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Short communication

In vitro activity of verapamil alone and in combination with fluconazole or tunicamycin against *Candida albicans* biofilms

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ABSTRACT

Calcium channels and pumps play important roles in morphogenesis, stress response and virulence in *Candida albicans*. We hypothesised that verapamil, a potent calcium channel blocker, may display an inhibitory effect on *C. albicans* biofilms. To test this hypothesis, the in vitro activity of verapamil was evaluated alone and in combination with fluconazole or tunicamycin against *C. albicans* biofilms using a 96-well microtitre plate model. As expected, verapamil exerted inhibitory activity against *C. albicans* biofilms. The combinations of verapamil/fluconazole and verapamil/tunicamycin yielded synergistic effects on biofilm formation and on pre-formed biofilms. Furthermore, verapamil alone or in combination with fluconazole or tunicamycin led to a significant decrease in the transcription level of *ALS3*, essential for biofilm development. Therefore, verapamil may be a potential agent to enhance the effect of antifungal drugs against *C. albicans* biofilms.

1. Introduction

Candida albicans is one of the most important opportunistic fungi in humans. It may cause mucocutaneous infections and life-threatening disseminated candidiasis in immunocompromised hosts [1]. Many *C. albicans* infections are closely associated with its ability to form biofilms on host mucosal surfaces or implanted medical devices [1]. *Candida albicans* biofilms, like biofilms formed by bacterial pathogens, are resistant to many antimicrobial agents, including azoles, the most commonly deployed class of antifungal drugs, and are responsible for its virulence [2]. Biofilm development begins with adherence of yeast cells to a substrate, a key process that requires the action of adhesins. Yeast cells then proliferate on the surface and switch to hyphae and pseudohyphae. As the biofilms develop, cells accumulate extracellular matrix and display high-level resistance to antifungal drugs [3]. To improve the efficacy of treatments for *C. albicans* infections, it will be necessary to explore new antifungal strategies against *C. albicans* biofilms in clinical practice.

There is abundant evidence to support the significance of calcium channels and pumps in morphogenesis, stress response and virulence in *C. albicans* [4,5]. However, their roles during *C. albicans* biofilm development remain to be investigated. It has been reported that calcium chelators such as ethylene diamine tetra-acetic acid (EDTA) disrupt *C. albicans* biofilms, indicating the importance of calcium homeostasis in *C. albicans* biofilm development [6,7]. Most recently, we further illuminated the role of the calcium pump Spf1 in *C. albicans* biofilm development [8]. These findings suggested that agents targeting the calcium homeostasis system may have potential inhibitory effects on *C. albicans* biofilms.

Verapamil belongs to the phenylalkylamine class of calcium channel blockers and is widely used in the treatment of hypertension and angina pectoris [9]. As an L-type voltage-gated calcium channel (VGCC) blocker, verapamil exerts inhibitory effects on the plasma membrane calcium channel Cch1 in *Saccharomyces cerevisiae*, an orthologue of mammalian VGCCs [10]. In this study, the in vitro activity of verapamil against *C. albicans* biofilms was evaluated. Furthermore, since fluconazole is one of the most important antifungal drugs in clinical practice, and tunicamycin is a nucleoside antibiotic that blocks *N*-linked glycosylation in fungal cells and inhibits *C. albicans* biofilms [11], the synergistic effects of verapamil in combination with both of these compounds on *C. albicans* biofilm formation and on pre-formed biofilms were examined. In addition, since both *ALS3* and *HWP1* play important roles in *C. albicans* biofilms [3], the effects of verapamil alone and in combination with fluconazole or tunicamycin on their expression levels were further investigated by real-time quantitative PCR (RT-qPCR) to explore how verapamil impacts on *C. albicans* biofilms.

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2. Materials and methods

2.1. Strain and drugs

Wild-type *C. albicans* strain DAY1 was generously provided by Prof. Dana Davis (University of Minnesota, Minneapolis, MN). Verapamil was obtained from the National Institutes of Food and Drug Control (Beijing, China). A stock solution of 50 mM verapamil was prepared in distilled water and was stored at −20 °C. Fluconazole was obtained as a powder from Sigma (St Louis, MO) and a stock solution of 10 mg/mL was prepared in dimethyl sulphoxide (DMSO). Tunicamycin was obtained from Bio Basic Inc. (Amherst, NY) and a stock solution of 1 mg/mL was prepared in DMSO. All stock solutions were stored at −20 °C. Subsequent dilutions of these drugs were made in RPMI-1640 (Invitrogen Co., Grand Island, NY) supplemented with uridine (final concentration 80 μg/mL; Sigma) and buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS) (final concentration 20 mM, pH 7.4; Sigma).

