Original article

The inhibitory effect of human beta-defensin-3 on *Candida glabrata* isolated from patients with candidiasis

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Abstract

Background: *Candida glabrata* is a common non-*albicans Candida* species found in patients with candidiasis and it sometimes develops antifungal resistance. Human beta-defensin-3 (hBD-3) is an antimicrobial peptide of immune system active against various types of microbes including *Candida* spp.

Objectives: To investigate antifungal activity of hBD-3 and its synergistic effect with a first-line antifungal agent on clinical isolates of *C. glabrata*.

Methods: *Candida* spp. were characterised in patients with candidiasis. The antifungal activities of hBD-3 and fluconazole against *C. glabrata* were evaluated using Broth microdilution assay. The synergistic activity of these two agents was determined by checkerboard microdilution and time-killing assays. The cytotoxicity of hBD-3 was evaluated using LDH-cytotoxicity colorimetric assay.

Results: Of 307 episodes from 254 patients diagnosed with candidiasis, *C. glabrata* was found in 21 clinical isolates. Antifungal susceptibility tests of *C. glabrata* were performed, fluconazole demonstrated an inhibitory effect at concentrations of 0.25-8 μg/ml but one antifungal resistant strain was identified (>64 μg/ml). hBD-3 showed an inhibitory effect against all selected strains at concentrations of 50-75 μg/ml and exhibited a synergistic effect with fluconazole at the fractional inhibitory concentration index (FICI) of 0.25-0.50. A concentration of 25 μg/ml of hBD-3 alone showed no cytotoxicity but synergistic activity was seen with fluconazole.

Conclusion: hBD-3 has antifungal activity against *C. glabrata* and synergistic effects with fluconazole at concentrations that alone, have no cytotoxicity. hBD-3 could be used as an

adjunctive therapy with first-line antifungal agents for patients with *C. glabrata* infection particularly those infected with fluconazole-resistant strains.

Introduction

Candidiasis is a frequent healthcare-associated infection and is widely recognized as a major opportunistic infection in patients with chronic diseases and immunodeficiency (Lockhart, 2014; McCarty, Pappas, 2016; Pappas et al., 2018). *Candida albicans* is the most common species found in these patients with candidiasis. However, recent studies have shown that non-albicans *Candida* spp. have been significantly increasing worldwide, particularly *Candida glabrata* (Diekema et al., 2012; Guinea, 2014; Tan et al., 2015). It has been reported that *C. glabrata* is the first or second most common non-albicans *Candida* spp., and it has a remarkable capacity to develop drug resistance to currently used antifungal agents (e.g. fluconazole) (Arendrup, Patterson, 2017; Healey et al., 2016; Pappas et al., 2018; Vale-Silva, Sanglard, 2015). Patients with candidiasis caused by *C. glabrata* shows higher morbidity than those infected with different *Candida* spp. (Kullberg, Arendrup, 2015). Therefore, many studies have been searching for novel strategies to improve the current treatment against *C. glabrata*. One area of research interest is a possible use of current antifungal agents combined with other antimicrobial agents such as human antimicrobial peptides (AMPs) (Hancock et al., 2016; Silva et al., 2014).

Human AMPs, also known as host defense peptides, are short cationic amphipathic peptides with diverse sequences produced by various types of immune cells (Hancock et al., 2016). These peptides demonstrate a wide range of activities not only in eliminating pathogens (e.g. bacteria, virus and fungi), but also in modulating immune functions of host cells and tissues. The

structure of AMPs provides an overall net positive charge that targets the negative charges on the microbial surface with electrostatic interactions leading to pore formation and disruption of the microbial cell membrane (Bobone, 2014; Scorzoni et al., 2017; Silva et al., 2014).

There are several types of human AMPs such as cathelicidin (LL-37), psoriasin (S100A7), dermcidin and defensins (Pazgier et al., 2006; Wang, 2014). Human β-defensins (hBDs), a type of defensin family, are composed of 4 different subtypes. hBDs are mainly produced by epithelial cells lining on the skin, airways, gastrointestinal and genitourinary tracts (Pazgier et al., 2006; Wang, 2008). It has been demonstrated that hBD-3 has the highest positive charge (+11) and this subtype shows a broad range of killing pathogens including bacteria, viruses, and fungi including *Candida* spp. (Hancock et al., 2016; Wang, 2014).

