RESEARCH ARTICLE

Synergistic effect of doxycycline and fluconazole against *Candida albicans* biofilms and the impact of calcium channel blockers

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Abstract

Candida albicans is a clinically important fungus and is capable of forming biofilms, which contributes to the emergence of fluconazole resistance. Here, sessile minimum inhibitory concentrations (SMICs) of fluconazole combined with doxycycline against biofilms of *C. albicans* were determined, and the results of SMICs were compared with minimum inhibitory concentrations (MICs) of planktonic cells. SMICs and MICs were determined by microdilution checkerboard method, and the interactions between two drugs were interpreted by two models of fractional inhibitory concentration index and the percentage of growth difference ($\triangle E$). For the biofilms formed over 4, 8, and 12 h, synergism was displayed by the combination of doxycycline(1–64 mg L^{-1}) and fluconazole, and the fluconazole SMIC reduced from 64–512 mg L^{-1} to 1–16 mg L^{-1} against all the tested isolates. Calcium homeostasis is an important factor in growth of *C. albicans*. In this study, the impact of calcium channel blocker on the drug combination was observed by plate streaking and determined by liquid methods quantitatively. Obvious enhancement of antifungal effect appeared by combination of three drugs. These results show us that fluconazole combined with doxycycline could be effective against *C. albicans* biofilm, and the combined antifungal mechanism is associated with calcium.

Introduction

Candida albicans is a common commensal organism of human skin and mucosal surfaces such as cornea, respiratory tract, oral cavity, bone marrow, intestines, bladder, and genital tract. However, it has emerged as a clinically important fungal pathogen that causes both mucosal and deep tissue infections (Pappas *et al.*, 2004) associated with a crude mortality rate of 62%(Gudlaugsson *et al.*, 2003). Azoles are one of several major options for invasive candidiasis, and fluconazole is used most commonly in azoles. However, long-term therapy with fluconazole facilitates the emergence of fluconazole resistance (Rex *et al.*, 1995). In addition, *C. albicans* commonly exist in biofilms and easily attach on both mucosal and plastic surfaces of indwelling devices (Hasan *et al.*, 2009). The biofilms are microbial communities that are distinct from their planktonic counterparts, and cells in biofilms bind tightly each other and display specific phenotypes (Ramage *et al.*, 2005). As the result, the resistance of biofilms to antifungal agent is increased compared with that of planktonic cells (Kuhn & Ghannoum, 2004).

Many approaches have been used to improve the effect of antimicrobial therapy for resistant isolates, and one of them is drug combination(Khan *et al.*, 2012). Synergism was observed when antifungal agents were combined with some nonantifungals, such as tacrolimus, cyclosporin A, and amiodarone (Guo *et al.*, 2008; Li *et al.*, 2008; Sun *et al.*, 2008). In addition, Fiori *et al.* demonstrated that doxycycline was synergistic with fluconazole against planktonic *C. albicans* at a concentration of 25 mg L^{-1} (Fiori & Van Dijck, 2012). Other reports showed that

high dose of doxycycline ($\geq$ 128 mg L^{-1}) in combination with traditional antifungal agents may be useful for the treatment of *C. albicans* biofilm infection (Miceli *et al.*, 2009), and synergistic effect was observed on biofilms of non-*albicans Candida* spp. with the concentration of 512 mg L^{-1} of doxycycline (El-Azizi, 2007). However, the effect of lower concentration of doxycycline in combination with fluconazole against biofilms of *C. albicans* needs to be investigated, and so does the distinction among the combined effects on *C. albicans* with different susceptibilities to fluconazole.

Calcium homeostasis is an important factor during normal growth of *C. albicans* and is associated with multiple aspects of fungal physiology, including morphogenesis, antifungal drug susceptibility, and virulence (Uppuluri *et al.*, 2008). The chelation of extracellular calcium or deletion of the calcium transporters that modulate cytosolic calcium could severely reduce expression of some calcium transport proteins (Mid1, Cch1, or Pmr1), which influence the normal functions of *C. albicans* (Brand *et al.*, 2009). Most of the combined antifungal activities are mediated by calcium regulation. Gupta *et al.* (2003) proved that antifungal activity of amiodarone is mediated by disruption of calcium homeostasis in *C. albicans*. In a preliminary study, we found that the combination of minocycline and fluconazole had synergistic effects on the biofilms of *C. albicans*, and benidipine (calcium channel blocker, antihypertensive, and anti-anginal drug used in a clinic) could significantly enhance this synergistic effect (Shi *et al.*, 2010). However, little is known about the effect of the calcium channel blocker combined with doxycycline and fluconazole.

In this study, we investigated the *in vitro* combined antifungal activity of doxycycline (1–64 mg L^{-1}) and fluconazole against biofilms of *C. albicans* with different susceptibilities (azole susceptible, azole-susceptible dose dependent, and azole resistant) by a microdilution checkerboard method, and such drug combination against planktonic *C. albicans* was also performed as a contrast to the result of biofilms. In addition, we evaluated the impact of calcium channel blocker (benidipine and nifedipine) on the drug combination of fluconazole and doxycycline against the planktonic cells and biofilms of *C. albicans* by plate streaking and liquid quantitative methods in order to find the clue for the further research on the synergistic mechanisms.

