Synergistic effect of fluconazole and doxycycline against *Candida albicans* biofilms resulting from calcium fluctuation and downregulation of fluconazole-inducible efflux pump gene overexpression

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*Candida albicans* biofilms are intrinsically resistant to antimicrobial agents. Previous work demonstrated that the antifungal activity of fluconazole against *C. albicans* biofilms is notably enhanced by doxycycline. In order to explore the synergistic mechanism of fluconazole and doxycycline, we investigated the changes of efflux pump gene expression, intracellular calcium concentration and cell cycle distribution after drug intervention in this study. The expression levels of *CDR1*, *CDR2* and *MDR1* were determined by real-time PCR, and the results showed that fluconazole alone could stimulate the high expression of *CDR1*, *CDR2* and *MDR1*, and the combination of doxycycline and fluconazole downregulated the gene overexpression induced by fluconazole. Intracellular calcium concentration was determined using Fluo-3/AM by observing the fluorescence with flow cytometry. A calcium fluctuation, which started 4 h and peaked 8 h after the treatment with fluconazole, was observed. The combined drugs also initiated a calcium fluctuation after 4 h treatment and showed a peak at 16 h, and the peak was higher than that stimulated by fluconazole alone. The cell cycle was measured using flow cytometry. Fluconazole alone and the combined drugs both induced a reduction in the percentages of S-phase cells and an elevation in the percentages of cells in the G2/M phase. The results of this research showed that the synergism of fluconazole and doxycycline against *C. albicans* biofilms is associated with blockade of the efflux pump genes *CDR1*, *CDR2* and *MDR1*, and stimulation of high intracellular calcium concentration. The findings of this study are of great significance in the search for new antifungal mechanisms.

INTRODUCTION

*Candida albicans* is able to adhere to surfaces of the medical material used for intravascular catheters, endotracheal tubes, artificial heart valves, joint prostheses, etc., resulting in the generation of biofilms. *C. albicans* biofilms are resistant to the majority of antifungics (Hawser & Douglas, 1995) and are therefore difficult to treat. Recently, synergism against *C. albicans* biofilms has been reported when high-dose doxycycline was combined with fluconazole, amphotericin B and caspofungin (Miceli *et al.*, 2009). In our previous research, we demonstrated that the antifungal effect of fluconazole could be sensitized by doxycycline at a concentration of 1–64 mg l⁻¹ against *C. albicans* biofilms (Gao *et al.*, 2013). However, as for research on the mechanism, Fiori & Van Dijck (2012) reported that iron depletion may account for the synergism of fluconazole and doxycycline against planktonic cells, and no other mechanism has been reported. Thus, the precise synergistic mechanism of fluconazole and doxycycline against *C. albicans* biofilms remains unknown and needs exploration.

The mechanisms responsible for drug resistance associated with *C. albicans* biofilms are complex. Recent research revealed that drug efflux pump genes were overexpressed...
during the course of *C. albicans* biofilm formation and development, which indicated that the efflux pump contributes to biofilm resistance (Ramage *et al.*, 2002; Watamoto *et al.*, 2011). The drug efflux in *C. albicans* is mostly related to the ATP-binding cassette (ABC) transporters and the major facilitator superfamily transporters. The ABC transporters are encoded by *Candida* drug resistance genes *CDR1* and *CDR2*, and the major facilitator superfamily transporters are encoded by the multidrug resistance gene *MDR1* (Albertson *et al.*, 1996; Sanglard *et al.*, 1997). Research has shown that azole resistance associated with overexpression of efflux pumps could be reverted by some modulators, such as ibuprofen, verapamil and sex hormones (Ford & Hait, 1990; Pina-Vaz *et al.*, 2005), which allows reversion of resistance through blocking the efflux pumps. Thus, here we investigated whether the synergism induced by fluconazole and doxycycline was associated with the blockade of efflux pump genes.

*C. albicans* has evolved a complex network of mechanisms in response to drug resistance and one of them is calcium homeostasis. In *C. albicans*, calcium homeostasis is essential in developmental and stress signalling pathways (Bader *et al.*, 2006). It has been reported that calcium-associated pathways are related to key pathogenic steps in fungi, including *C. albicans* (Reedy *et al.*, 2010). Research revealed that the antifungal effects of some agents are associated with calcium homeostasis disturbance. In our previous studies, the combination of fluconazole and minocycline caused a significant increase of intracellular calcium (Shi *et al.*, 2006). It has been reported that calcium-associated mechanisms in *C. albicans* biofilms and drug interventions.