This study therefore aimed to investigate the effect of hBD-3 alone and in combination with fluconazole on *C. glabrata,* including a drug resistant strain, isolated from patients with *C. glabrata* infection.

Materials and Methods

The following reagents were used in this study: human beta-defensin-3 (Peptide Institute, Japan); fluconazole, MOPS 3-(N-Morpholino) propane sulfonic acid buffer, fibronectin, bovine serum albumin (Sigma-Aldrich, USA); RPMI 1640 media with glutamine and phenol red, bovine collagen type I (Gibco, USA); yeast extract (Lab-M, UK); bacteriological peptone, dextrose bacteriological grade and sabouraud dextrose agar (Oxoid, UK); chloramphenicol (Acumedia™, USA); cycloheximide (BD BBL™, USA); agar powder (Himedia, India); bronchial epithelial cell

growth medium bullet kit without GA-1000 (Lonza, Switzerland); LDH-cytotoxicity colorimetric assay kit II (Biovision Inc, USA).

Data collection

We obtained all retrospective data of patient records from the principal diagnosis, comorbidity, and complication of identified candidiasis from the Mycology laboratory records at the tertiary-care King Chulalongkorn Memorial Hospital (KCMH), from 1 January 2017 to 31 December 2017. This study was approved by the Institutional Review Board (IRB No. 262/61), Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The retrieved data included specimen collection date, lab number, age, gender, specimen source, species name, and patient diagnosis. *Candida* spp. isolated from the same patient/location within 7 days were considered as species arising from the same infection episode, whilst isolates from patients/samples >7 days after the first isolation were considered as distinct episodes of infection.

Yeast Identification

All collected specimens were initially examined using 10% KOH (Potassium hydroxide) solution under light microscopy. Each specimen was cultured for up to 4 weeks until fungal colonies were observed in: Sabouraud dextrose agar (SDA); SDA with chloramphenicol; SDA with chloramphenicol plus cycloheximide, at both 37℃ and 25℃ depending on the type of specimen. All positive colonies were visualised by light microscopy after lactophenol cotton blue staining. Vitek 2® XL (BioMérieux) with YST cards which is an automated colorimetric biochemical assay, were used for yeast identification. Detain in brief (Aj Mee) (Posteraro et al., 2015; Valenza et al., 2008).

Fungal culture

C. parapsilosis ATCC 22019 and *C. krusei* ATCC 6258 were used as laboratory quality controls (QC) for testing the antifungal drug susceptibility of *C. glabrata* clinical isolates. The *C. glabrata* S3304, S3650 and F52615 isolates randomly chosen for this study were collected from the respiratory tract of patients with candidiasis. The *C. glabrata* F2018C isolate from the blood of the patients was identified as fluconazole-resistant while the *C. glabrata* ATCC 2001 strain was used as a standard species. All strains and isolates were grown on yeast peptone dextrose agar (YPD; 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, 1.5% w/v agar) at 35ºC for 48 h prior to experiments.

Antifungal susceptibility testing

Stock solutions of fluconazole and hBD-3 were prepared in sterile water and frozen at -20ºC before use. Final concentrations of fluconazole ranged from 0.125 to 64 μg/ml and hBD-3 ranged from 0.019 to 100 μg/ml, diluted in RPMI-1640 media based on the Clinical and Laboratory Standards Institute (CLSI) broth microdilution M27-A3 method (Institute, 2017). Minimum inhibitory concentrations (MICs) of *C. glabrata* to fluconazole and hBD-3 were measured using the CLSI broth microdilution M27-A3 method (Institute, 2017). The lowest concentration of each drug that produced a measurable decrease in turbidity after 24 h incubation compared to drug-free growth control were recorded as 50% of MIC. The MIC breakpoints used for interpretation of fluconazole were followed by CLSI M27-S4E guidelines: MIC $\leq$ 32 μg/ml, susceptible-dose dependence (S-DD); MIC $\geq$ 64 μg/ml, resistance (R). All experiments were performed in biological triplicates.

Fungicidal testing

Minimum fungicidal concentrations (MFCs) of *C. glabrata* to hBD-3 were determined using the spread plate technique. Cell suspension of each $MIC_{50} \pm 1$ concentration was diluted and plated onto SDA plates at 35°C for 24 h. The concentrations of the peptides that resulted in no viable colonies were recorded as MFCs. All experiments were performed in biological triplicates.