2.2. In vitro inhibitory effect of verapamil on Candida albicans biofilm formation

Candida albicans biofilms were formed on polystyrene, flat-bottom, 96-well microtitre plates (Corning Inc., Corning, NY) under static conditions. Briefly, 50 μL of RPMI-1640 medium containing verapamil ranging from 1280 mg/L to 5 mg/L was added to the wells in the first nine columns of the microtitre plate, and 50 μL of RPMI-1640 medium without verapamil was added to the wells in the tenth column for positive control for biofilm formation. Then, 50 μL of *C. albicans* DAY1 cell suspensions (2×10^6 cells/mL) was added to each well of the abovementioned 10 columns. To each well of the last two columns, 100 μL of RPMI-1640 medium was added to serve as a negative control for subsequent analysis. The plate was covered with its lid, sealed with Parafilm and incubated at 37 °C for 24 h. The medium was then removed and the plate was washed three times with phosphate-buffered saline (PBS). Metabolic activities of biofilms were assessed by the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] reduction assay as described by Pierce et al. [11]. Briefly, 100 μL of XTT (Sigma) solution (1 μM prepared in 10 mM menadione–acetone solution) was added to each pre-washed well. The plates were incubated in the dark at 37 °C for 2 h, then 90 μL of XTT solution in each well was transferred to a new microtitre plate and XTT reduction was assessed by determining the absorbance at 490 nm on a microplate reader (Model 680; Bio-Rad, Philadelphia, PA).

2.3. In vitro inhibitory effect of verapamil on pre-formed Candida albicans biofilms

Pre-formed *C. albicans* biofilms were prepared as before. The medium was then removed and wells were washed three times with sterile PBS. Then, 100 μL of RPMI-1640 medium containing verapamil ranging from 1280 mg/L to 5 mg/L was added to the wells of the first nine columns, and 100 μL of RPMI-1640 medium containing no verapamil was added to each well of the tenth column to serve as a positive control. The last two columns were kept empty to serve as negative controls. The plate was covered and incubated at 37 °C for a further 24 h and was washed three times with PBS. The metabolic activities of biofilms were also measured by the XTT reduction assay.

2.4. In vitro activity of antifungal combinations against Candida albicans biofilms

To test the effects of verapamil, fluconazole and tunicamycin alone and of the antifungal combinations verapamil/fluconazole and verapamil/tunicamycin on *C. albicans* biofilm formation, dilutions of verapamil (1280–5 mg/L), fluconazole (1280–5 mg/L) and tunicamycin (128–0.25 mg/L) were examined alone and in combination in a chequerboard format. Biofilms were formed in the wells of microtitre plates and were assessed using the XTT reduction assay. The minimum inhibitory concentration of drug associated with a 50% reduction in XTT readings compared with the no-drug control wells (MIC_{50}) was determined [12]. Drug combination interactions were calculated by determining the fractional inhibitory concentration index (FICI) [13]. $FICI_A$ was calculated as the MIC of drug A in the combination divided by the MIC of drug A alone, and $FICI_B$ was calculated as the MIC of drug B in the combination divided by the MIC of drug B alone. The FICI of the combination of drug A and drug B ($FICI_{AB}$) was calculated as follows: $FICI_{AB} = FICI_A + FICI_B$. Interpretation of the FICI was as follows: FICI < 0.5, synergistic; 0.5 ≤ FICI ≤ 4.0, no interaction; and FICI > 4.0, antagonistic [14]. To test the effects of these drugs alone and in combination on pre-formed biofilms, biofilms were formed as before and were treated for 24 h with these drugs at a series of concentrations alone and in combination in a chequerboard format. Biofilm activity was then assessed and the FICI against pre-formed biofilms was calculated as mentioned above.

2.5. Real-time quantitative PCR

For detecting the expression levels of *ALS3* and *HWP1*, *C. albicans* DAY1 cells were incubated in RPMI-1640 medium at 37 °C for 4 h. Drugs alone or in combination were then added to the cultures at the following final concentrations: verapamil at 160 mg/L; fluconazole at 16 mg/L; tunicamycin at 1 mg/L; verapamil at 20 mg/L + fluconazole at 1 mg/L; and verapamil at 5 mg/L + tunicamycin at 0.25 mg/L. Cultures without drugs served as the control. Cells were further incubated at 37 °C for 2 h and were then harvested for RNA extraction. Cell total RNA was isolated using Total RNA Extraction Kit (Tiangen, Beijing, China) following the manufacturer's instructions. Total RNA was treated with DNase I (Takara, Dalian, China) and was reversely transcribed with oligo(dT) primers using M-MLV Reverse Transcriptase (Promega, Beijing, China) with 30 min at 37 °C, 1 h at 42 °C and 15 min at 72 °C. RT-qPCR reactions were performed using TranStart Green qPCR SuperMix (Transgene, Beijing, China) in quintuplicate using the primers ALS3-5RT (5′-CTCATTACACCAACCATACA-3′) and ALS3-3RT (5′-GGATTCTGTGGTTGTAGTAT-3′) for *ALS3* and the primers HWP1-5RT (5′-TGTCTACACTACATTCTGTC-3′) and HWP1-3RT (5′-AGGAATAGATGGTTGTGAAC-3′) for *HWP1*. The *ACT1* gene was used as the endogenous control [15]. The PCR protocol consisted of a primary denaturation step at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 20 s, annealing at 58 °C for 20 s and extension at 68 °C for 30 s. Results were analysed using iQ™5 software (Bio-Rad, Hercules, CA).

2.6. Statistical analysis

Each experiment was performed with five replicates for each condition tested. The inhibitory effect of drug treatments was compared with the controls (untreated) and the significance of the difference ($P < 0.05$ or $P < 0.01$) was assessed using Student's *t*-test (SPSS v19.0 software; IBM, Armonk, NY).

3. Results and discussion

3.1. Verapamil displays inhibitory effects on biofilm formation and pre-formed biofilms

Since verapamil is a potential calcium channel blocker in *C. albicans*, we hypothesised that it may display inhibitory activity