**METHODS**

**Strains and media.** The tested *C. albicans* strains CA1 and CA10 were isolated from different clinical specimens in Qianfoshan Hospital, Jinan, China. The strains were fluconazole susceptible (CA1; MIC 0.5 mg l⁻¹) and fluconazole resistant (CA10; MIC 512 mg l⁻¹). In our previous studies, we determined the biofilm MICs of CA1 (fluconazole alone, 128 mg l⁻¹; doxycycline alone, 512 mg l⁻¹; fluconazole in combination, 2 mg l⁻¹; doxycycline in combination, 16 mg l⁻¹) and CA10 (fluconazole alone, 512 mg l⁻¹; doxycycline alone, 312 mg l⁻¹; fluconazole in combination, 4 mg l⁻¹; doxycycline in combination, 32 mg l⁻¹) (Gao *et al.*, 2013). The isolates were maintained in YPD medium (1% yeast extract, 2% peptone, 2% glucose) (Ding Guo Biological) at 70°C. They were propagated and subcultured on YPD solid medium at least twice to ensure purity and viability before each experiment.

**Antimicrobial agents.** Fluconazole was provided by Cheng Chuang Pharmaceutical and doxycycline was purchased from An Bei Ka Pharmaceutical. They were dissolved in distilled water and DMSO, respectively. The concentration of stock solution of fluconazole was 2.56 g l⁻¹ and the concentration of stock solution of doxycycline was 6.4 g l⁻¹. Stock solutions were kept at −70°C until use.

**Preparation of *C. albicans* biofilms and drug interventions.** *C. albicans* biofilms were formed on polystyrene 12-well plates. Cells were first suspended in RPMI-1640 (Gibco-BRL) supplemented with glucose to a final concentration of 2% and buffered with 0.165 M MOPS (Ding Guo Biological) to pH 7.0, standardized to a cell density of 1.0 x 10⁵ cells ml⁻¹. Then, 1 ml cell suspensions were pipetted into microtitre plates and incubated overnight at 37°C. In our previous works, we used *C. albicans* biofilms that formed over 4, 8, 12, and 24 h, and we discovered that the overnight biofilm was mature enough to evaluate the drug effect (Gao *et al.*, 2013). Each well of the plates was washed with PBS (pH 7.2–7.4; Solarbio Science & Technology) to remove planktonic cells and then the biofilm was obtained. *C. albicans* biofilms were incubated with drugs (4 mg l⁻¹ fluconazole and 8 mg l⁻¹ doxycycline) for 24 h. The cells were then washed with PBS and processed for research on the synergistic mechanism.

**RNA extraction, cDNA synthesis and quantitative real-time (RT)-PCR.** Total cellular RNA was extracted using the hot phenol method (Schmitt *et al.*, 1990). Briefly, 12 ml AE buffer (50 mM sodium acetate pH 5.2, 10 mM EDTA) was used to suspend the frozen cells at room temperature, then 800 µl 25% SDS and 12 ml acid phenol (Fisher Science) were added. The cells were incubated with vortexing each minute for 10 min at 65°C and cooled on ice for 5 min; then they were centrifuged for 15 min at 11 952 g. Supernatants were transferred to a new tube, mixed with 15 ml chloroform and centrifuged at 200 g for 10 min. The aqueous layer was transferred to a new tube containing 1 vol. 2-propanol and 0.1 vol. 2 M sodium acetate (pH 5.0), and the mixture was centrifuged at 17 211 g for 35 min at 4°C. The precipitation was resuspended in 10 ml 70% ethanol and the mixture was centrifuged at 17 211 g for 20 min at 4°C. The RNA pellet was resuspended in diethylpyrocarbonate-treated water. RNA concentrations were quantified spectrophotometrically and the samples were stored at −80°C until use.