Checkerboard microdilution assay

The fractional inhibitory concentration (FIC) between fluconazole and hBD-3 was assessed by checkerboard assay according to CLSI broth microdilution M27-A3 method (Institute, 2017) (Refs), and recorded visually as for MIC measurements. The FIC index (FICI) was calculated using the sum of the FICs of each drug tested. The FIC of each drug was determined using the MIC of each drug when used in combination, divided by the MIC of each drug when used alone at 24 h. The FICI values were interpreted as follows: FICI $\leq$ 0.5, synergistic; 0.5 < FICI $\leq$ 1, additive; 1 < FICI $\leq$ 4, no interaction (indifferent); FICI > 4, antagonistic (Denardi et al., 2017; Shrestha et al., 2015b). All experiments were performed in biological triplicates.

Time-killing assay

C. glabrata F2018C at 10^3 cells were incubated in RPMI-1640 medium alone (growth control), or medium with 0.5x MIC of fluconazole alone (32 μg/ml), 0.5x MIC of hBD-3 alone (25 μg/ml), or a combination between 32 μg/ml of fluconazole and 25 μg/ml of hBD-3. The yeast cell suspensions were incubated at 35°C in 200 rpm shaking incubator. At 0, 4, 8, 12 and 24 h incubation, aliquots of 100 μl from each tube were serially diluted and 100 μl of each dilution was plated onto SDA plates. Colony counts were determined after 48 h of incubation at 35ºC, and assessed according to the following criteria: CFU/ml of combination decrease ≥ 2 $\log_{10}$ compared to the most active drug = synergistic; CFU/ml of combination increase ≥ 2 $\log_{10}$ compared to the

least active drug = antagonism (Shrestha et al., 2015a; Shrestha et al., 2015b). This experiment was performed in three biological triplicates.

Cell lines and culture

Human bronchial epithelial BEAS-2B cells (ATCC) were cultured in a pre-coated tissue culture flask, as recommended by ATCC (0.01 mg/ml fibronectin, 0.03 mg/ml bovine collagen type I, 0.01 mg/ml bovine serum albumin) with Bronchial Epithelial Cell Growth Medium (BEGM) Medium at 37ºC in a 5% CO_2 incubator. The cells were serially passaged before reaching confluence, and experiments were conducted with subconfluent cells at 3^{rd}-5^{th} passage in the proliferative phase at 70–80% confluence.

Cytotoxicity test

Cytotoxicity of hBD-3 was measured using the LDH-Cytotoxicity Colorimetric Assay. Briefly, 10^4 BEAS-2B cells were seeded with 50 µl of DMEM in a pre-coated 96-well plates. hBD-3 at concentrations of 25, 50, 75 and 100 µg/ml were added into the plate and the cells were incubated in 5% CO_2 incubator at 37ºC for 24 h. The culture medium was collected and transferred into a 96 well-plate and incubated with the LDH Reaction Mix for 30 min at room temperature. The absorbance of all controls and samples were measured using a 450 nm filter and the percentage of cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{test sample} - \text{low control}}{\text{high control} - \text{low control}} \times 100$$

Statistical analysis

All data were analyzed using GraphPad Prism software version 8. Comparison between groups performed with a one-way ANOVA with post-hoc Bonferroni's multiple comparisons test.

Data were expressed as mean ± SEM, and differences with a p-value of <0.05 were considered statistically significant.

Results

Demographic data

There were total 307 episodes of candidiasis from 254 patients and *C. glabrata* accounted for 21 episodes from 21 patients (6.8%) (Figure 1). Of patients with *C. glabrata* infection, 15 patients were male (71.4%) and 6 patients were female (28.57%). The mean age of the patients was 69 ± 16.75 years old (Table 1). The clinical isolates were collected from the respiratory tract, skin and nails, urine and blood (Table 2).

The inhibitory effect of human beta-defensin-3 on the growth of *Candida glabrata*

To evaluate the susceptibility to fluconazole of each *C. glabrata* isolates, the MIC values of fluconazole were measured prior to hBD-3 susceptibility tests. Of 21 clinical isolates from 21 patients, 5 strains were no longer reserved in the laboratory stock during the period of this study. Therefore, antifungal susceptibility tests using *C. glabrata* clinical isolates (n=16) compared to *C. glabrata* ATCC 2001 were performed. This showed that all 16 strains were inhibited by fluconazole with the MICs ranging from 1-8 μg/ml (susceptible-dose dependent; S-DD), except for one clinical isolate of *C. glabrata* (F2018C), that showed an MIC of up to 64 μg/ml (fluconazole-resistant; R) (Table 3). Nevertheless, most strains of *C. glabrata* were susceptible to the second line antifungal agents; caspofungin and amphotericin B.

