The Inhibitory Effect of a Macrocyclic Bisbibenzyl Riccardin D on the Biofilms of Candida albicans

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Biofilm formation plays a key role in the life cycles and subsistence of many microorganisms. The human fungal pathogen Candida albicans has a high propensity to develop biofilms and resulted resistant to traditional antifungal agents. Biofilms are composed of a mixture of cell types, including yeast, pseudohyphal and hyphal cells, and hyphae are a prominent feature of biofilms. Riccardin D is a macrocyclic bisbibenzyl isolated from the liverwort Dumortiera hirsute in our laboratory. In the present investigation, the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay and live/dead cell staining were employed for evaluating the effects of riccardin D on C. albicans biofilms. The results demonstrated that riccardin D can interfere with the biofilm formation. To investigate whether this effect was due to the inhibition of hyphae formation, morphological observation and real-time reverse transcriptase polymerase chain reaction (RT-PCR) were employed for evaluating the effects of riccardin D on the hyphae formation and the expression of hyphae specific genes. The results showed that the hyphae formation was strongly inhibited and the mRNA expression levels of hyphae specific genes were downregulated after riccardin D treatment. We concluded that riccardin D interfered with the biofilm formation of C. albicans through downregulating the expression of hyphae specific genes and inhibiting the formation of hyphae.

Key words  Candida albicans; hyphae; biofilm; riccardin D

Candida albicans is a major human pathogen, causing both mucosal and deep tissue infections. During recent years the Candida species, have become one of the most common agents of hospital-acquired infections.1) Especially problematic is the fact that Candida can form biofilms on the surface of inert or biological surfaces, and this phenotype is associated with infections at both the mucosal and systemic sites.2) It is estimated that biofilms might be involved in 65% of infections.3) Biofilms are structured microbial communities in which the cells bind tightly to a surface and become embedded in a matrix of extracellular polymeric substances produced by these cells.4) The development of C. albicans biofilms in vitro proceeds through an early phase, in which yeast cells populate a substrate, an intermediate phase, in which pseudohyphal and hyphal cell types are produced, and a maturation phase, in which continued cell growth is accompanied by accumulation of an extracellular matrix.5) Direct observation has shown clearly that hyphae are a component of C. albicans biofilms,6,7) and it has been shown that hyphae defective mutants are defective in producing a substantial biofilm.8) The cellular communities formed on device surfaces have characteristic architectural and phenotypic properties distinct from their planktonic counterparts.9) One of the most important characteristics of biofilms is a high level of resistance to antimicrobial drugs, which can be up to 1000-fold greater than planktonic cells. Biofilms of C. albicans have been shown to be resistant to the azole drugs and amphotericin B.9,10) As a consequence, new antifungal agents are needed, especially those that are effective against biofilms. Plant derived antimicrobials have been shown to inhibit microbial biofilm production and some of them are proved to be active in treating fungal biofilms.11,12)

Bisbibenzyl compounds, exclusively occurred in liverworts,13) have proven to be very effective against different clinically important fungi, and it is also active against those Candida isolates displaying high levels of fluconazole resistance.14,15) In this study, we demonstrate the inhibitory effect of riccardin D (Fig. 1), a macrocyclic bisbibenzyl isolated from Chinese liverwort Dumortiera hirsute,16) on the biofilm formation of C. albicans through downregulating the expression level of hyphae related genes and inhibiting the formation of hyphae.

MATERIALS AND METHODS

Organism and Growth Conditions Eight clinical isolates of C. albicans (CA1, CA2, CA3, CA10, CA127, CA132, CA138, and CA139) used in this study were donated by Qianfoshan Hospital of China. CA1, CA2, CA3, CA127 and CA132 were sensitive to fluconazole (MIC<0.25 \( \mu \)g/ml), while CA10, CA138 and CA139 were resistant to fluconazole (MIC=128 \( \mu \)g/ml). C. albicans ATCC10231 was used as a quality control. The isolates were grown on YPD (1% yeast extract, 2% tryptone, 2% dextrose) agar plates at 30 °C.

Antifungal Susceptibility Test of Riccardin D The in vitro minimal inhibitory concentrations (MICs) of riccardin D for each test isolate were determined by broth microdilution method according to the methods approved by the National Committee for Clinical Laboratory Standards (CLSI M27-A3) using RPMI 1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS), and the pH was adjusted to 7.0. The stock solution of ric-
Riccardin D was 20480 µg/ml in dimethylsulfoxide (DMSO) in −20 °C. Riccardin D was serially diluted (twofold) in RPMI 1640 medium, and 100 µl aliquots were dispensed into microtiter plates for a final drug concentration ranging from 0.125 to 256 µg/ml. The final concentration of DMSO in the assay did not exceed 3%. Then, 100 µl of yeast inoculum containing 1×10^5 yeast cells/ml was added to each well. The plates were incubated at 30 °C and read at 24 h by visual inspection and spectrophotometry at 490 nm. The MIC was defined as the lowest drug concentration that reduced growth by 90% compared to drug-free controls.