The cDNA was synthesized using the First-stand cDNA Synthesis SuperMix (TransGen Biotech) according to the manufacturer’s instructions. All of the reagents, as well as 2 µg cellular RNA, were added to a final volume of 20 µl, and then the samples were incubated at 42°C for 30 min and 85°C for 5 min. The synthesized cDNA samples were stored at −20°C until use. The PCR primers used in this assay were designed and synthesized by Sangon Biological Engineering Technology and Services (Shanghai, China) and the specificity of each primer was checked by comparing its sequence with...
the C. albicans database by BLAST at the National Center for Biotechnology Information. The sequences of the primers are listed in Table 1. The RT-PCR was carried out with an ABI ViiA 7 (Applied Biosystems) sequence detection system using SYBR Green 1 (Cwbiotech) in duplicate for three separate experiments. An aliquot of 25 μl PCR mix was used for each gene and the cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence intensities were quantified by using StepOne software (Applied Biosystems). The expression of the ACT housekeeping gene was used as a control and the relative quantities of the target genes were analysed using the comparative ΔΔCt method (Pfaffl, 2001). The comparison of data among four groups (control, fluconazole-alone, doxycycline-alone and fluconazole combined with doxycycline) was determined by one-way ANOVA, followed by Dunnett’s test using SPSS Statistics software and significance was defined as P<0.01.

Intracellular calcium detection. The calcium-sensitive indicator Fluo-3/AM (Biotium) was used to evaluate the intracellular calcium (Wang et al., 2011); it is readily hydrolysed to Fluo-3 free acid and becomes activated in cytoplasm when passively loaded into cells. Fluo-3/AM was dissolved in 20 % Pluronic F-127 (Biotium) DMSO becomes activated in cytoplasm when passively loaded into cells. Fluo-3/AM (Biotium) was used to evaluate the intracellular calcium levels. Intracellular calcium detection. Fluo-3/AM at a final concentration of 5 μM for 30 min at 37 °C. The cells were then washed three times with D-Hanks buffer (KCl 0.4 g, KH2PO4 0.06 g, NaCl 8.0 g, NaHCO3 0.35 g, Na2HPO4.7H2O 0.09 g dissolved in 1 l distilled water). Calcium levels were determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson) with excitation at 488 nm and emission at 525 nm.

Cell cycle analysis. DNA content was quantified with the DNA-specific fluorescent dye propidium iodide (PI). The C. albicans strains were first washed twice with PBS and then fixed with cold 70 % ethanol (in PBS) at 4 °C overnight. The cells were centrifuged at 1000 g and washed twice with PBS. RNase A (final concentration 0.2 mg ml⁻¹) was added to the cells and the mixture was allowed to react at 50 °C for 2 h. After the reaction, PI (final concentration 0.05 mg ml⁻¹) was added and the samples were stained for 30 min in the dark. DNA content was determined by a FACScan flow cytometer (Becton Dickinson) with excitation at 488 nm and emission at 630 nm. The data were analysed using WINMDI 2.9 software (Joseph Trotter, The Scripps Institute, La Jolla, CA, USA).

RESULTS

Expression of genes encoding efflux pumps in response to combined drugs

The gene expression results for CA10 are shown in Fig. 1. Fluconazole significantly increased CDR1 expression compared with the control group by 389 % (P<0.01), and the combination of fluconazole and doxycycline downregulated the CDR1 high expression significantly compared with fluconazole challenge alone by 65 % (P<0.01). Expression of CDR1 was downregulated on doxycycline challenge compared with the control group by 54 % (P<0.01). CDR2 expression was depressed by doxycycline and increased by fluconazole challenge (P<0.01), 59 and 57 %, respectively. The drug combination downregulated CDR2 expression to a significant extent compared with fluconazole alone by 81 % (P<0.01). MDR1 overexpression was observed with the intervention of fluconazole alone and the combination treatment dampened this transcriptional response significantly by 47 % (P<0.01). The efflux pump gene expression of the fluconazole-susceptible strain CA1 was also tested in this study. For CA1 biofilms, fluconazole alone could stimulate the overexpression of CDR1, CDR2 and MDR1, and the combination of doxycycline and fluconazole downregulated this gene overexpression. Therefore, no significantly different results were observed compared with CA10 (data not shown).

Intracellular Ca2+ levels change upon drug combination

The intracellular Ca2+ level following treatment with the doxycycline and fluconazole drug combination was determined by the fluorescent calcium indicator Fluo-3/AM, and the results for CA10 are shown in Fig. 2. In the control and doxycycline-alone groups, the intracellular levels of Ca2+ were rather low and changed very little over time. Treatment of the cells with fluconazole alone resulted in an apparent calcium fluctuation, which started 4 h and peaked 8 h after the treatment. More complex changes were observed when the cells were challenged with the doxycycline and fluconazole drug combination. In the first 4 h, the intracellular level of Ca2+ was as low as the control group, and then an obvious increase in fluorescence was observed and lasted for 4 h. The increase became slower after 8 h challenge and showed a peak at 16 h. Moreover, we observed that this peak was higher than that stimulated with fluconazole alone (P<0.01).