To evaluate the antifungal inhibitory effect of hBD-3, an antifungal susceptibility test was performed as for fluconazole. The result showed that hBD-3 inhibited the growth of all randomly

chosen *C. glabrata* isolates with the MICs ranging from 50-75 µg/ml (Table 4). Furthermore, MFC of *C. glabrata* ATCC 2001 was 50 µg/ml while MFCs of both *C. glabrata* F2018C and S3304 were 75 µg/ml. However, the MFCs of *C. glabrata* S3650 and F52615 were > 100 µg/ml.

The synergistic effect of human beta-defensin-3 and fluconazole on fluconazole-resistant *Candida glabrata*

As previous experiments showed that hBD-3 inhibited the growth of all chosen isolates of *C. glabrata* at the concentration of 50-75 µg/ml, checkerboard assays were performed and the result showed that the MICs of fluconazole decreased to 8 µg/ml and the MICs of hBD-3 were 6.25 µg/ml (Figure 2). The fractional inhibitory concentration index (FICI) values ranged from 0.25 to 0.50. The FICI values suggested that both hBD-3 and fluconazole showed synergistic effects on fluconazole-resistant isolates of *C. glabrata*.

To confirm this synergistic effect of hBD-3 and fluconazole against fluconazole-resistant *Candida glabrata*, time-killing assays were performed. The time-killing assays revealed that the growth of fluconazole-resistant *C. glabrata* was significantly decreased at 2 $\log_{10}$ in CFU/ml compared to single drug treatment and the untreated group (Figure 3). This result confirmed that hBD-3 showed a synergistic effect with fluconazole to inhibit the growth of fluconazole-resistant *C. glabrata*.

Human beta-defensin-3 cytotoxicity towards human cells

To assess the cytotoxic effect of hBD-3 on human cells, LDH-cytotoxicity colorimetric assays were performed. The result demonstrated that hBD-3 at a concentration of 25 µg/ml showed no significant cytotoxicity on BEAS-2B when compared with untreated control cells (Figure 4a).

Additionally, this cytotoxicity of hBD-3 together with fluconazole at a concentration of 32 µg/ml on BEAS-2B was not significantly different to that of untreated cells (Figure 4b).

Disscussion

hBDs contain a high content of cationic residues clustered near the carboxyl terminus which is essential for their antimicrobial property (Wang, 2014). These cationic residues induce electrostatic interactions leading to disruption of the cell membrane of pathogens (Pazgier et al., 2006). As hBD-3 is a member of the hBD family that contains the highest positive charge even in high salt concentrations (+11 net charge) (Wang, 2014) and broad-spectrum antibiotic activity (Joly et al., 2004; Krishnakumari et al., 2009; Schneider et al., 2005; Sudheendra et al., 2015), our study first investigated whether hBD-3 inhibited growth of *C. glabrata* (Pappas et al., 2018; Vale-Silva, Sanglard, 2015).

Of all of the *Candida* spp. isolated from patients with candidiasis at KCMH, *C. albicans* was the most common species found, whilst *C. tropicalis* and *C. glabrata* were the leading cause of non-albicans Candida infection in our population, which is similar to a previous study (Tan et al., 2015). The current treatment of candidiasis, including *C. glabrata* infections, are azoles (as the first choice of treatment), caspofungins or echinocandins; however, their adverse drug reactions and side effects need to be carefully considered, particularly in patients with underlying illnesses (e.g. renal and liver diseases) (Pappas et al., 2018). Moreover, *C. glabrata* is capable of developing drug resistance to the antifungal agents (Diekema et al., 2012; Pappas et al., 2018; Vale-Silva, Sanglard, 2015).

In our study, we found that one of the 16 isolates (6.25%) of *C. glabrata* was resistant to fluconazole. Interestingly, our results demonstrated that hBD-3 showed inhibitory effects against all randomly chosen *C. glabrata* clinical isolates, including this fluconazole-resistant isolate (Table 4). However, the inhibitory concentrations of hBD-3 on each strain varied (50-75 μg/ml), probably due to differences in cell membrane structure that affect the total net charge of *C. glabrata* (Hollmann et al., 2018; Marr et al., 2006). Furthermore, cell wall thickness and other resistance mechanisms of *C. glabrata* (e.g. efflux pump overexpression and drug target alteration) may play roles in drug resistance, which resulted in different fungicidal activity of hBD-3 (Arendrup, Patterson, 2017; Déry, Hasbun, 2011).

