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

Yuan Gao,1,2 Hui Li,1 Shuyuan Liu,1 Xiang Zhang3 and Shujuan Sun4

1School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong Province, PR China
2Department of Pharmacy, First Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, PR China
3Department of Pharmacy, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong Province, PR China
4Department of Pharmacy, Qianfoshan Hospital Affiliated to Shandong University, Jinan, Shandong Province, PR China

*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 antimycotics (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, amphoterin 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 MDRI (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). In addition, a significant inhibition of fungal growth was found when calcium channel blockers (benidipine and nifedipine) were combined with fluconazole and doxycycline in this study.

Recent research has shown that cell cycle arrest (Jung *et al.*, 2007) and calcium homeostasis disturbance contribute to biofilm resistance (Ramage *et al.*, 2002; Watamoto *et al.*, 2011). 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). In addition, a significant inhibition of fungal growth was found when calcium channel blockers (benidipine and nifedipine) were combined with fluconazole and doxycycline (Gao *et al.*, 2013), which suggested that synergism may be related to the change of cytoplasmic calcium. In order to confirm this hypothesis, the cytosol calcium concentration was determined when *C. albicans* sessile cells were treated with fluconazole and doxycycline in this study.

Recently, with the emergence of widespread resistance to antifungal drugs, several therapeutic strategies have been explored, including targeting the cell cycle. Much of the current study has led to our understanding of the antifungal mechanism associated with cell cycle arrest. For example, amphoterocin B has already been demonstrated to induce apoptosis through arresting the cell cycle (Phillips *et al.*, 2003). The antifungal effects of amphotericin B, terpenoids and plagiclin E were also reported and were associated with cell cycle arrest (Jung *et al.*, 2007; Wu *et al.*, 2010; Zore *et al.*, 2011). As a consequence, cell cycle arrest was considered as one of the antifungal pathways.

Therefore, in this study we investigated the expression of the resistance genes CDR1, CDR2 and MDRI, the intracellular calcium concentration, and the distribution of the cell cycle in *C. albicans* biofilms after exposure to the doxycycline and fluconazole drug combination. Any discovery could be of great significance in finding new antifungal mechanisms.

**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, 512 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 × 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...
Intracellular calcium detection. The calcium-sensitive indicator Fluo-3/AM (Biotium) was used to evaluate the intracellular calcium levels change upon drug combination. The intracellular Ca$^{2+}$ 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 Ca$^{2+}$ 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 Ca$^{2+}$ 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 ($P<0.01$).

**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).

**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>GTTAGGTCTAAAGTGCAAGCTATC</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GTTTGGTCAATTACAGCAAGTCACAA</td>
</tr>
<tr>
<td>CDR1</td>
<td>F</td>
<td>TGCCAAGAATCTGCTCAAGAAT</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTGCAACACCCCATACCTCAGCT</td>
</tr>
<tr>
<td>CDR2</td>
<td>F</td>
<td>AGTGTGAACCAGACAGACTCA</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AGCCATTACCACCAAAAAAC</td>
</tr>
<tr>
<td>MDR1</td>
<td>F</td>
<td>GCTGCTACTACTGCTTCTGGTG</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGAAAACCAAACCGAACTAC</td>
</tr>
</tbody>
</table>
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
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.

ACKNOWLEDGEMENTS

We are grateful to Shandong Provincial Natural Science Foundation and Department of Science and Technology of Shandong Province, China (ZR2011HL049, 2010GWZ20217 and 2013GSF11848) for financial support and we thank the Medicinal Research Central

Fig. 3. Effects of fluconazole and doxycycline on the cell cycle of C. albicans biofilms. The cells exposed to fluconazole and doxycycline were collected, fixed with cold 70% ethanol and incubated in PBS containing RNase A. PI was added and allowed to stain for 30 min in the dark. The DNA contents were measured with FACSscan flow cytometry. DOX, doxycycline (8 mg l\textsuperscript{–1}); FLC, fluconazole (4 mg l\textsuperscript{–1}).
Laboratory of Qianfoshan Hospital for technical assistance as well as the Clinical Microbiology Room of Qianfoshan Hospital for providing the isolates used in this study.

REFERENCES


