In vitro Antifungal Effect of Caspofungin-Fluconazole Sequential Treatment Against Mixed *Candida albicans* and *Candida glabrata* Biofilm

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Abstract

Candidiasis is the most prevalence opportunistic fungal infection of humans, and invasive *Candida* infections remain an important cause of morbidity and mortality, especially in hospitalized and immunocompomised patients. Recent investigation revealed that simultaneous combination of caspofungin and fluconazole appeared to affect the quantity and cell architecture of *C. albicans* and *C. glabrata* mixed biofilm *in vitro*. The objective of this study was to further investigate the effect of caspofungin (CAS) sequentially combined with fluconazole (FLU) on the vitality and quantity of mixed *C. albicans* and *C. glabrata* biofilm. Viability of biofilms was evaluated by 2,3-bis(2-methoxy-4-nitro-5-

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sulfophenyl)-5[(phenylamino)carbonyl)]-2H-tetrazolium hydroxide (XTT) assay and biomass of biofilm was assessed by crystal violet assay. The inhibitory effects of sequential CAS-FLU combinations on the biomass of biofilm were significantly declined when mixed biofilms were pretreated with FLU for a longer period of time over 3 h ($p < 0.001$), however, the vitality of FLU-pre exposed mixed biofilms in response to CAS-FLU combination treatment were not different. The biomass reduction effect of CAS-FLU combinations was dependent on the sequence of initial of drugs combination and the pre-exposure time. Conclusion, sequential CAS-FLU combinations treatment had an impact on the biomass but not viability of mixed *C. albicans* and *C. glabrata* biofilm.

Keywords: biofilm; *Candida albicans*; *Candida glabrata*; Caspofungin; Fluconazole

1. Introduction

There has been a significant increase in the incidence of fungal infections with yeast of the genus *Candida* becoming the fourth most common causes of nosocomial blood stream infections (Wisplinghoff *et al*., 2004). Candidiasis has been attributed to *Candida albicans*; however, infections caused by non-*albicans Candida* (NAC) species, such as *C. glabrata*, *C. tropicalis* and *C. parapsilosis* are increasingly being recognized and continuously reported (Horn *et al*., 2009; Muadcheingka *et al*., 2015). Clinical study showed that *C. albicans* and *C. glabrata* were often coisolated in infection (Redding *et al*., 2004) and the degree of infection was worsened by the presence of both species (Coco *et al*., 2008).

Biofilms are organized communities of microorganisms that grow and embed in a self-produced extracellular polymeric matrix on an abiotic or biotic surface. *Candida* species are able to attach to polymeric surfaces and generate a biofilm structure, protecting the organisms from the host defenses and antifungal drugs (Chandra *et al*., 2001b; Ramage *et al*., 2001). Treatments of this infection are restricted due to limited classes of antifungal agents. For the last 20 years, triazole drugs have commonly been used to treat fungal infections including those caused by *C. albicans*. Fluconazole (FLU) disturbs synthesis of ergosterol in cell membrane resulting in growth arrest (Taff *et al*., 2013). FLU is generally effective against candidiasis but its use may be limited by the increasing prevalence of *Candida* species with acquired or intrinsic resistance (Flevari *et al*., 2013). The echinocandins represent a novel class of antifungal agent which has been introduced to clinical practice. Caspofungin (CAS) is a fungicidal, water-soluble semisynthetic echinocandin that inhibits the synthesis of β-1,3-glucan, the major structural component of *Candida* cell walls, resulting in osmotic instability and fungal cell lysis. CAS is recommended as first-line treatment for candidemia/invasive candidiasis in all patient (Flevari *et al*., 2013).

Due to their distinct mechanisms of action, several studies on the activity of antifungal combinations against *Candida* biofilms have been studied in order to improve the