Table 1. Forward (F) and reverse (R) primers used in RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>Primer sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>F</td>
<td>GGTTAGGTCTAAATGGCGATCATC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGTGTGTCATTACCGACGTTCAAA</td>
</tr>
<tr>
<td>CDR1</td>
<td>F</td>
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<tr>
<td></td>
<td>R</td>
<td>CTCCGCAACCCGATCTCACTC</td>
</tr>
<tr>
<td>CDR2</td>
<td>F</td>
<td>ATGCCTGAAAGCCAGACTCA</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AGCCATACCCGACAAAACA</td>
</tr>
<tr>
<td>MDR1</td>
<td>F</td>
<td>GCTGCTACCTAGTCTGTTG</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGGAAAGCGCAACGGACTAC</td>
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</table>
Results were normalized against ACT housekeeping gene expression and are shown as the mean ± SD of three independent experiments. DOX, doxycycline (8 mg l⁻¹); FLC, fluconazole (4 mg l⁻¹). *P<0.01 versus control group.

Effects of combined drugs on the cell cycle

The cell cycle was analysed to explore how the combined drugs inhibited cell proliferation and the results for CA10 are shown in Fig. 3. In the control group, the percentages of cells in different phases were all evenly distributed (between 30 and 35%). The result for treatment with doxycycline alone was similar to the control group. An obvious difference in the cell cycle distribution was observed when the cells were exposed to fluconazole alone. The results show a reduction in the percentage of S-phase cells (from 34.6 to 6.0%) and an elevation in the percentage of cells in the G₂/M phase (from 32.7 to 71.8%), indicating that fluconazole induced cell cycle arrest within the G₂/M phase in C. albicans biofilms. For the combined group, the cell percentages of S phase and G₂/M phase were similar to those of the fluconazole-alone group (6.4 and 66.1%, respectively). However, the combination of doxycycline and fluconazole resulted in cell arrest in the G₂/M phase to a weaker extent compared with the fluconazole-alone group.

DISCUSSION

Drug combination has recently been used to improve the effect of fluconazole therapy for C. albicans biofilms (Bink et al., 2011). In previous studies, we found that fluconazole susceptibility against C. albicans biofilms could be increased by doxycycline (Gao et al., 2013). In order to understand the synergistic effect of fluconazole and doxycycline, the possible mechanisms were analysed in this study.

In our previous study, the synergistic effect of fluconazole and doxycycline was found to change with the sensitivities of the C. albicans isolates tested (six azole-resistant strains, one azole-susceptible dose-dependent strain, and three azole-resistant strains), and a stronger effect was observed on resistant strains compared with susceptible strains (Gao et al., 2013). The efflux pump was considered as one of the common fungal resistance mechanisms (Ramage et al., 2002), and thus the mRNA levels for CDR1, CDR2 and MDR1 were analysed in this study. We found that the expression levels of CDR1 and CDR2 were downregulated slightly when biofilms were exposed to doxycycline alone. Fluconazole alone can obviously upregulate the expression levels of CDR1 and CDR2, and thus gene overexpression was downregulated by the fluconazole and doxycycline drug combination. These results indicated that doxycycline alone has an influence on CDR1 and CDR2 expression, and the blockade of the two efflux pumps could be achieved when doxycycline is combined with fluconazole. In addition, MDR1 was upregulated by fluconazole alone and the combination treatment dampened this overexpression significantly. No significant variation of MDR1 expression was induced by doxycycline alone. These results demonstrated that MDR1 overexpression could be induced by fluconazole alone and this overexpression could be reversed by fluconazole combined with doxycycline. A study of the synergistic mechanism of tetracycline and amphotericin B in C. albicans demonstrated that the tetracycline effect on amphotericin B was independent of efflux pump gene expression, including CDR1, CDR2 and MDR1 (Oliver et al., 2008). However, the results of this study showed that the combination of fluconazole and doxycycline induced the...