In order to combat the clinical problems associated with the evolution of drug-resistant fungi, many previous studies have demonstrated the efficacy of different combinations of groups of antifungal agents or combined with other drugs both *in vitro* and *in vivo* in order to increase the inhibitory effects on drug-resistant fungi (Campitelli et al., 2017; Scorzoni et al., 2017). For instance, combination therapy between azoles or amphotericin B and chemotherapeutic agents (e.g. flucytosine) showed synergistic effects on drug-resistant cryptococcosis and candidiasis by increasing an uptake of flucytosine, that inhibits nucleic acid synthesis, after cell membrane damage (Campitelli et al., 2017; Scorzoni et al., 2017). Furthermore, the combination of azoles and some modified antibiotics (e.g. tobramycin and aminoglycosides) that are used to inhibit bacterial protein synthesis, and also showed synergistic effects on *Candida* spp. (Shrestha et al., 2015a; Shrestha et al., 2015b). As a synergistic effect of azoles combined with hBD-3 has never been reported, our study explored this drug combination on fluconazole-resistant *C. glabrata* clinical isolate and found that hBD-3 showed a synergistic effect with fluconazole by reducing the MICs of fluconazole and hBD-3 of fluconazole-resistant *C. glabrata* (Figure 2-3). We propose

that hBD-3 may induce cell membrane permeability of *C. glabrata*, potentially by induction of pore formation or micellization on the membrane which then promotes fluconazole intracellular uptake (Pasupuleti et al., 2012; Silva et al., 2014). Increased uptake of fluconazole, can then inhibit the activity lanosterol 14 alpha-demethylase enzyme which normally converts lanosterol to ergosterol on fungal cell membrane: this disruption then alters cell membrane integrity (Berkow, Lockhart, 2017; Pappas et al., 2018; Scorzoni et al., 2017).

In order to measure cytotoxicity of these drugs on human cells, we performed a LDH-Cytotoxicity Colorimetric Assay to investigate the cytotoxicity of fluconazole, hBD-3 and their combination on human bronchial epithelial cells. Although hBD-3 alone had dose-dependent cytotoxic effects (Figure 4a), we did not find any cytotoxic activity at the concentration of combination drugs that we used, and we found these had a synergistic effect (Figure 4b). Our study, showing this selective cytotoxicty effect on different cells may be explained by differences in the proprerties of the target membranes. For example, it may be that hBD-3 (at ≤ 25 μg/ml with or without fluconazole) has an affinity for the negatively charged hydrophobic lipids in the cell membrane of *C. glabrata* that is greater than for the zwitterionic (neutral) amphiphilic components on the human cell membrane: this may explain selectivity of hBD-3 for antimicrobial activity (Hollmann et al., 2018; Maturana et al., 2017; Sudheendra et al., 2015). In addition, yeast cell walls possess phosphorylated mannosyl side chains, further enhancing their anionic surface charge (Lipke, Ovalle, 1998) that possibly helps the cationic hydrophobic surface of hBD-3 to bind and insert into the *C. glabrata* cell membrane (Maturana et al., 2017; Sudheendra et al., 2015).

In conclusion, hBD-3 showed an inhibitory effect on fluconazole-resistant *C. glabrata* and a synergistic effect together with fluconazole. The combination of these two agents at concentrations that were inhibitory for *C. glabrata*, had no cytotoxicity towards human epithelial

cells and probably could be used as an adjunctive treatment in patients with *C. glabrata* infection. Nonetheless, future studies should investigate the mechanisms underlying these synergistic effects.

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Authorship contributions

TI performed the experiments; TI, AT, SV, CK and SWE co-wrote the manuscript; DC designed the study and co-wrote the manuscript.