Materials and methods

Candida albicans isolates and medium

Ten *C. albicans* strains isolated from different clinical specimens with invasive fungus infection were tested in this study. They were identified according to standardized mycological methods (Pfaller *et al.*, 2010). The susceptibilities of tested isolates to fluconazole were determined by broth dilution test according to the CLSI M27-A3 standard (Clinical. aLSI, 2008), and the break points are described as follows: minimum inhibitory concentration (MIC) $\leq$ 8 mg L^{-1} (susceptible),MIC $\geq$ 64 mg L^{-1} (resistant), 16 and 32 mg L^{-1} (susceptible dose dependent)(Pfaller *et al.*, 2008a). The strains were azole susceptible (CA1, CA5, CA12, CA14, CA15, CA135), azole-susceptible dose dependent (CA137), and azole resistant (CA10, CA16, CA129), and they were maintained at −70 °C. The *C. albicans* (ATCC 10231) were used as quality control strains.

The isolates were propagated on yeast–peptone–dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar; Ding Guo Biological Co. Ltd., China) at 35 °C and subcultured at least twice before experiments to ensure purity and viability. The cells were washed and diluted in sterilized phosphate-buffered saline (PBS; pH 7.2–7.4; Solarbio Science & Technology Co. Ltd., China). The liquid medium used for susceptibility testing was RPMI 1640 (GIBCO BRL, Life Technologies, Woerden, the Netherlands) with the pH adjusted to 7.0. The medium was supplemented with dextrose to a final concentration of 2% and 0.165 M morpholinepropanesulfonic acid (MOPS; Ding Guo Biological Co. Ltd.) (Diekema *et al.*, 2009).

Drugs

The powders of fluconazole, doxycycline, and calcium channel blocker (benidipine and nifedipine) were used in this study. Fluconazole was provided by Cheng Chuang Pharmaceutical Co. Ltd., China; doxycycline and calcium channel blocker were purchased from the An Bei Ka Pharmaceutical Co. Ltd., China. Fluconazole was dissolved at a 2.56 g L^{-1} stock solution with distilled water. Doxycycline was prepared at a 6.4 g L^{-1} stock solution with dimethyl sulfoxide. Benidipine and nifedipine were dissolved at 2.56 g L^{-1} stock solutions with dimethyl sulfoxide, respectively, and the content of dimethyl sulfoxide in each assay was kept below 0.5%. These stock solutions were kept in storage at −70 °C until use.

Determination of MICs of planktonic cells

The interaction between doxycycline and fluconazole against ten *C. albicans* strains was tested according to methods of the CLSI M27-A3 (Clinical. aLSI, 2008) by a microdilution checkerboard technique in 96-well plates. The concentration of yeast suspension was standardized to 1×10^3 cells mL^{-1} in each well of plates with RPMI 1640 medium. The final concentration of fluconazole ranged from 1 to 512 mg L^{-1} for R and S-DD isolates (CA10, CA16, CA129, and CA137) and from 0.03125 to 16 mg L^{-1} for S isolates (CA1, CA5, CA12, CA14, CA15,

and CA135). The final concentration of doxycycline ranged from 0.5 to 32 mg L^{-1} for all strains. The control wells were filled with yeast suspension and RPMI 1640 without antifungal agents. The 96-well plates were incubated at 35 °C for 48 h. A colorimetric reduction assay was carried out with 2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tet razolium hydroxide (XTT; Sigma) (Melo *et al.*, 2007). The values of optical density (OD) were measured at 490 nm with a microtiter plate reader (Thermolabsystems Multiskan MK3). The MIC endpoint was defined as the lowest drug concentration that caused an 80% inhibition compared with that of drug-free growth control (Pfaller *et al.*, 2008b). Each testing was carried out in duplicate and repeated three times on different days. The MIC of control strain ATCC 10231 was within the prescript of CLSI.