Fig. 1. Relative transcriptional expression of resistance genes in C. albicans biofilms in response to fluconazole and doxycycline. CDR1, CDR2 and MDR1 expression levels were analysed by RT-PCR. Results were normalized against ACT housekeeping gene expression and are shown as the mean ± SD of three independent experiments. DOX, doxycycline (8 mg l⁻¹); FLC, fluconazole (4 mg l⁻¹). *P<0.01 versus control group.

Fig. 2. Effects of fluconazole and doxycycline on Ca²⁺ elevation in C. albicans biofilms. The cells were exposed to fluconazole and doxycycline for up to 24 h. At the indicated times, aliquots of cells were taken for staining with Fluo-3/AM. Ca²⁺ levels were determined by observing the fluorescence using flow cytometry. Each point represents the mean ± SD of three independent experiments. DOX, doxycycline (8 mg l⁻¹); FLC, fluconazole (4 mg l⁻¹).
inhibition of expression of efflux pump genes CDR1, CDR2 and MDR1. The possible reason for the discrepancy is that different antifungal agents (amphotericin B and fluconazole, respectively) were combined and different states of the isolates (planktonic cells and biofilms) were tested.

In fungi, calcium is a basal nutrient for the regulation of several processes as well as a vital second messenger in cell growth (Bader et al., 2006). Evidence has shown that cytosolic calcium entry in yeast cells is critical for survival under stresses (Cruz et al., 2002). It has been demonstrated that calcium influx is responsible for the fungicidal activity of amiodarone (Gupta et al., 2003). Furthermore, in previous research, we found that the synergistic effect of fluconazole and doxycycline could be enhanced by calcium channel blockers (benidipine and nifedipine) (Gao et al., 2013), which suggested that the combined drug-mediated growth inhibition seems to be related to calcium disturbance. In this study, intracellular calcium concentrations were detected to determine whether intercellular calcium changes in C. albicans biofilms could be induced by the combination of fluconazole and doxycycline. We observed that there was an increase of intracellular calcium levels after the 4 h intervention of fluconazole and it reached a peak at 8 h. An intracellular calcium burst was also induced by the combined drugs, which was more durable and stronger than that induced by fluconazole alone. Excessive intracellular calcium is known to be toxic (McConkey & Orrenius, 1997), therefore we speculate that one of the synergistic mechanisms is the high intracellular calcium level stimulated by fluconazole and doxycycline. A rise in cytoplasmic calcium has been shown to be responsible for Saccharomyces cerevisiae apoptosis (Pozniakovsky et al., 2005). Studies have suggested that H₂O₂-induced C. albicans biofilm apoptosis is associated with calcium ions and the downstream calcineurin/Crz1p/CaMCA1 pathway (Lu et al., 2011). However, whether the combined drug-induced high intracellular calcium level has a relationship with apoptosis needs further study.

The cell cycle is an evolutionarily conserved process in all eukaryotic cells, and it is used to control growth and division (Jacks & Weinberg, 1996). Cell cycle arrest could be induced by many agents in yeast, including amphotericin B (Phillips et al., 2003), amiodarone (Zhang & Rao, 2007), paclitaxel (Foland et al., 2005), etoposide (Sabourin et al., 2003) and camptothecin (Kauh & Bjornsti, 1995). In order to explore whether the drug combination of fluconazole and doxycycline induced the cell cycle arrest of C. albicans biofilms, the effect of the combined drugs on cell proliferation was determined. The results showed that increases in the percentage of cells in G₂/M phase and decreases in the percentage of cells in S phase were promoted by fluconazole alone. The drug combination of fluconazole and doxycycline caused arrest in the G₂/M phase to a weaker extent compared with the fluconazole-alone group. Those results suggested that fluconazole alone could cause cell cycle arrest and that the synergism was independent of cell cycle arrest.

This study discovered the potential synergistic mechanisms of fluconazole and doxycycline against C. albicans biofilms. The results showed that the synergism of fluconazole and doxycycline against C. albicans biofilms is associated with the blockade of efflux pump genes CDR1, CDR2 and MDR1. Furthermore, we discovered that stimulation of a high intracellular calcium concentration was induced by the combined drugs. The change of the calcium level presented here will open a new perspective for the discovery of synergistic mechanisms, and provide a clue for the development of drug combinations and new antifungal agents against C. albicans biofilms.

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