Conflict of interest disclosures

None

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Table 1. Demographic data of 21 patients with *C. glabrata* infection

Characteristics	**No. of patients (%)**
Mean age ± SD, years old (range)	69 ± 16.75 (17-97)
Gender	
Male	15 (71.43)
Female	6 (28.57)

Table 2. Infection area distribution of *Candida glabrata* (21 episodes)

Characteristics	**No. of episodes (%)**
Area	
Respiratory tract	15 (71.42)
Skin and nail	3 (14.28)
Urine	2 (9.52)
Blood	1 (4.76)

Table 3. Antifungal susceptibility tests of clinically isolated *Candida glabrata* (n=16) and a standard strain (ATCC 2001)

Fungal isolates	**MIC (µg/ml)**		
	Fluconazole	**Caspofungin**	**Amphotericin B[a]**
Candida glabrata ATCC 2001	1 (S)	0.125 (S)	0.5
1. *Candida glabrata* F2018C	> 64 (R)	0.25 (I)	1
2. *Candida glabrata* N7160	1 (S-DD)	0.25 (I)	0.5
3. *Candida glabrata* W98278	0.5 (S-DD)	0.25 (I)	1
4. *Candida glabrata* B46945	0.5 (S-DD)	0.25 (I)	0.5
5. *Candida glabrata* B06048	0.25 (S-DD)	0.25 (I)	0.5

6. *Candida glabrata* T04267	1 (S-DD)	0.25 (I)	0.5
7. *Candida glabrata* B03989	1 (S-DD)	0.25 (I)	0.25
8. *Candida glabrata* F52615	8 (S-DD)	0.25 (I)	0.5
9. *Candida glabrata* B5804	1 (S-DD)	0.25 (I)	0.25
10. *Candida glabrata* S6169	1 (S-DD)	0.25 (I)	0.25
11. *Candida glabrata* F3530	0.5 (S-DD)	0.25 (I)	0.25
12. *Candida glabrata* B9392	8 (S-DD)	0.125 (S)	0.25
13. *Candida glabrata* S3650	4 (S-DD)	0.125 (S)	0.25
14. *Candida glabrata* S3304	4 (S-DD)	0.125 (S)	0.5
15. *Candida glabrata* S5866	1 (S-DD)	0.125 (S)	0.5
16. *Candida glabrata* S5843	1 (S-DD)	0.125 (S)	0.25

[a] No Amphotericin B interpretive guidelines are available for testing of *Candida* species.

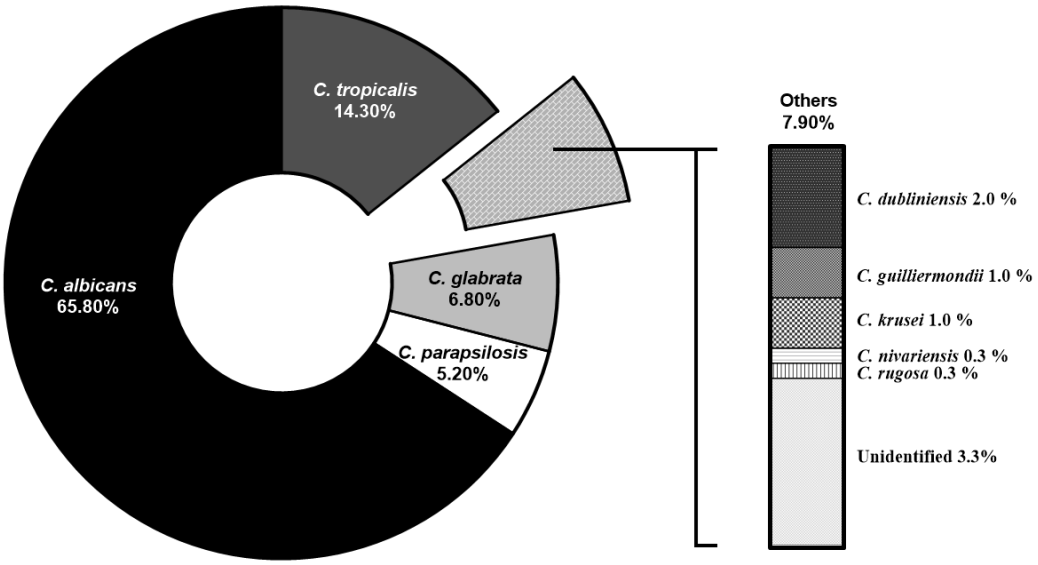
S-DD; susceptible-dose dependent, S; susceptible, I; intermediate, R; resistant

Table 4. Antifungal susceptibility tests of fluconazole and hBD-3 against randomly chosen *Candida glabrata* isolates

Fungal isolates	**MIC (µg ml^{-1})**		**MFC (µg ml^{-1})**
	Fluconazole	**hBD-3[a]**	**hBD-3**
Candida glabrata ATCC 2001	1 (S-DD)	50	50
Candida glabrata F2018C	> 64 (R)	50	75
Candida glabrata S3304	4 (S-DD)	50	75
Candida glabrata S3650	4 (S-DD)	75	>100
Candida glabrata F52615	8 (S-DD)	50-75	>100

[a] No hBD-3 interpretive guidelines are available for testing of *Candida* species.