activity against *Candida* biofilms and *Candida* biofilm-associated infections (Kontoyiannis *et al*., 2004; Pesee *et al*., 2016; Sarkar *et al*., 2014). In refractory cases of invasive fungal infections, combinations of triazoles and echinocandins have been studied as promising therapies to reduce high attributable mortality rates (Kontoyiannis *et al*., 2004). However, clinical fungal infections were usually ascribed to two or more fungal pathogens co-inhabited in certain cites. Moreover, not all fungal pathogens could live together to form mixed biofilms. According to previous studies, *Candida albicans* could co-exist with *C. glabrata* in oral cavity (Pathak *et al.*, 2012; Silva *et al.*, 2011). In addition, our recently study revealed that simultaneous combination between CAS and FLU was appeared to affect the quantity and cell architecture of mixed *C. albicans* and *C. glabrata* biofilm (Pesee *et al*., 2016). Nevertheless, sequential therapy investigation demonstrated that pre-exposure of single species *C. albicans* biofilms with FLU lead to a significant decrease of the efficacy of CAS (Sarkar *et al*., 2014). Therefore, this study aimed to further investigate the impact of sequential therapy with CAS and FLU on the vitality and biomass of mixed *C. albican* and *C. glabrata* biofilm.

2. Materials and Methods

2.1 Organisms

C. glabrata DMST46683 and *C. albicans* ATCC10231 were kindly supported by Department of Medical Sciences, Ministry of Public Health, Thailand. Stock cultures were divided into small portions and stored at - 80 ^{o}C in 20 % glycerol tryptone soil broth.

2.2 Antifungal agents

CAS was provided by Merck Sharp & Dohme Limited, and FLU was supported by Hetero Thailand Limited. Both were pharmaceutical grade and obtained in powder form in sterile vial. CAS and FLU were reconstituted in sterile distilled water before tested.

2.3 Culture condition

Microorganisms were grown in Sabouraud dextrose broth (SDB) (Difco Laboratories, Detroit, MI). Briefly, fifty milliliters of SDB medium was inoculated with a loopful of *Candida* from thawed stock cultured and incubated on the orbital shaker (Stuart, SI500, UK) at 37 ^{o}C for 24 h. Cells were harvested and counted using hematocytometer. Cells were resuspended in RPMI 1640 without sodium bicarbonate supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS) (Gibco, USA) (Bachmann *et al*., 2002), and then, the suspension was adjusted to 10^{6} cells/mL with RPMI 1640.

2.4 Biofilm formation

Mixed *C. albicans* and *C. glabrata* biofilms were performed on polystyrene, flat bottom, 96-well microtiter plates (Corning Incorporated, Corning, N.Y.) as previously described (Pesee *et al*., 2016). Briefly, mixed biofilms of *C. albicans* and *C. glabrata* at the 1 : 1 ratio were formed by pipetting 50 µl of each standardized cell suspensions into selected wells of the microtiter plate and incubating the

plate for 24 h at 37 °C. Biofilm formation in each well was evaluated by direct observation under inverted microscope (Nikon, Japan) before tested. After biofilm formation, the medium was discarded and non-adherent cells were removed by thoroughly washing the biofilms three times in sterile phosphate-buffered saline (PBS), the inhibitory effects of antifungal drugs were then tested.

2.5 Effect of the order of initiation of CAS-FLU and pre-exposure time on the antifungal activity of combinations treatment at the minimum inhibitory concentration

Mature biofilms were treated with either CAS (= CAS-pretreated) or FLU (= FLU-pretreated) at the concentration of 1 x MIC. The MIC of CAS against mixed *C. albicans* and *C. glabrata* biofilm is 0.56 µg/mL, and MIC of FLU is 309 µg/mL (Pesee *et al*., 2016). After mixed biofilms were incubated with the first drug for 1-6 h, the second drug was added to the pretreated biofilm as a CAS-FLU combination. Biofilm wells without drug were prepared for controls. Monotherapy of biofilms with CAS or FLU were also performed. The microtiter plates were incubated for 24 h at 37 °C. Triplicated wells were included in each group for all experiment, and triplicated experiments were performed.

2.6 Assay of biofilm viability

The vitality of mixed biofilm was determined by using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino) carbonyl)]-2H-tetrazolium hydroxide (XTT) colorimetric assay following the manufacturer's protocol. Briefly, 50 µl of XTT (1 mg/mL; Thermo Fisher Scientific, USA) containing 5 mM Phenazine methosulfate (PMS) (5 µl/mL) was added to wells of 96 microtiter plate. The plates were incubated for 2 h, and the optical density (OD) at 450 nm was measured by a microtiter plate reader (Opsys MR, Dynex, USA).