**Effect of Riccardin D on the Hyphal Formation of C. albicans**  
C. albicans SC5314 [1×10^6 cells/ml in RPMI 1640 medium (or RPMI 1640 medium plus 5% fetal bovine serum, FBS)] was incubated with riccardin D at 37 °C without shaking. Hyphae induction for microscopic quantification was carried out in 6 well flat bottom non-tissue culture-treated plastic plates. After the incubation, the ratio of hyphae cells was counted microscopically. At the same time, the ratio of the hyphal cells was measured. Each experimental condition was tested in triplicate on a given day, and each experiment was repeated on multiple days. For each replicate, over 200 cells per well were counted, and the percentage of cells growing hyphae was determined from an average of three replicate wells.

**Biofilm Formation and Antifungal Susceptibility Testing of C. albicans Biofilms**  
A loopful of the culture was transferred to 20 ml YPD liquid medium and incubated overnight at 30 °C in a shaking incubator. Prior to use in the biofilm experiments, blastospores were harvested and washed twice in sterile 0.1 M phosphate-buffered saline (PBS, pH 7.4). The cells were then suspended in RPMI 1640 medium, counted in a hemocytometer, and adjusted to the desired cell density (1.0×10^6 cells/ml). Riccardin D, a macrocyclic bis(bibenzyl) isolated from Chinese liverwort D. hirsute in our laboratory, was dissolved in DMSO as a 20480 µg/ml stock solution.

Biofilms were formed in the wells of microtiter plates as essentially described previously.17) Biofilms were formed by pipetting 100 µl of the standardized cell suspensions into selected wells of the commercially available presterilized, polystyrene, flat-bottomed, 96-well microtiter plates (Corning Incorporated, Corning, NY, U.S.A.) and incubating the plate for 24 h at 37 °C. After biofilm formation, the medium was discarded and nonadherent cells were removed by washing the biofilms three times in sterile PBS. Riccardin D was then added to selected wells by making 2-fold serial dilutions in RPMI 1640. The biofilms were then incubated in the presence of the antifungal agent riccardin D for 24 h. Untreated biofilms containing RPMI 1640 were included to serve as negative controls for each isolate. The antifungal effects were monitored by using a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay as previously described.12) Briefly, XTT (Sigma) was prepared as a saturated solution at 0.5 mg/ml in Ringer’s lactate and filter sterilized and stored at −70 °C. Prior to each assay, menadione (10 mM prepared in acetone; Sigma) was added to obtain a final concentration of 1 µM. A 100 µl aliquot of XTT-menadione was then added to each well, and microtiter plates were incubated in the dark for 2 h at 37 °C. The colorimetric change (a reflection of the metabolic activity of cells within the biofilm) was measured in a BIO-TEK ELX800 microtiter plate reader at 490 nm. The antifungal effect was measured by comparing the reduction in the mean absorbance of the riccardin D treated wells to that of the riccardin D-free control. The experiment was performed in triplicate.

**Live/Dead Cell Visualization in Biofilms Using Fluorescence Microscopy**  
Biofilms of C. albicans strain CA10 were formed in six-well cell culture plates by adding 4 ml of 10^6 colony forming unit (CFU)/ml cell suspensions and incubating them at 37 °C for 1 h. In order to investigate the inhibitory activity of riccardin D on biofilm formation, concentrations ranging from 8 to 32 µg/ml of riccardin D was added after 2 h of cell attachment then the cultures were incubated for 24 h. After 24 h incubation, the biofilms were washed and stained with fluorescein diacetate (FDA) and propidium iodide (PI) as described previously.19) Cell viability can be assessed using fluorescent microscopy because healthy cells with an intact membrane stain fluorescent green, whereas those with damaged membranes stain fluorescent red.

**Relative Quantification by Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)**  
C. albicans SC5314 cultures were diluted to an initial concentration of 1×10^6 cells/ml in 10 ml prewarmed RPMI 1640 medium plus 5% FBS. Each tube received 64 µg/ml riccardin D or DMSO control and was incubated at 37 °C for 15 h. The total RNAs were respectively isolated using the hot phenol method as described elsewhere.20) Gene expression levels of CDC35, PDE2, ALS3, EFG1 and HWP1 were measured using Eppendorf Mastercycler ep realRiccardin Dx Real Time PCR System. Primer sequences used for amplification of specific genes are listed in Table 1. 18S ribosomal RNA (rRNA) served as the internal control.