S-DD; susceptible-dose dependent, R; resistant



Characteristics	No. of patients (%)
Mean age ± SD, years old (range)	69 ± 16.75 (17-97)
Gender	
Male	15 (71.43)
Female	6 (28.57)

Figure 1. Demographic data of 307 episodes from 254 patients with candidiasis at KCMH in 2017. Isolated *C. glabrata* accounted for 21 episodes from 21 patients (6.8%)

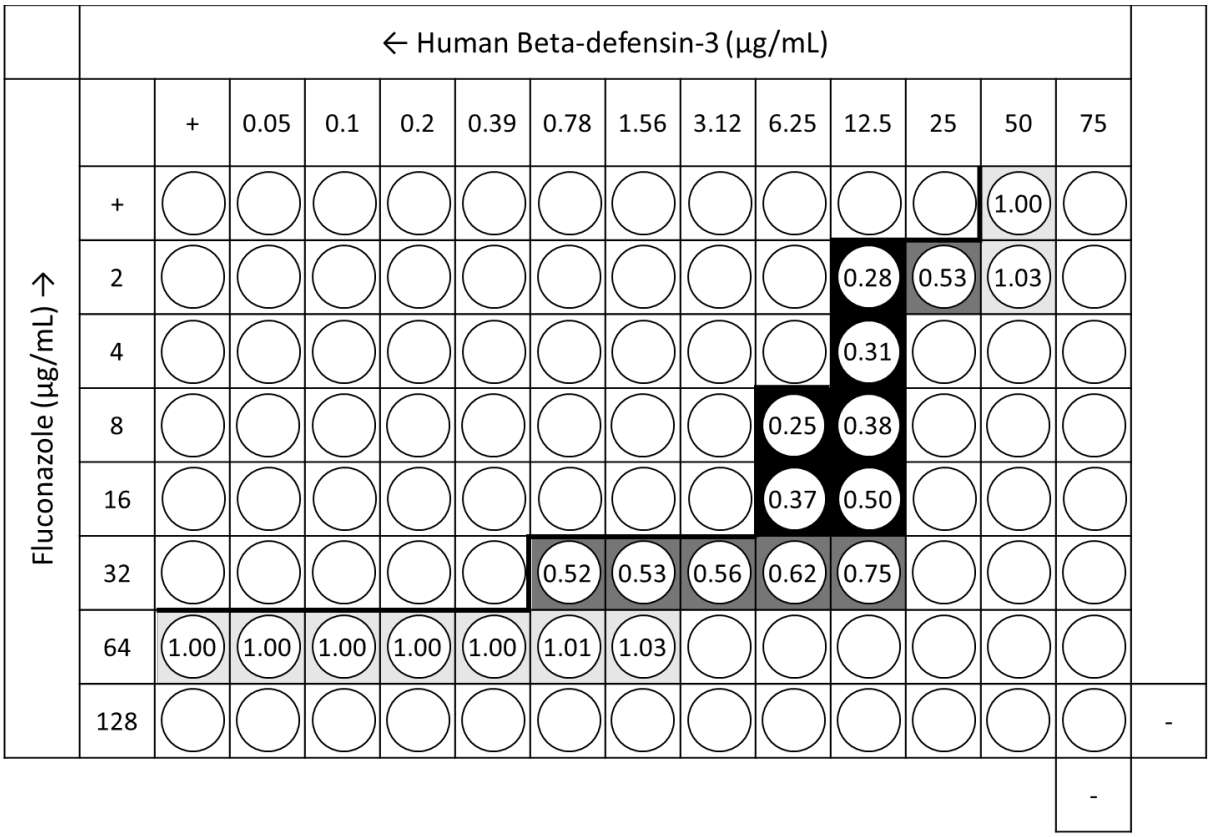


Figure 2. Checkerboard microdilution assay of hBD-3 and fluconazole against fluconazole-resistant *C. glabrata* F2018C. *C. glabrata* were incubated with combinations of fluconazole (2-128 µg ml^{-1}) and hBD-3 (0.5-75 µg ml^{-1}) for 24 h. Synergistic effect (black); Additive effect (dark grey), and No interaction (light grey) on *C. glabrata* growth. FICI was indicated each well