2.7 Biomass of biofilm quantitation

The biomass of biofilm was determined after 24 h of incubation at 37 °C with antifungal agents using crystal violet (CV) assay as described previously. (Pesee *et al*., 2016) Briefly, the medium was aspirated from each well and the adherent cells were washed twice with PBS. One hundred microliters of 99 % methanol were added to each well and fixed for 15 minutes. Wells were air dried after methanol discarded, and 100 µl of 0.5 % (w/v) CV solution was added. The excess CV was then removed with sterile distilled water after 20 minutes incubation. Finally, bound CV was released by adding 150 µl of 33 % acetic acid. The acetic acid was gently pipetted to completely solubilize the CV for 1 minute, and plate was read using a microtiter plate reader (Opsys MR, Dynex, USA) at 590 nm.

2.8 Statistical analysis

The effects of CAS-FLU combination on the viability and biomass of mixed biofilms were measured by comparing the reduction in the mean absorbance of the antifungal-challenged biofilm to that of the unchallenged biofilm as control and expressed as the percentage of biofilm reduction following formula:

$$\% \text{ Biofilm reduction} = [(OD_{control} - OD_{test}) \div OD_{control}]$$

x 100.

The average values of the triplicated wells were used in the data analysis to calculate the mean ± standard deviation (SD) of all experiments performed under the same conditions. The analyses were performed by using GraphPad Prism version 5 (GraphPad Software, Inc.). Differences between mean values of the percentage of biofilm reduction of FLU-pretreated and CAS-pretreated mixed biofilm at various pre-exposure times were assessed by Two-way analysis of variance (2-way ANOVA). When monotherapy with CAS or FLU was compared, differences between mean values were assessed by one-way ANOVA with Tukey's multiple comparison post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Viability of mixed biofilm in response to the CAS-FLU sequential combination

A limited number of *in vitro* studies thus far have evaluated the effects of sequential therapy of CAS followed by triazole against *Candida* spp. biofilm. As this study aimed to investigate the impact of sequential treatment of CAS and FLU against mixed *C. albicans* and *C. glabrata* biofilm, thus, comparisons of the efficacy of sequential therapy of CAS and FLU against single species biofilm were not included here.

The effects of sequential CAS-FLU combination on the viability of CAS-pretreated mixed biofilm were not significantly different compared to those of FLU-preincubated mixed biofilm at every pre-exposure time (Figure 1). The percentage of XTT reduction of 1 h FLU- and 1 h CAS-pretreated mixed biofilm in response to CAS and FLU combination were 66.49±4.15 (means±SD) and 62.83±12.53, respectively. However, the inhibition effects of sequential CAS-FLU combination on the vitality of FLU- and CAS-pretreated mixed biofilm were significantly greater than those of monotherapy with FLU ($p < 0.0001$), but not different to those of monotherapy with CAS. The efficacy of CAS treatment on the vitality of mixed biofilm was significantly higher than that of FLU treatment as well ($p < 0.0001$). The percentage of XTT reduction of mixed biofilm with FLU and CAS treatment alone were 25.24±10.94 and 62.21± 9.63, respectively.

The equivalent inhibition effects of sequential CAS-FLU combination on the vitality of mixed biofilm compared to that of CAS given alone found here were similar to previous study in planktonic *C. albicans* cells exposed to CAS at the concentrations of 0.2 and 0.4 µg/mL for 4 hours followed by FLU at the concentrations ranged from 0.008-4.0 µg/mL (Barchiesi *et al*., 2004). However, the inhibition effects of CAS-FLU combination on the vitality of mixed biofilm were significantly greater than those of FLU treatment alone. The different mechanisms of drug action could be the explanation for these results, as FLU interferes with ergosterol biosynthesis results in disruption of the cell membrane leading to growth inhibition of the

fungus, while CAS inflicts cell wall damage and cell death through inhibition of fungal cell wall synthesis. The enhanced vitality inhibition effect of CAS following administration of FLU compared to that of FLU treatment given alone would suggest a feedback regulation between ergosterol and cell wall biosynthesis. The influence of ergosterol depletion to cell wall remodeling has been previously suggested in *C. albican* (Pfaller *et al*., 1992). It is possible that exposure of mixed *Candida* biofilm to FLU could result in downregulation of cell wall remodeling, thus augmenting the effects of subsequent use of CAS. Conversely, CAS-induced fungal cell wall alterations could affect FLU entry or efflux, thus the inhibition effect of FLU following administration of CAS could be enhanced when compared to that of FLU monotherapy.