**Results**

**Antifungal Susceptibility Test**  
Riccardin D was evaluated for antifungal properties with broth microdilution method against 8 isolates of Candida albicans and SC5314. Results showed that riccardin D displayed a moderate antifungal activity with the MIC₉₀ at 16 µg/ml against all these C. albicans isolates.

**Effect of Riccardin D on Growth Form of C. albicans**  
C. albicans was cultured in RPMI 1640 medium (or RPMI 1640 medium plus 5% FBS) at 37 °C. After 6 h and 15 h in-

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**Table 1. Gene-Specific Primers Used for Relative Quantification Real-Time RT-PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Tₑₒ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS3-F</td>
<td>5’-AGGTGTTCCTGGTTTCG-3’</td>
<td>55.7</td>
</tr>
<tr>
<td>ALS3-R</td>
<td>5’-TGATAGGGAAGTTG-3’</td>
<td>53.7</td>
</tr>
<tr>
<td>CDC35-F</td>
<td>5’-TCATACGGGGTATTTAC-3’</td>
<td>53.7</td>
</tr>
<tr>
<td>CDC35-R</td>
<td>5’-CTCTATCACCGGCATTTC-3’</td>
<td>57.8</td>
</tr>
<tr>
<td>PDE2-F</td>
<td>5’-ACACACACACTACTAC-3’</td>
<td>57.8</td>
</tr>
<tr>
<td>PDE2-R</td>
<td>5’-AAAAATGTGTTCCGTCGTC-3’</td>
<td>53.7</td>
</tr>
<tr>
<td>EFG1-F</td>
<td>5’-TATGCCCGACAAACAACTG-3’</td>
<td>57.8</td>
</tr>
<tr>
<td>EFG1-R</td>
<td>5’-TTGTTGTCCTGCTGTCGTC-3’</td>
<td>57.8</td>
</tr>
<tr>
<td>HWP1-F</td>
<td>5’-TGTTGATTCCATATTCGGG-3’</td>
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</tr>
<tr>
<td>HWP1-R</td>
<td>5’-CAAATAGACGCCGAC-3’</td>
<td>55.75</td>
</tr>
<tr>
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<tr>
<td>18S rRNA-R</td>
<td>5’-AGGCTCTAAGCGCATCA-3’</td>
<td>57.8</td>
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cubation, *C. albicans* cells were photographed (Figs. 2, 3). The results showed that *C. albicans* suspended in RPMI 1640 medium (or RPMI 1640 medium plus 5% FBS) grew in hyphal form. When riccardin D was added to the medium, *C. albicans* grew in yeast forms. As shown in Fig. 3, the ratio of hyphal cells was 98.5% in the absence of riccardin D and 69.3% or 18.2% in the presence of 32 μg/ml or 64 μg/ml riccardin D when cultured in RPMI 1640 medium plus 5% FBS. The ratio of hyphae formation was significantly inhibited by riccardin D. These findings suggested that riccardin D regulated the hyphae transformation of *C. albicans* cells.

**In Vitro Activity of Riccardin D against Preformed C. albicans Biofilms**

Riccardin D was used to treat adherent cell populations with different concentrations (4, 8, 16, 32, 64 μg/ml) to determine the inhibitory activity against *C. albicans* biofilms. A sharp reduction in the metabolic activity of cells within the biofilms was measured using XTT, when preformed biofilms were exposed to riccardin D. When the effect of riccardin D on biofilms was tested at 16 μg/ml, the metabolic inhibition of various clinical fluconazole sensitive and resistant isolates caused by riccardin D ranged from 33 to 80.3%. Although susceptibility of biofilms to riccardin D differed among clinical isolates, there was no obvious correlation between riccardin D susceptibility and fluconazole sensitive and resistant strains (Fig. 4).

**Live/Dead Cell Visualisation in Biofilm Using Fluorescence Microscopy**

We found that the identification of live and dead cells stained by FDA-PI was unequivocal. Intense green fluorescence resulted from FDA binding to metabolically active cells, while the red color was due to PI-staining the dead cells. Treatment of *C. albicans* CA10 with various concentrations of riccardin D showed that there was a dose-dependent effect in inhibiting biofilm formation. Almost all *C. albicans* in the control showed an intense green fluorescence, indicating the mature biofilms were viable. After incubation with riccardin D at a concentration of 8 μg/ml there were fewer cells although a majority of which were still green (Fig. 5). With riccardin D treatment at a concentration of 32 μg/ml, there were also much fewer cells compared to the control and almost all of the remaining cells stained red after 24 h. All of these findings demonstrate the ability of riccardin D not only to penetrate the biofilm, but also to inhibit its formation and actively kill the *C. albicans* cells.