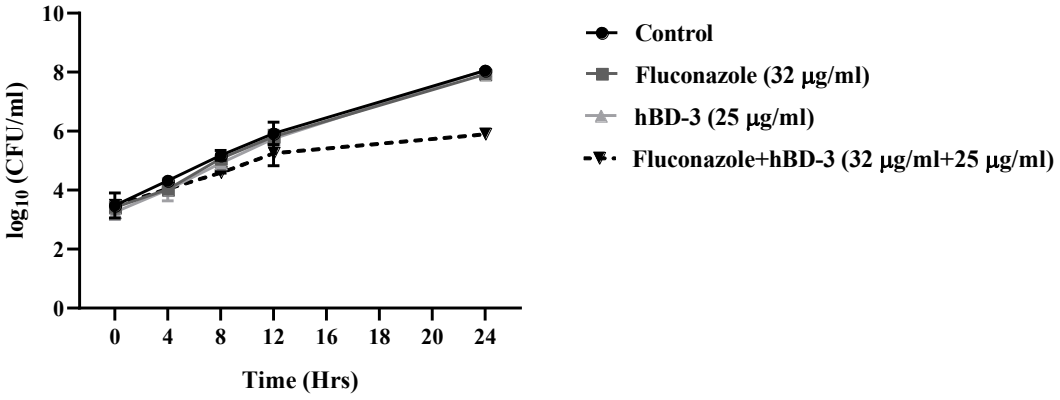


Figure 3. Time-killing assay of hBD-3 alone and hBD-3 in combination with fluconazole against fluconazole-resistant *C. glabrata* isolate (F2018C). *C. glabrata* were incubated with fluconazole at 32 µg/ml (dark gray square), hBD-3 at 25 µg ml^{-1} (light gray triangle), a combination of fluconazole and hBD-3 (black triangle), and control or vehicle (black circle) for 24 h.

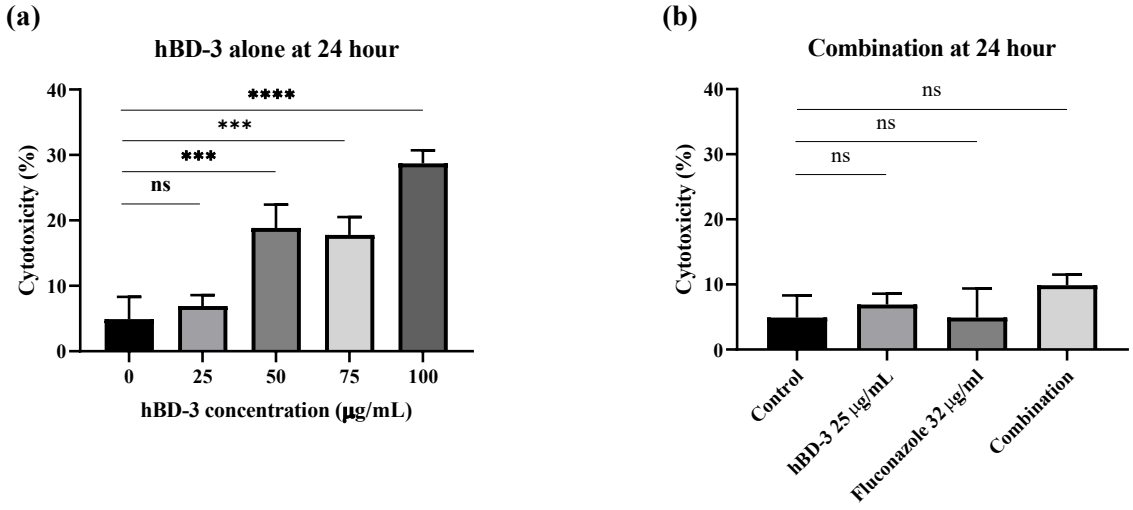


Figure 4. Cytotoxicity of hBD-3 alone or hBD-3 in a combination with fluconazole on BEAS-2B cells. BEAS-2B cells were co-cultured with different concentrations of hBD-3 (25-100 µg ml^{-1}) (a) or in a combination with fluconazole (b) at 37°C in a 5% CO_2 incubator for 24 h. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, Student's t-test. ns: not significance