Our previous study obviously detected the greater number of colony-forming units of *C. glabrata* than those of *C. albicans* in mixed *C. albicans* and *C. glabrata* biofilm (Pesee *et al*., 2016). Therefore, it seems possible that the equivalent efficacy of CAS-FLU combinations to reduce the viability of mixed biofilm compare to CAS alone may be the result from the effect of combinations on viability of *C. glabrata* cells rather than *C. albicans* in mixed biofilm. These findings were supported by previous study revealed an absence of decreasing in the efficacy of CAS on viability of *Candida* when *C. glabrata* biofilm was firstly treated with FLU for 24 hours followed by another 24 hours of CAS treatment, while there was a significant decrease in the efficacy of CAS on viability of FLU-pretreated *C. albicans* biofilim (Sarkar *et al*., 2014).

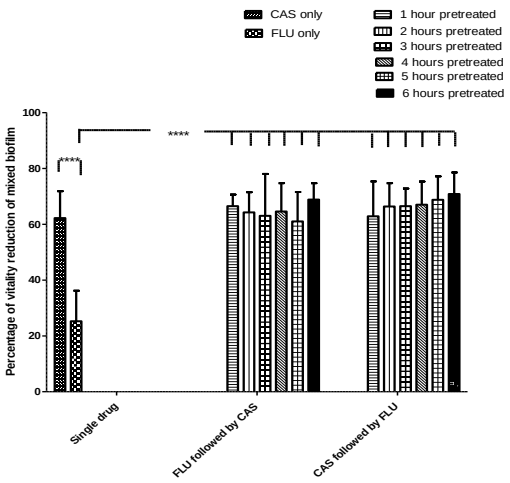

Figure 1 Inhibition effects of sequential combinations of CAS and FLU at the concentration as 1MIC on the reduction of vitality of mixed *C. albicans* and *C. grabrata* biofilm measured by XTT assay. Bars represent means±SD from three experiments**.** **** showed statistically significant different at $p < 0.0001$ among experiment groups using One-way ANOVA.

3.2 Impact of the sequence and pre-exposure time of CAS-FLU combination against biomass of mixed biofilm

XTT colorimetric reduction assay can quantify viability of *Candida* cell in biofilm based on metabolic activity. A biofilm is composed of several cell layers and biofilm cells are enclosed in an exopolymeric matrix, this may limit access to nutrients and oxygen, resulting in possible alterations in cellular metabolic activity. If this is

the case, the XTT assay, which is based on metabolic activity, may not determine accurately the number of cells. While crystal violet assay stained both living and dead cells as well as biofilm extracellular matrix. Therefore, CV staining was further evaluated to quantify total biomass of mixed *C. albicans* and *C. glabrata* biofilm in response to sequential CAS-FLU treatment.

Of interest, the efficacy of sequential CAS-FLU combination on the biomass of mixed *C. albicans* and *C. glabrata* biofilms was affected by both the order of combination drug and the duration of pre-exposure time. In contrast to what we observe with CAS followed by FLU regimen, the inhibitory effects of CAS-FLU combinations on FLU-pretreated mixed biofilm were dependent on the length of preincubation time of FLU. The inhibition activity of CAS-FLU combination on the biomass of mixed biofilm were continuously decreased when biofilm was pretreated with FLU for a longer period of time (1 to 3 h) ($p < 0.001$) (Figure 2). The percentage of biomass reduction in response to combinations when mixed biofilms were preincubated with FLU for 1 h was 64.66±8.07 (means±SD), and significantly declined when biofilms were preincubated with FLU for 3 h (16.07±32.00), and 4 h (-16.89±50.85). However, the percentages of biomass reduction of FLU-pretreated biofilm in response to CAS-FLU were indifferent from those of single drug administration. Although CV staining is a simple and reliable method for total biomass quantification (Jogalekar *et al.*, 2014), its low reproducibility to give repeatable results is a weakness of this assay. These issues may contribute to some large variations among the percentages of biofilm reduction of FLU-pretreated biofilms presented here.