**Relative Quantification by Real-Time PCR**

Relative quantification real-time PCR revealed *C. albicans* hyphae related genes *EFG1, HWP1, ALS3* were downregulated while
Biofilms are highly organized communities of cells, possessing unique developmental characteristics that differ from free-floating planktonic cells. *C. albicans* biofilm development occurs in three phases, as observed with *in vitro* models.\(^{19,20}\) During the early phase, yeast cells adhere to the surface of the support and begin to divide and form a layer of microcolonies. During the intermediate phase, continued yeast cell growth is accompanied by extracellular material production and initial differentiation to produce elongated pseudohyphae and hyphae. Finally, a maturation phase occurs, in which the amount of extracellular material increases, and the network of pseudohyphae and hyphae embedded in this matrix grows in parallel to assemble a biofilm. Direct observation has shown clearly that hyphae are a component of *C. albicans* biofilms,\(^{6,7}\) and it has been shown that hyphae defective mutants are defective in producing a substantial biofilm.\(^{8}\) In this study, we demonstrated riccardin D, a macrocyclic bisbibenzyl from the liverwort *D. hirsute*, displayed moderate antifungal activity against *C. albicans* isolates with MIC\(_{90}\) at 16 \(\mu g/ml\) against and this compound showed no toxicity to normal RPE1, LO2 cell lines at concentrations more than 20 \(\mu g/ml\) (data not shown). We also demonstrated that riccardin D can penetrate the biofilm and inhibit its formation (Figs. 4, 5) and inhibited metabolic activity of a mature biofilm. This activity was dependent on the riccardin D concentration and there was no obvious difference of drug action on fluconazole sensitive and resistant strains. From these results, we concluded that riccardin D has a function in inhibition the biofilm formation at dose dependent manner. To investigate the mechanism of this inhibition effect, the effect of riccardin D on the formation of *C. albicans* hyphae was measured. The results showed that addition of riccardin D can inhibit the formation of hyphae (Figs. 2, 3). When the cells were cultured in RPMI 1640 medium, almost all the cells grew in yeast form after 16 \(\mu g/ml\) riccardin D treatment, while when 5% FBS was added to the RPMI 1640 medium, almost all the cells grew in yeast form when addition of riccardin D up to 64 \(\mu g/ml\). This result suggested that the inhibition of the FBS induced hyphae formation required higher concentration of riccardin D maybe due to FBS is a strong hyphae inducer.

In *C. albicans* hyphae formation was stimulated by the Ras1-cAMP-Efg1 signalling pathway. In this pathway, the small GTPase Ras1, when activated, stimulates adenylate cyclase (*CDC35*) leading to the generation of a cAMP signal that promotes PKA-mediated activation of transcription factor(s), including *EFG1*, that induce the expression of the hyphae specific genes such as *HWP1, ALS3* and then mediate the yeast-to-hypha transition.\(^{21}\) This process is generally under negative feedback regulation by phosphodiesterases (PDEs) that degrade cAMP to AMP.\(^{22}\) Ramage et al. have shown that the hyphal regulatory gene *EFG1* is required for normal biofilm growth.\(^{12,16}\) Surface adhesin *ALS3* and hyphae specific gene *HWP1* have a pivotal role in biofilm formation. Reduced hyphae production by the *efg1* mutant certainly contributes to the biofilm defective. To elucidate the effect of riccardin D on hyphae formation signals in *C. albicans*, in present investigation, the expression level of the genes in this pathway was measured and the results showed that *HWP1, ALS3* and *EFG1* was downregulated and the PDE2 and *CDC35* were upregulated after riccardin D treatment. These results suggested that riccardin D upregulated the expression of PDE2 and stimulated the degradation of cAMP. The lower level of intracellular cAMP decreased the expression of downstream genes, *EFG1, HWP1* and *ALS3* and then inhibited the formation of hyphae. The upregulation of *CDC35* maybe was due to the negative feedback of the reduced levels of intracellular cAMP.

In conclusion, we demonstrated that riccardin D can inhibit biofilm formation of *C. albicans* through inhibiting the hyphae formation as a result of downregulating the expression of hyphae specific genes.

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