Determination of sessile minimum inhibitory concentrations (SMICs) of biofilms

Biofilms were formed as described by Ramage *et al.* (2001) on 96-well plates in modified cell suspensions being 1×10^3 cells mL^{-1}, and we demonstrated that biofilm could form with this cellular density over at least 4-h cultivation. The biofilms were formed over four time intervals (4, 8, 12, and 24 h) at 37 °C by pipetting 100 μL of the standardized cell suspension into selected wells of 96-well plate. At each time point, the biofilms were washed three times gently with sterile PBS to remove the planktonic yeast. Fluconazole and doxycycline were then added to the biofilms in serially double-diluted concentrations. The final concentration of fluconazole in wells ranged from 1 to 512 mg L^{-1} for resistant isolates (CA10, CA16, CA129), from 0.5 to 256 mg L^{-1} for susceptible dose-dependent isolates (CA137), and from 0.25 to 128 mg L^{-1} for susceptible isolates (CA14, CA135, CA1, CA5, CA15, CA12). The final concentration of doxycycline in wells ranged from 1 to 64 mg L^{-1} for all strains. The control wells were filled with RPMI 1640 without antifungal agents. Then the whole system was incubated for a further 48 h at 35 °C. A colorimetric reduction assay was carried out with XTT according to the protocol of Melo *et al.* (2007). The absorbance was measured with a microtiter plate reader (Thermolabsystems Multiskan MK3) at 490 nm. The drug concentration that brought about a reduction in absorption by 80% contrasted to control well was reported as the SMIC endpoint. Each testing was carried out in duplicate and repeated three times on different days.

Drug interaction interpretation

In this study, the drug combination interaction was interpreted according to two models based on Loewe additivity (LA) and Bliss independence (BI) theories, respectively (Prichard & Shipman, 1990; Polak, 1999; Sun *et al.*, 2009), including the fractional inhibitory concentration index (FICI) and the percentage of growth difference ($^{\triangle}E$). In FICI model, the difference between effective concentration of drugs alone and in combination was compared. In $^{\triangle}E$ model, the difference between estimated combined effects based on the effects of the individual drugs and those obtained from the experiment was compared.

FICI model

The nonparametric model FICI was defined as follows: $FICI = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B$, where MIC_A and MIC_B are the MICs of the drugs A and B used alone, and C_A and C_B are the MICs of the drugs A and B used in combination. The interpretation of the FICI was as Odds (Odds, 2003) suggested that: the value of $FICI \leq 0.5$ revealed synergy, $0.5 < FICI \leq 4$ revealed no interaction, and $FICI > 4$ represented antagonism.

ΔE model

The nonparametric model ΔE was based on the BI theory, where E is the percentage of growth. $E_i = E_A \times E_B$, where E_i is the predicted percentage of growth of the combination of drugs A and B, and E_A and E_B are growth percentage of drugs A and B used alone. Interaction between two drugs is described by the difference (ΔE) between the predicted and measured percentages of growth at various combinations. In the nonparametric approach described by Prichard *et al.* (1991, 1993), E_A and E_B are obtained directly from experimental data. When the average value of ΔE was positive and the 95% confidence interval (CI) among the three replicates did not include 0, SS (statistically significant) synergy was claimed; when the average value of ΔE was negative and the 95% CI did not include 0, SS antagonism was claimed.

To summarize the interaction between two drugs, the sums of the percentages of all SS synergistic (SSYN) and SS antagonistic (SANT) interactions were calculated. Statistically values of < 100% meant a considered weak interaction, those of 100–200% meant a considered moderate interaction, and those of > 200% meant a considered strong interaction (Meletiadis *et al.*, 2003).

The impact of calcium channel blocker on drug combination

The impact of calcium channel blocker (benidipine and nifedipine) on drug combination was investigated by

plate-streaking method firstly. The strains used in this experiment were CA1, CA12, CA137, and CA10. The concentration of yeast suspension was standardized to 1×10^5 cells mL^{-1} with PBS. Melting YPD agar was put into the culture plates with the volume of 20 mL. Drugs were added into the medium, and the final concentration was 2 mg L^{-1} for fluconazole, 32 mg L^{-1} for doxycycline, 16 mg L^{-1} for benidipine, and 8 mg L^{-1} for nifedipine. The yeast suspension was spread onto the solidified medium. At last, the plates were incubated at 35 °C for 24 h.

In order to explore the impact of calcium channel blocker on the drug combination of fluconazole and doxycycline and determine how this impact varies as the concentration of calcium channel blocker changes, a quantitative analysis in 96-well plates was performed. Planktonic cells and biofilms were prepared in the 96-well plates as described above. A given concentration of fluconazole and doxycycline was chosen in order to distinguish the difference in the impact of calcium channel blocker with various concentrations. The solution of benidipine and nifedipine was diluted with RPMI 1640 to the concentrations of 1, 2, and 4 mg L^{-1} and 4, 6, and 8 mg L^{-1}, respectively. And 1 mg L^{-1} for benidipine and 4 mg L^{-1} for nifedipine are minimum effective concentrations as a result of our preliminary experiments. The 96-well plates were incubated for 48 h at 35 °C. Activities of cells in planktonic cells and biofilms were assessed by XTT assay (Melo *et al.*, 2007). OD value was measured by a microtiter plate reader (Thermolabsystems Multiskan MK3) at 490 nm. Each testing was carried out in duplicate and repeated three times on different days. Statistical significance of the results was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test.