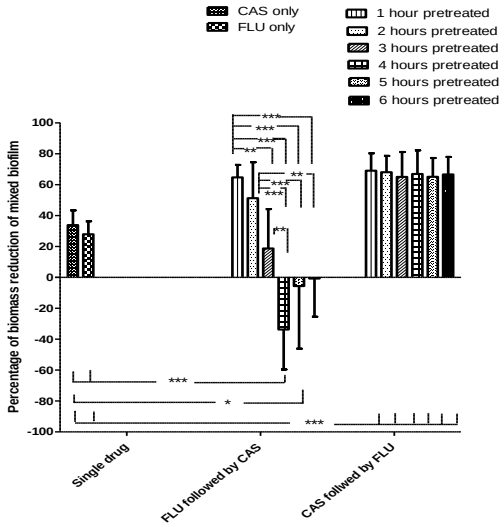

Figure 2 The percentage of biomass reduction of mixed *C. albicans* and *C. glabrata* biofilm following sequential combinations of CAS and FLU at concentration as 1 MIC measured by crystal violet assay. Bars represent means±SD from three experiments. * showed statistically significant different at $p < 0.05$, ** showed statistically significant different at $p < 0.01$, *** showed statistically significant different at $p < 0.001$ among experiment groups using One-way ANOVA.

Although the inhibition effect of CAS-FLU combination on the biomass reduction of CAS-pretreated biofilm at every pre-exposure

time were significantly greater than those of single drug treatment ($p < 0.001$), preincubation of mixed biofilm with CAS for 1 to 6 h before FLU exposure did not alter the efficacy of CAS-FLU combinations on the biomass reduction. The percentage of biomass reduction in response to combinations when mixed biofilms were preincubated with CAS for 1 h was 69.00±11.24 (means±SD), while those of monotherapy with CAS or FLU- were 33.85± 9.45 and 27.90±8.38, respectively. Recently, a study indicated an effective reduction in the biomass of the *C. glabrata* biofilm after CAS exposure, with a minimal reduction of 70 % of the biofilms (Rodrigues *et al*., 2018). In addition, the β-1,3 glucan concentrations were statistically significantly reduced in the biofilm matrices of *C. glabrata* (Rodrigues *et al*., 2018). The β-1,3 glucans are a group of specific polysaccharides from the cell walls of *Candida* species that are also recognised as major constituents of the biofilm matrices of this genus (Chandra *et al*., 2001a; Kuhn *et al*., 2002). The biofilm matrices of *Candida* provide the protection against physical and chemical environmental attack, such as by drugs. These polymers make it difficult for drugs to diffuse into the biofilm cells, which make the biofilms recalcitrant to antifungals. Therefore, the greater efficacy to reduce biomass in response to CAS-FLU combination of CAS-pretreated mixed biofilm might be the results from the mechanism of action of the echinocandins, which affects the cell wall and the matrix composition. Pre-exposure of mixed biofilm with CAS would inhibit β-1,3 glucan synthesis of cell wall and also impair production of the critical matrix component of mixed biofilm. Then, the protection mechanism against antifungal drugs of biofilm would be depleted resulting in the increased susceptibility of biofilm to the subsequent administration of FLU. Thus, the inhibition effects of CAS-FLU combinations on biomass would be augmented when compared to those of monotherapy.

The inefficacy of CAS-FLU combination against 4-6 h FLU-pretreated mixed *Candida* biofilm demonstrated here may be explained by the compensatory mechanism of cell wall after fluconazole treatment. Since fluconazole treatment results in increased fluidity of the plasma membrane, and it seems possible that this could indirectly affect cell wall integrity. The cell wall of *Candida* spp. is dynamic and its response to environmental changes plays a critical role in host–pathogen interactions. In fungi, β-(1,3)-glucan and chitin from a primary scaffold that is responsible for structural integrity and shape of the cell wall. Fluconazole treated-*Candida* revealed a significant increase in cell wall chitin (Pfaller *et al*., 1992). The increase in cell wall chitin is most likely due to degradation of chitin synthesis secondary to ergosterol depletion in the cell membrane. In addition, it was shown by *in vitro* experiments that treatment of *C. albicans* with low level of echinocandins elevated chitin content, and that this response protects the cells from cell wall damage due to inhibition of β-(1,3)-glucan synthesis (Walker *et al*., 2013). Therefore, it is possible that

increasing of membrane fluidity and drug permeability caused by fluconazole may induce the cell wall compensatory mechanisms that activate chitin synthesis, and that this response might contribute to a potential mechanism of tolerance to caspofungin.

