role in stabilizing fungal protoplasts. In the medium containing sorbitol, the antifungal activity of the drugs that specifically inhibit the fungal cell wall will be reversed. \(^{38}\) Similarly, the antifungal action of drugs that target ergosterols or SPLs can also be attenuated by exogenous addition of ergosterols or PHS. The effects of myriocin on the cell wall and ergosterol of *C. albicans* have not been reported, but there have been literatures to evaluate the action mode of natural products through sorbitol and ergosterol addition experiments. For example, geraniol and citral do not exert antifungal effect by affecting cell wall.
and ergosterol, carvanol can bind to ergosterol but does not affect cell wall, and isuegenol, the antifungal active ingredient of laurus nobilis linnaeus essential oil, has effects on both cell wall and ergosterol. In this study, the MIC value of myriocin in the presence of sorbitol, ergosterol or PHS was 2, 16 or more than 128 times higher than that in the absence, respectively. These results suggested that myriocin has slight effects on the cell wall, while its effects on ergosterol and SPLs are more prominent, especially on SPLs. It has been reported that myriocin specifically inhibits SPLs de novo biosynthesis by inhibiting the enzyme serine palmitoyl transferase. Our results laterally confirmed the specific inhibitory effects of myriocin on SPLs.

Furthermore, the damage of myriocin to the cell membrane of C. albicans was revealed by PI staining. PI is a fluorescent probe that selectively penetrates the damaged cell membrane and binds to DNA to increase the fluorescence intensity. PI staining can reflect the effect of myriocin on the cell membrane permeability of C. albicans. In this study, the proportion of fluorescent cells stained by PI was detected by FCM. The results confirmed that PI could enter the cells under the action of myriocin, indicating that myriocin could damage the cell membrane and the proportion of damaged cells was positively correlated with the concentration and contact time of myriocin. The surface changes of C. albicans cells, such as shrinkage, deformation, depression, bulge, and exudation were observed intuitively via SEM. Combined with FCM and SEM analysis, the damage to C. albicans cell membrane caused by myriocin was a dynamic process, which was positively correlated with the concentration and contact time.

Rodrigues et al. found that myriocin alone did not harm the insect model Galleria mellonella, while myriocin combined with C. albicans resulted in 7% larval survival rate after 48 hours, compared to 33% in the C. albicans group. These results suggested that myriocin, despite its powerful anti-fungal activity, increased the susceptibility to infection by inhibiting the insect's immune system. Under the induction of C. albicans, pro-inflammatory cytokines accumulate in tissue cells before being released into the serum, so high inflammatory responses tend to occur in the
later stage of infection. Therefore, we speculate that myriocin, which has the dual activity of immunosuppression and fungal inhibition, might benefit the host in the late stage of infection.

It was found that immunosuppressants have certain inhibitory activity against fungi in vitro, among which calcineurin inhibitors are the most prominent. Calcineurin is involved in many physiological activities of fungi, including morphology, pathogenicity and drug resistance formation. Immunosuppressants (such as FK506 and CsA) can inhibit calcineurin by binding to FKBP12 prolyl isomerase and cyclophilin. And the FKBP12 are different between fungi and human. Consequently, designing specific calcineurin inhibitors without immunosuppressive activity according to this difference is a new direction for the development of antifungal drugs. To improve the specificity against fungal enzymes and avoid side effects of host, myriocin needs further chemical modifications. Our next task is to filter 130 myriocin analogues to find out 2~3 compounds with excellent antifungal activity but without immunosuppressive activity, to correlate their antifungal mechanism with structural characteristics, to establish structure-activity relationships and complete the evaluation and preliminary application.

Biofilm is a powerful virulence factor of Candida, and its minimum inhibitory concentration can be as high as 1000 times that of planktonic cells. Biofilms can be formed on all implantable medical devices (such as catheters, pacemakers, dentures, etc.), and can also be colonized on biological surfaces (such as mucous membranes and epithelial cells). Once fungal cells disperse from the biofilm and flow into the bloodstream, it is possible to cause life-threatening colonization and clarity of parenchyma organs, leading to serious transmitted infections. The clinically relevant more virulent drug-resistant strain was selected in order to face the problem of clinical invasive fungal infection. Inhibition of biofilm will greatly reduce the virulence and transmission of fungi, which is of great significance for the control and treatment of infection. By crystal violet staining and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) reduction assays,
Perdoni et al. observed that the biomass and metabolic activity of Aspergillus fumigatus biofilms gradually decreased with the increase of myriocin concentration.\(^{53}\) These results are consistent with our research, which further illustrates the inhibitory effect of myriocin on fungal biofilm.

Extracellular matrix is a physical barrier that prevents the penetration and diffusion of antimicrobial agents through biofilms and provides a stable environment for microorganisms. The extracellular matrix contributes a lot to the resistance of biofilm. EPS are the main components of the extracellular matrix of biofilms, and its quantity is related to the formation of biofilm.\(^{54-56}\) Our results confirmed the inhibitory and scavenging ability of myriocin to EPS, which is of great significance to reduce fungal resistance, improve the efficacy of antifungal drugs and reduce adverse reactions. Comparing the two sets of data in Fig 4a and Fig 5a, there was no direct linear relationship between them, which indicated that EPS inhibition was only one part of the biofilm inhibition effect of myriocin on \(C.~albicans\).