Results

MICs of fluconazole in combination with doxycycline

The results of the tested drugs alone and in combination against planktonic *C. albicans* were summarized in Table 1. It showed that doxycycline had little effects on all strains when it was used alone; however, a potent antifungal effect was observed when in combination with fluconazole. The effects varied against strains with different susceptibilities to fluconazole. For resistant strains, an obvious synergism was observed. The MIC of fluconazole was reduced nine and seven dilutions for CA16 and CA10 with 8 mg L^{-1} of doxycycline, respectively, and the MICs of fluconazole were reduced six dilutions for CA129 in the presence of 2 mg L^{-1} of doxycycline. For susceptible dose-dependent strains (CA137), the MIC was reduced four dilutions in the presence of 1 mg L^{-1} of doxycycline. However, for susceptible strains, little synergism was observed, and antagonism was observed in a few wells. The differences in the MICs reduction against different isolates suggested that the synergism mediated by doxycycline was related to the susceptibilities of *C. albicans*. The growth percentage of each drug combination was calculated by OD values of the drug-containing wells compared with those of the drug-free control wells. Synergism was consistently interpreted by FICI and ΔE models for three resistant isolates and one susceptible dose-dependent isolate. However, different interpretations were found by these two models in six susceptible isolates.

Table 1. Combined antifungal effect of fluconazole alone and in combination with doxycycline against planktonic *Candida albicans*

	MICs (mg L^{-1})								
	Drug alone		Drug in combination		FICI model		ΔE model		
Strains	FLC	DOX	FLC	DOX	Median	INT	∑SYN (%)	∑ANT (%)	INT
CA14	0.25	512	0.25	0.5	1.001	IND	162.87	−236.67	ANT
CA135	0.25	512	0.25	0.5	1.001	IND	208.69	−279.58	ANT
CA1	0.5	512	0.5	0.5	1.001	IND	107.91	−368.63	ANT
CA5	1	512	1	0.5	1.001	IND	158.42	−259.62	ANT
CA15	1	512	1	0.5	1.001	IND	156.43	−252.64	ANT
CA12	8	512	8	0.5	1.001	IND	106.45	−215.56	ANT
CA137	16	512	1	1	0.064	SYN	1894.67	−15.31	SYN
CA129	64	512	1	2	0.020	SYN	2041.34	−6.43	SYN
CA10	512	512	4	8	0.023	SYN	1327.06	−30.20	SYN
CA16	512	512	1	8	0.018	SYN	1686.22	−7.72	SYN

MICs were read as the lowest concentrations that produced an 80% reduction in growth compared with that of the drug-free control in planktonic cells. The values of MICs were the median of three times. FICI and ΔE were two drug interaction models. For the ΔE model, ∑SYN and ∑ANT were the sums of the percentages of all synergistic and antagonistic interactions.

FLC, fluconazole; DOX, doxycycline; INT, interpretation; SYN, synergism; ANT, antagonism; IND, indifference.

SMICs of fluconazole in combination with doxycycline

Ten isolates with different susceptibilities to fluconazole were studied in this experiment. A consistent synergistic effect of fluconazole and doxycycline was interpreted by FICI and ΔE models against *C. albicans* biofilms formed over 4, 8, and 12 h. However, as the biofilms matured and its complexity increased, the synergism weakened and was scarcely observed on the biofilms that formed over 24 h (not shown). For each strain, the different combined antifungal effect appeared and was shown in Table 2.

The impact of calcium channel blocker on drug combination

The impact of calcium channel blocker (benidipine and nifedipine) on drug combination of doxycycline and fluconazole was evaluated by plate-streaking method, and the results against four isolates were shown in Fig. 1. The fungi in control group grew well as expected. Little effect was observed in single-agent group including doxycycline, benidipine, and nifedipine, and a similar result was obtained when doxycycline was combined with benidipine and nifedipine. In order to evaluate the effect of the combined drugs, low concentration of fluconazole was used

Table 2. Combined antifungal effect of fluconazole alone and in combination with doxycycline against biofilms of *Candida albicans*