The responses of organisms to stress have been recognized as antifungal resistance mechanism of biofilms. The induced resistance to CAS seen subsequent to FLU treatment might be related to the induction of cellular stress response. Yeast cells encounter a wide range of stress during growth and, as a consequence, adaptation to stress including oxidative and osmotic stress is essential for continued survival and replication. Stress responses have become more fully realized as defined mechanisms of antifungal resistance. Osmotic and oxidative stress responses are two cellular responses that play a crucial role in fungal virulence and antifungal susceptibility. Osmotic stress leads to rapid water loss, cell size reduction and a fall in turgor pressure, while, when exposed to oxidative stress, it encounters reactive oxygen species (ROS) related by polymorphonuclear leukocytes (PMNLs) and macrophages (Klipp *et al*., 2005; Mavor *et al*., 2005; Kuhn *et al*., 2012). Stress adaptation is crucial for *C. albicans* virulence as it increases the survival of this pathogen (Arana *et al*., 2007; Patterson *et al*., 2013). Recently study revealed that *C. albicans* responded to osmotic (NaCl) stress by producing a polysaccharide and protein rich exopolymeric matrix, whereas oxidative (H_2O_2) stress induced sub-lethal oxidative stress and enhanced the extracellular DNA content (Pemmaraju *et al*., 2016). The architecture of the *C. albicans* biofilm topology visualized under a SEM depicted extensive biofilm formation with an amorphous extracellular matrix enclosing yeast cells and germ tubes when subjected to osmotic stress, compared to oxidative stress and the control (Pemmaraju *et al*., 2016). Moreover, previous study demonstrated that exposure of *C. albicans* biofilm to FLU for 22 h and further incubated in antifungal-free medium demonstrated marked overexpression of *SKN1* after removal of fluconazole, which could be related to biofilm regrowth (Nailis *et al*., 2010). Treatment of the biofilm with FLU first would result in disruption of the cell membrane leading to osmotic stress. *Candida* biofilm might respond by producing an extensive polysaccharide and protein rich exopolymeric matrix for survival, as well as by regrowth of the biofilm. Stress adaptation then stabilizes the cell in the presence of drug and allows it to develop more profound resistance mechanisms over time.

Our results from CV and XTT assay might suggest that the order of initiation of FLU and CAS in sequential therapy impact on the amount of extracellular matrix (ECM) rather than the vitality of *Candida* cells in the mixed *C. albicans* and *C. glabrata* biofilm. Previous observations reported that biofilms with increased matrix polymers display increased resistance to antifungals (Mukherjee *et al*., 2004; Hawser *et al*., 1998) suggesting that the ECM might play a central role in the resistance of *C. albicans* to a subset of antifungals. Additional,

previous investigations revealed that FLU bind to β-1,3 glucans of the fungal cell wall as well as of the biofilm ECM (Nett *et al*., 2007). The physical interaction between FLU and glucan might act as a drug sponge to prevent subsequent antifungal from reaching biofilm cells. Thus, CAS treatment lately might not completely reach the site of action, which may be associated with lower biofilm susceptibility of FLU-pretreated mixed *Candida* biofilms. However, mechanisms of sequential CAS/FLU combination resistance against *Candida* mixed biofilm related to extracellular matrix are needed to be determined in the future. In addition, these observations were made using only one strain of *C. albicans* and *C. glabrata.* The observations of both CV and XTT assays have confirmed that an individual strain of clinical isolates and laboratory reference *Candida* species and strains has unique biofilm mass and activity (Alnuaimi *et al*., 2013). Therefore, before the benefit of sequential CAS and FLU therapy for candidiasis is accepted, study using clinical isolates of *C. albicans* and *C. glabrata* in both single-species and multi-species combinations should be further investigated.

4. Conclusion

As in the case of short exposure to CAS or FLU, both sequence of drugs and the duration of pre-exposure time affected on the biomass of mixed *C. albicans* and *C. glabrata* biofilm. However, sequential treatment with CAS and FLU has no impact on the vitality reduction of mixed *C. albicans* and *C. glabrata* biofilm.

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