CSH is an intrinsic property of the external cell wall what make \(Candida\) spp. cells close to each other and induce aggregation. This force causes adhesion molecules to bind strongly to host receptors and eventually to adhere irreversibly to mucous membranes, implanted medical devices, or other substrates. Adhesion is the first and important step in the formation of biofilm. Therefore, hydrophobicity might be one of the factors promoting biofilm formation. In addition, due to the presence of CSH, the cell behavior and adhesion ability are influenced by it, and the decreased hydrophobicity will lead to the restriction of yeast colonization.\(^{32,57}\) The effect of myriocin on \(C.~albicans\) CSH has not been reported, but the role of natural products in CSH has been evaluated. For example, Eugenia uniflora Extract can reduce the CSH of Candida spp. (such as \(C.~albicans\), \(Candida\) tropicalis, \(Candida\) glabrata, \(Candida\) parapsilosis).\(^{32}\) Shirley et al. found that aucubin significantly inhibited the biofilm formation, biofilm metabolic activity and CHS of \(C.~albicans\) at high concentration (61~244 \(\mu g/ml\)), while Baicalein inhibited the growth and biofilm formation by reducing the hydrophobicity of cell surface.\(^{54}\) In our study, \(C.~albicans\) was treated with different concentrations of myriocin. The results showed that the higher the
concentration of myriocin, the lower the CSH. It can be speculated that inhibiting the
adhesion of *C. albicans* and then reducing biofilm is one mechanism of myriocin in
reducing the pathogenicity of *C. albicans*.

Previous studies have shown that myriocin reduces the ability of fungi to form
biofilms mainly by reducing the level of lipid rafts and hyphal formation.\(^5^3,\)\(^5^8-6^0\) However, the formation of biofilm is a complex process, which depends on a variety
of factors. Our study indicated that myriocin inhibited biofilm through inhibiting EPS
and CSH, which was a useful supplement to the inhibiting mechanism of myriocin on
biofilm.

Drug combinations is one of the common methods in clinical treatment of severe
and refractory infections, which has certain advantages in expanding antibacterial
spectrum, reducing drug toxicity, preventing drug resistance or increasing efficacy. The
development of new antifungal agents is a long-term work, which requires a lot of time
and energy. In addition to developing new antifungal agents, drug combinations
provides a promising choice for the treatment of infections caused by drug-resistant
fungi. There have been many reports about combined fungistasis.\(^6^1\) For example,
some immunosuppressants (such as calmodulin inhibitors, glucocorticoids, etc.) can
reverse fungal resistance to fluconazole, and allicin combined with fluconazole can
reduce the biofilm formation of *C. albicans*.\(^4^6,\)\(^6^2\) In this study, myriocin combined
respectively with VCZ and amphotericin B were used to observe the growth of *C.
 albicans* CAU-01, CAB-04 and BNCC186382. The results showed that the FICI
values of the two combinations were between 0.5 and 1, indicating that the two
combinations had additive fungistasis effect on *C. albicans*, which could reduce the
dosage of antifungal drugs and avoid the adverse reactions and drug resistance caused
by high concentration of drugs. Non-*Candida albicans* (NCA) infection is also a
problem that can’t be ignored, especially in intensive care units.\(^6^3,\)\(^6^4\) The
combination of myriocin with VCZ or AMB showed additive activity in vitro against
*C. tropicalis* CTS-01 and *C. glabrata* CGU-01. Therefore, the combination of
myriocin and VCZ or AMB may provide a new alternative for the treatment of fungal
infections.
5. Conclusions

In conclusion, myriocin had significant antifungal activity against *C. albicans*, and the antifungal mechanisms might be cell wall and membrane damage. In addition, myriocin effectively inhibited and eliminated biofilms, and its mechanism may be the inhibitory effect on EPS and CSH.

With the emergence of drug-resistant strains, new antifungal compounds have become the focus of attention. Myriocin is expected to be developed as a novel drug for controlling fungal infection, which is worth further study to understand its pharmacology effects, toxicity and application. Myriocin can be used as an alternative prototype for the production of new and future antifungal drugs, thus contributing to the existing treasure house of antifungal products and providing a new direction for the treatment of clinical fungal infections.