		SMICs (mg L^{-1})								
		Drug alone		Drug in combination		FICI model		ΔE model		
Strains	Time (h)	FLC	DOX	FLC	DOX	Median	INT	∑SYN (%)	∑ANT (%)	INT
CA14 (S)	4	64	512	1	2	0.020	SYN	2743.05	−13.56	SYN
	8	128	512	1	4	0.016	SYN	2693.04	−12.53	SYN
	12	128	512	1	8	0.023	SYN	2945.93	−30.45	SYN
CA135 (S)	4	64	512	1	4	0.023	SYN	3523.64	−3.45	SYN
	8	128	512	1	8	0.023	SYN	2745.24	−12.35	SYN
	12	128	512	1	8	0.023	SYN	2689.51	−13.64	SYN
CA1 (S)	4	128	512	1	4	0.016	SYN	2834.65	−9.43	SYN
	8	128	512	1	16	0.039	SYN	2456.21	−17.43	SYN
	12	128	512	2	16	0.047	SYN	2047.87	−20.56	SYN
CA5 (S)	4	128	512	1	8	0.023	SYN	3024.74	−10.45	SYN
	8	128	512	2	8	0.031	SYN	2134.95	−10.32	SYN
	12	128	512	4	16	0.062	SYN	1943.65	−14.53	SYN
CA15 (S)	4	128	512	1	4	0.016	SYN	2431.90	−5.74	SYN
	8	128	512	2	16	0.047	SYN	1794.34	−22.43	SYN
	12	128	512	4	16	0.062	SYN	1647.86	−21.60	SYN
CA12 (S)	4	64	512	2	4	0.039	SYN	1732.53	−31.23	SYN
	8	128	512	4	16	0.062	SYN	1346.86	−42.53	SYN
	12	128	512	8	32	0.125	SYN	1023.58	−38.82	SYN
CA137 (SDD)	4	256	512	2	8	0.023	SYN	1953.42	−24.64	SYN
	8	256	512	4	8	0.031	SYN	1830.54	−32.54	SYN
	12	256	512	8	16	0.062	SYN	1689.43	−31.84	SYN
CA129 (R)	4	256	512	1	16	0.035	SYN	1632.54	−24.35	SYN
	8	256	512	4	16	0.047	SYN	1253.43	−31.05	SYN
	12	256	512	8	16	0.062	SYN	1043.25	−52.53	SYN
CA10 (R)	4	512	512	1	16	0.033	SYN	1632.53	−45.23	SYN
	8	512	512	2	32	0.066	SYN	1023.58	−50.34	SYN
	12	512	512	4	32	0.070	SYN	846.74	−86.97	SYN
CA16 (R)	4	512	512	1	32	0.064	SYN	1245.35	−36.86	SYN
	8	512	512	2	32	0.066	SYN	821.34	−73.45	SYN
	12	512	512	16	64	0.156	SYN	723.42	−67.93	SYN

Time indicated the incubation period of biofilm formation. SMICs were read as the lowest concentrations that produced an 80% reduction in growth compared with that of the drug-free control. The values of SMICs were the median of three times. FICI and ΔE were two drug interaction models. For the $^{\triangle}E$ model, ∑SYN and ∑ANT were the sums of the percentages of all synergistic and antagonistic interactions.
INT, interpretation; FLC, fluconazole; DOX, doxycycline; SYN, synergism; S, azole-susceptible; SDD, azole-susceptible dose-dependent; R, azole--resistant.

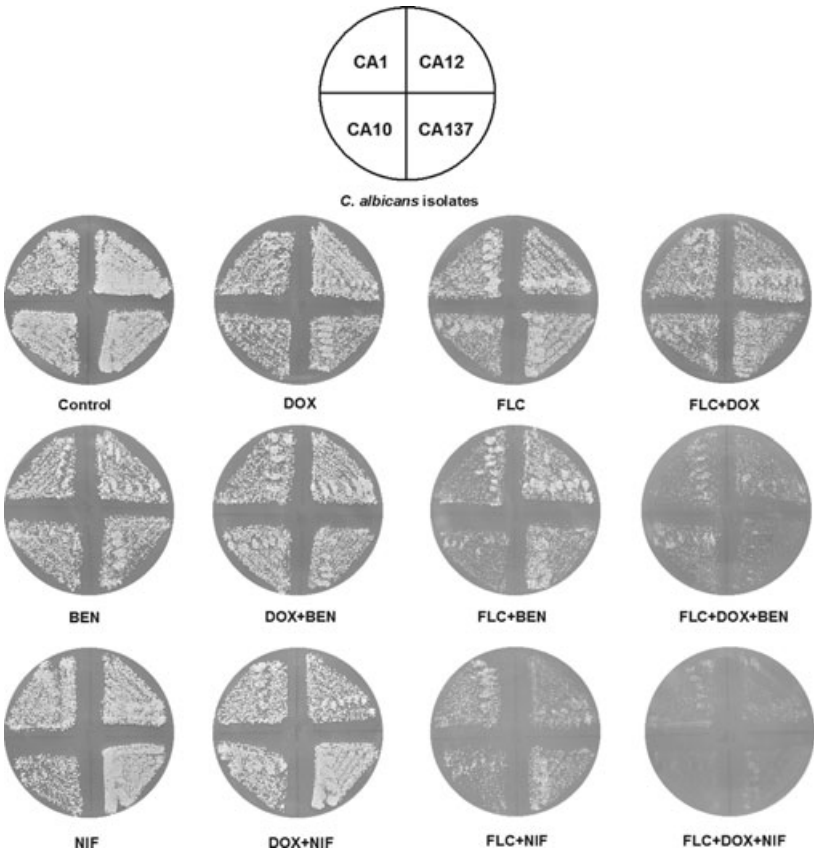

Fig. 1. Effect of calcium channel blocker on the combination of fluconazole (FLC) and doxycycline (DOX) against *Candida albicans*. The tested isolates on the plate were CA1, CA12, CA137, and CA10. The concentrations were 2 mg L^{-1} for FLC, 32 mg L^{-1} for DOX, 16 mg L^{-1} for benidipine(BEN) and 8 mg L^{-1} for nifedipine (NIF).

in the plate-streaking test, so no obvious effect was observed when fluconazole was used alone against isolates with different susceptibilities. The addition of doxycycline or calcium channel blocker (benidipine and nifedipine) with fluconazole inhibited the fungal growth compared with fluconazole used alone, and the impact of calcium channel blocker (especially nifedipine) seemed stronger than doxycycline. Furthermore, we were surprised to find that addition of calcium channel blocker to drug combination of doxycycline and fluconazole enhanced the effect dramatically, the growth of the fungi was almost inhibited completely by the three drug combination of fluconazole, doxycycline, and nifedipine, and the effect was observed on both fluconazole-resistant and fluconazole-susceptible strains.

To explore the impact of different concentrations of calcium channel blocker on the drug combination of fluconazole and doxycycline, a liquid analysis was carried out quantitatively. Planktonic cells and biofilms of four strains (CA10, CA16, CA129, and CA137) were studied in this test. As shown in Fig. 2, a reduction in growth percentage was displayed when benidipine was added to drug combination of doxycycline and fluconazole, and progressive reductions were achieved with benidipine concentration increased. In planktonic cells (a), the effect of benidipine on the resistant strain of CA10 and CA16 is more apparent than that on CA129 and CA137, while in biofilms (b), no significant differences among four isolates were found. Compared with biofilms, sharper reductions in the growth percentage in planktonic cells appeared after the addition of benidipine. As shown in Fig. 3, decrease in growth percentages was caused by addition of nifedipine to drug combination of fluconazole and doxycycline, and the higher the concentration of nifedipine added, the better the effect achieved. In planktonic cells (a), the growth percentage reduction in CA16 was less than that in CA10, CA129, and CA137, while in biofilms (b), the effect of nifedipine on CA10 and CA16 was more apparent than that on CA129 and CA137. For the two calcium channel blockers, the antifungal activity was similar against the biofilms formed over different time intervals.

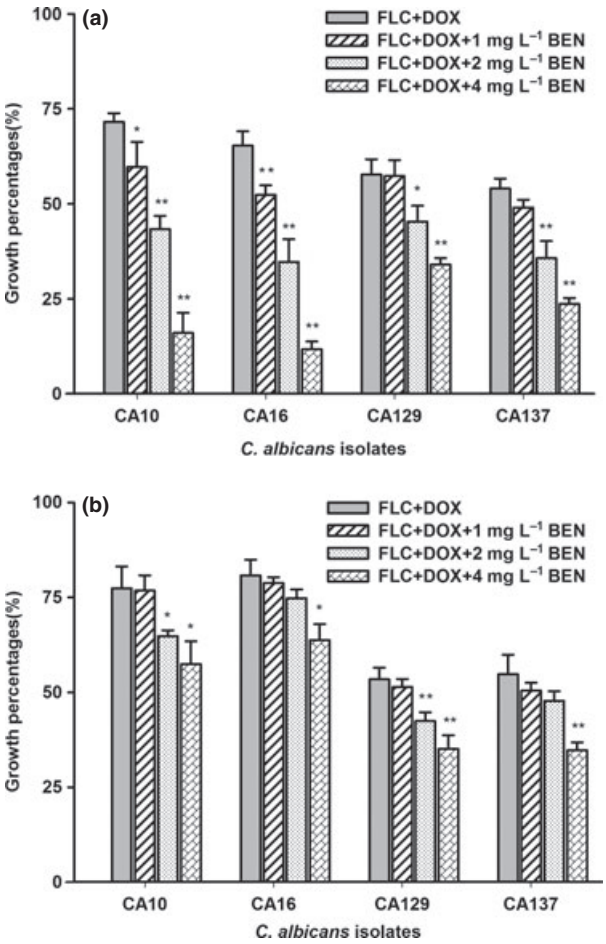

Fig. 2. Effect of different concentrations of benidipine (BEN) on the drug combination of fluconazole (FLC) and doxycycline (DOX). The chart bar meant the growth percentages of *Candida albicans* including CA10, CA16, CA129, and CA137. The growth percentages of the yeast suspension incubated in RPMI 1640 without antifungal agents were considered as 100%. (a) The effect of BEN on drug combination of FLC and DOX against planktonic cells; (b) the effect of BEN on drug combination of FLC and DOX against biofilms. In planktonic cells, the concentrations of FLC were 1 mg L^{-1} for CA10 and CA16, 1 mg L^{-1} for CA129 and CA137, and the concentrations of DOX were 4 mg L^{-1} for CA10 and CA16, 0.5 mg L^{-1} for CA129 and CA137. In biofilms, the concentrations of FLC were 2 mg L^{-1} for CA10 and CA16, 2 mg L^{-1} for CA129 and CA137, and the concentrations of DOX were 16 mg L^{-1} for CA10 and CA16, 4 mg L^{-1} for CA129 and CA137. The concentrations of BEN were 1, 2, and 4 mg L^{-1}. *P* values are effect of FLC, DOX and BEN compared to that of FLC and DOX. **P* < 0.05; ***P* < 0.01.