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Authors' Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
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Table 1 The MICs of antifungal drugs against strains. VCZ: Voriconazole, AMB: Amphotericin B. \(^*p<0.05\), all the myriocin-related data of *C. albicans* strains were compared with CAU-01.

| Species             | Strains | MIC (µg/ml) |       |       |       |
|---------------------|---------|-------------|-------|-------|-------|
|                     |         | Myriocin    | VCZ   | AMB   |       |
| *C. albicans*       | CAU-01  | 0.5         | 64    | 0.5   |       |
|                     | CAU-02  | 0.25        | 4     | 0.125 |       |
|                     | CAB-03  | 1\(^*\)     | 16    | 0.0625|       |
|                     | CAB-04  | 0.5         | 256   | 0.0625|       |
|                     | CAS-05  | 0.5         | 0.5   | 0.5   |       |
|                     | CAS-06  | 1\(^*\)     | 1     | 0.5   |       |
|                     | CAV-07  | 1\(^*\)     | 0.125 | 0.25  |       |
|                     | CAV-08  | 0.5         | 0.25  | 0.125 |       |
| BNCC 186382         |         | 0.5         | 0.125 | 0.125 |       |
| *C. tropicalis*     | CTS-01  | 0.5         | 64    | 1     |       |
|                     | CTS-02  | 0.5         | 2     | 1     |       |
|                     | CTV-03  | 0.25        | 1     | 2     |       |
|                     | CTV-04  | 0.5         | 2     | 2     |       |
| *C. krusei*         | CKW-01  | 4           | 2     | 0.25  |       |
|                     | CKW-02  | 2           | 2     | 0.5   |       |
| *C. glabrata*       | CGU-01  | 0.125       | 0.5   | 0.5   |       |
|                     | CGU-02  | 0.25        | 1     | 0.5   |       |
| *C. parapsilosis*   | CPU-01  | 1           | 0.5   | 0.5   |       |
|                     | CPU-02  | 2           | 1     | 0.5   |       |
| BNCC 336515         |         | 4           | 0.25  | 2     |       |
| *Cryptococcus*      | CNB-01  | 0.5         | 0.125 | 0.125 |       |
| neoformans          | CNB-02  | 0.5         | 0.25  | 0.25  |       |
Table 2. MIC values (μg/ml) of drugs in the absence and presence of sorbitol, ergosterol and phytosphingosine (PHS) against *C. albicans* CAU-01

| Drugs       | Sorbitol |    | Ergosterol |    | PHS |    |
|-------------|----------|----|------------|----|-----|----|
|             | presence |    | presence   |    |     |    |
| Myriocin(µg/ml) | 1        | 0.5| 8          | 0.5| >64 | 0.5|
| AMB(µg/ml)  | 0.5      | 0.5| 64         | 0.5| 0.5 | 0.5|
Table 3. Inhibitory effect of combined drugs.

VCZ: voriconazole; AMB: amphotericin B

| Strains             | Items                     | Myriocin+VCZ | Myriocin+AMB |
|---------------------|---------------------------|--------------|--------------|
|                     |                           | Myriocin     | VCZ          | Myriocin     | AMB          |
| C. albicans CAU-01  | MIC alone (µg/ml)         | 0.5          | 64           | 0.5          | 0.5          |
|                     | MIC in combination (µg/ml)| 0.25         | 2            | 0.25         | 0.007813     |
|                     | FICI                      | 0.53         | 0.52         |              |
| C. albicans CAB-04  | MIC alone (µg/ml)         | 0.5          | 256          | 0.5          | 0.0625       |
|                     | MIC in combination (µg/ml)| 0.25         | 128          | 0.25         | 0.03125      |
|                     | FICI                      | 1            | 1            |              |
| C. albicans BNCC 186382 | MIC alone (µg/ml)      | 0.5          | 0.125        | 0.5          | 0.125        |
|                     | MIC in combination (µg/ml)| 0.25         | 0.0625       | 0.25         | 0.03125      |
|                     | FICI                      | 1            | 0.75         |              |
Fig 1 The molecular formulas of myriocin
Fig 2 Quantitative fluorescence data of *C. albicans* CAU-01 for stained with PI after exposure to myriocin (a) at different concentrations for 3h; (b) at MIC for different time.

Data of triplicate trials are expressed as mean ± SE. *p<0.05*, all the data were compared with control.
Fig 3 Scanning electron microscope images of *C. albicans* CAU-01 cells
(A and a) blank control, (B and b), (C and c) and (D and d) exposed to myriocin at 4×MIC for 3h, 6h and 12h, respectively.
Indications of arrows: 1 the rough surface; 2 uneven surfaces; 3 collapse; 4 bulge; 5 cells with visibly shrinkage; 6 exudation of contents; 7 cystic bulge
Fig 4. Effects of myriocin on biofilm of *C. albicans* CAU-01.

(a) Inhibition effects on biofilm formation; (b) Clearance effects on mature biofilms.

Date are expressed as mean ± SE.
Fig 5. Effects of myriocin on extracellular polysaccharides (EPS) of *C. albicans* CAU-01.

(a) Inhibition effects on EPS secretion; (b) Eradication effects on secreted EPS.
Date are expressed as mean ± SE.
Fig 6. Effects of myriocin on cell surface hydrophobic (CHS) of *C. albicans* CAU-01.

Data of triplicate trials are expressed as mean ± SE. *p<0.05*, all the data were compared with control.