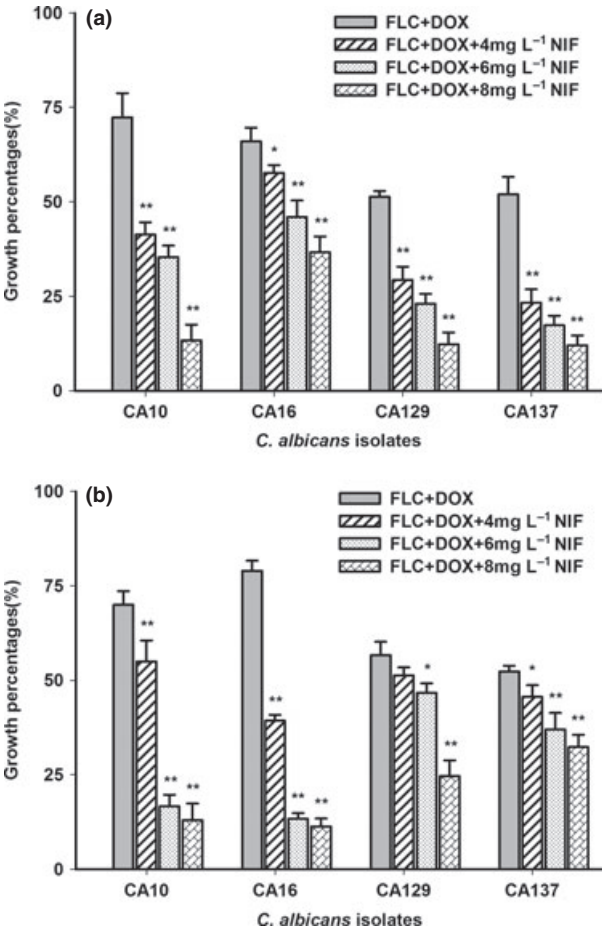

Fig. 3. Effect of different concentrations of nifedipine (NIF) on the drug combination of fluconazole (FLC) and doxycycline (DOX). The chart bar meant the growth percentages of *Candida albicans* including CA10, CA16, CA129, and CA137. The growth percentages of the yeast suspension incubated in RPMI 1640 without antifungal agents were considered as 100%. (a) The effect of NIF on drug combination of FLC and DOX against planktonic cells; (b) the effect of NIF on drug combination of FLC and DOX against biofilms. In planktonic cells, the concentrations of FLC were 1 mg L^{-1} for CA10 and CA16, 1 mg L^{-1} for CA129 and CA137, and the concentrations of DOX were 4 mg L^{-1} for CA10 and CA16, 0.5 mg L^{-1} for CA129 and CA137. In biofilms, the concentrations of FLC were 2 mg L^{-1} for CA10 and CA16, 2 mg L^{-1} for CA129 and CA137, and the concentrations of DOX were 16 mg L^{-1} for CA10 and CA16, 4 mg L^{-1} for CA129 and CA137. The concentrations of NIF were 4, 6, and 8 mg L^{-1}. *P* values are effect of FLC, DOX and NIF compared to that of FLC and DOX. **P* < 0.05; ***P* < 0.01.

Discussion

The marked increase in the emergence of fluconazole-resistant *C. albicans* isolates is on account of various reasons including decreased permeability of membranes, interference with target enzyme lanosterol 14a-demethylase, decreased accumulation of antifungal agents by enhanced efflux systems, qualitative or quantitative change in ergosterol, and biofilm formation. Doxycycline is a tetracycline derivative, which is considered to be an antibacterial agent; however, it was found to act synergistically with antifungal agents against *C. albicans*. Oliver *et al.* (2008) showed that tetracycline can alter antifungal drug susceptibility of amphotericin B and terbinafine in

C. albicans and other pathogenic fungi. Miceli *et al.* (2009) demonstrated that combination of high-dose doxycycline and azoles showed synergism against biofilms of *C. albicans* and non-*albicans Candida* spp., and a recent study showed that addition of relatively low concentration of doxycycline has a impact on prevention of fluconazole tolerance against planktonic *C. albicans* (Fiori & Van Dijck, 2012). Here we characterize the antifungal activity of fluconazole in combination with a given concentration of doxycycline (1–64 mg L^{-1}) against *C. albicans* biofilms, and in contrast, the drug combination against planktonic *C. albicans* was also performed. The results of microdilution checkerboard method showed that fluconazole and doxycycline had strong synergism against planktonic cells in fluconazole-resistant isolates. However, almost no synergy was observed in fluconazole-susceptible isolates, and discrepancy in interpretations made by FICI and $\triangle E$ models appeared perhaps because of the different theories that they depended on. Nevertheless, both FICI and $\triangle E$ are still useful approaches for analyzing the interactions between different drugs in spite of some disagreement. As for biofilms, significant synergistic effect of fluconazole in combination with doxycycline has emerged against almost all *C. albicans* biofilms that formed over three time intervals (4, 8, and 12 h). In addition, a weaker combined antifungal effect of fluconazole and doxycycline against *C. albicans* biofilms was observed compared with planktonic cells, and the combined efficacy decreased with the prolonged time of biofilm formation. At the same time, we found that SMICs of fluconazole alone increased compared with MICs in almost all isolates.

Calcium is an important second messenger in developmental and stress signaling pathways of *C. albicans* (Yang *et al.*, 2011). Uppuluri *et al.* (2008) documented that combinations of calcineurin inhibitor (tacrolimus and cyclosporin A) and fluconazole could prevent or eradicate biofilm-associated cells in *C. albicans*. Gamarra *et al.* (2010) suggested that the drug synergism of amiodarone and fluconazole is likely mediated by Ca^{2+} stress, and they pointed out that amiodarone causes the hyperexpression of vacuole Ca^{2+} pumps and calcineurin pathway genes. Benidipine is a non-selective calcium channel blocker that blocks Ca^{2+} channels including L- and T-type (Abe *et al.*, 2011), and it is used commonly as antihypertensive and anti-anginal drug in clinical therapy. The preliminary study in our laboratory has found that the calcium channel blocker including benidipine altered the antifungal effect of fluconazole in combination with minocycline (Shi *et al.*, 2010). In order to evaluate the potent relationship between calcium channel blocker and synergistic effect mediated by fluconazole and doxycycline, benidipine and nifedipine (a selective L-type calcium channel blocker) (Rouet *et al.*, 2012) were added to drug combination in this study. In a plate-streaking method, a surprising result was discovered that a sharp inhibition of fungal growth appeared when calcium channel blocker was added into drug combination of fluconazole and doxycycline, and the effect of nifedipine is better than that of benidipine. In contrast, obvious weakening of fungal growth was observed when nifedipine was combined with fluconazole, and this antifungal activity was more obvious than that brought about by combination of fluconazole and doxycycline. In order to determine the exact impact of calcium channel blocker, a liquid method was undertaken quantitatively using planktonic cells and biofilms of the four *C. albicans* isolates. Enhancement of antifungal activity was observed by addition of benidipine and nifedipine to drug combination of fluconazole and doxycycline, and sharper reduction in fungal growth percentage was presented with the higher concentration of calcium channel blocker. In planktonic cells, better effects of benidipine were achieved against the isolates with stronger resistance to fluconazole, while no obvious regularity was found with nifedipine. In biofilms, the effects of benidipine on all four isolates were similar, while the effect of nifedipine is more obvious on the isolates with stronger resistance to fluconazole (Figs 2 and 3). These findings disclosed that the doxycycline-mediated synergism can be enhanced by benidipine and nifedipine, which suggested that the blocking of calcium channel can promote the combined effect of fluconazole and doxycycline. Fiori and collaborators reported that doxycycline-mediated growth inhibition can be reversed by externally added iron, which indicates that iron depletion may account for the synergism (Fiori & Van Dijck, 2012). In this study, it is possible that calcium homeostasis may account for the synergism since calcium is another indispensable ion in *C. albicans*. However, further study is needed to investigate the relationship of various effects caused by different calcium channel blockers and the potential mechanisms involved in the expression of some proteins and genes, such as Ca^{2+} transporters of *C. albicans*, including pumps (Pmr1 and Pmc1), channels (Cch1, Mid1, and Yvc1), exchangers(Vcx1), and transcription factor (RIM101) involved in the cation overload response (Cui *et al.*, 2009).

Candida albicans biofilms are complex microbial communities consisting of yeast, hyphae, and pseudohyphae (Sudbery *et al.*, 2004). The structure of biofilms maintains stable, and they are highly resistant against most commonly used antimycotics. Ramage *et al.* (2002) demonstrated that *C. albicans* biofilm resistance is multifactorial and cannot be explained by one mechanism alone, and expression of genes encoding efflux pump including CDR1, CDR2, and

MDR1 was upregulated during the formation and development of *C. albicans* biofilms. Our study showed that in the presence of doxycycline and fluconazole, a great growth inhibition against resistant *C. albicans* and *C. albicans* biofilms appeared. The relationship between this synergistic effect and resistance mechanism including efflux pump genes needs further study.

In conclusion, this study suggested that the antifungal effect of fluconazole could be sensitized by doxycycline against *C. albicans* biofilms and resistant *C. albicans*, and the synergism of this combination can be enhanced by calcium channel blocker. This result is of great significance in looking for new antifungal agents and provides a reliable basis to further exploration of calcium-mediated antifungal mechanism. The mechanisms including the expression of Ca^{2+} transporters and efflux pump genes in *C. albicans* are under research in our laboratory now.

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Authors' contributions

Y.G. and C.Z. contributed equally to this work.

Conflict of interest

We declare that we have no conflict of interest.

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