(1-aryloxy-2-hydroxypropyl)-phenylpiperazine derivatives suppress Candida albicans virulence by interfering with morphological transition

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Summary
Clinical treatment of Candida albicans infections has become more difficult due to the limited development of antifungal agents and the rapid emergence of drug resistance. In this study, we demonstrate the synthesis of a series of piperazine derivatives and the evaluation of their inhibitory activity against C. albicans virulence. Thirty-four (1-aryloxy-2-hydroxypropyl)-phenylpiperazine derivatives, including 25 new compounds, were synthesized and assessed for their efficacy against the physiology and pathogenesis of C. albicans. Several compounds strongly inhibited the morphological transition and virulence of C. albicans cells, although they did not influence the growth rate of the fungal pathogen. A leading novel compound, (1-(4-ethoxyphenyl)-4-(1-biphenylol-2-hydroxypropyl)-piperazine), significantly attenuated C. albicans virulence by interfering with the process of hyphal development, but it showed no cytotoxicity against human cells at a micromolar level. These findings suggest that (1-aryloxy-2-hydroxypropyl)-phenylpiperazine derivatives could potentially be developed as novel therapeutic agents for the clinical treatment of C. albicans infections by interfering with morphological transition and virulence.

Introduction
Candida albicans has become the most frequently encountered fungal pathogen, causing mild mucosal infections and superficial but often persistent oral or vaginal candidiasis in humans (Poulain, 2015). It can also induce life-threatening systemic infections known as candidemia in immunocompromised patients (Miller et al., 2001; Pappas et al., 2003). C. albicans has a distinguishing feature, the yeast-to-hyphae dimorphism, which is the most important virulence factor that enables C. albicans to infect humans (Madhani and Fink, 1998; Brown and Gow, 1999; Staib et al., 2000). During the initial stage of infection, C. albicans cells exhibit a planktonic yeast morphology that is avirulent, and a subsequent transition from yeast to hyphae leads to tissue invasion in patients (Sudbery et al., 2004; Saville et al., 2003; Lo et al., 1997; Finkel and Mitchell, 2011). This ability to switch between yeast and hyphae is indispensable for the pathogenesis of C. albicans (Noble et al., 2017).

The clinical treatment of candidiasis caused by C. albicans relies on limited drugs, which are usually composed of three major classes: polyenes, azoles and echinocandins (Odds et al., 2003; Pierce et al., 2013). However, problems such as selectivity, toxicity and the
development of resistance have led to an emergent need for new strategies and novel drugs to efficiently treat *C. albicans* infections (Chandra *et al.*, 2001; Ramage *et al.*, 2002; Tobudic *et al.*, 2010). Several categories of natural products and synthetic chemicals have been screened and evaluated for their efficacy against *C. albicans* adhesion and morphological transition in recent years. Carvone and perillaldehyde were found to inhibit the formation of *C. albicans* filamentous structures (McGeady *et al.*, 2002). Quorum sensing molecules such as farnesioic acid and cis-2-dodecenoic acid (BDSF) inhibit *C. albicans* hyphae formation and appear to play a key role in regulating the *C. albicans* morphological transition (Oh *et al.*, 2001; Kim *et al.*, 2002; Boon *et al.*, 2008; Deng *et al.*, 2010). The recently identified small molecule filastatin exhibits excellent inhibitory activity against the cell adhesion, morphogenesis and pathogenesis of *C. albicans* (Fazly *et al.*, 2013).

Piperazine derivatives were reported to exhibit extensive pharmacological activities, such as antifungal, antioxidative and antitumor activities and the inhibition of cardiovascular disease (Huang *et al.*, 2015, 2016; Gettys *et al.*, 2017). These previous findings motivated us for the further exploration of the pharmacological activity of piperazine derived compounds. In this study, we reported the chemical synthesis of 34 piperazine derivatives, including some novel compounds, for the first time and investigated their ability to inhibit *C. albicans* virulence. Some derivatives showed excellent efficacy in preventing yeast-to-hyphae transition, biofilm formation and virulence but did not interfere with the growth rate of *C. albicans* cells. Intriguingly, these compounds were nontoxic to human cells, even at a high concentration. Together, our methods focus on evaluating multiple pathogenesis-related functions that do not involve the death of fungal pathogen cells to promote the development of novel antifungal therapeutics against *C. albicans*.

**Results**

**Chemical synthesis**

Piperazine derivatives 1c-34e were synthesized following the methods outlined in Fig. 1 (Pollard and Christie, 1958; Huang *et al.*, 2014). Phenol, 2,4-dichlorophenol and 4-hydroxybiphenyl were the initial precursors, which were then converted into epoxides through nucleophilic substitution with epichlorohydrin, followed by substitution with different piperazines in 2-propanol to yield the target compounds 1c-34e (Fig. 1, Table S1). The derivatives were characterized based on ESI-MS, HRESI-MS and $^1$H NMR spectral data, the results of which were completely consistent with their depicted structures (Fig. 1, Table S1).
Piperazine derivatives attenuate C. albicans virulence

To determine the effects of piperazine derivatives on the pathogenicity of C. albicans, we then investigated whether these compounds influenced C. albicans virulence in a cell line. Cytotoxicity was measured by quantifying the release of lactate dehydrogenase (LDH) into the supernatants of cultured A549 cells. It was found that most derivatives showed no toxic effects on A549 cells at a final concentration of 100 μM (Fig. 4A). However, the exogenous addition of certain piperazine derivatives led to a reduction in C. albicans cytotoxicity to A549 cells (Fig. 4B). Although 2,4-dichlorophenol derivatives exhibited strong inhibition of biofilm formation, their toxicity to the cell line was also very high (Fig. 4A). Compounds 1c, 2c, 8c and 28e were highly effective at attenuating C. albicans cytotoxicity by more than 80% when they were supplemented at a final concentration of 100 μM, while they exerted no toxic effects on the cell line at 8-h postinoculation (Fig. 4A and B). Considering that compounds 2c and 8c only weakly inhibited hyphae formation in C. albicans, compounds 1c (1-(4-methoxyphenyl)-4-(3-phenoxy-2-hydroxypropyl)-piperazine) and 28e (1-(4-ethoxyphenyl)-4-(1-biphenyl-2-hydroxypropyl)-piperazine) were then selected for further investigation. Interestingly, compounds 1c and 28e also inhibited the virulence of other clinical isolated Candida species (Fig. S1). However, some commercialized piperazine compounds showed no inhibition on C. albicans virulence even when they were added at a final concentration of 400 μM (Fig. S2).

Fig. 1. Chemical synthesis of piperazine compounds 1c-34e.
A. NaOH, 0–60°C.
B. 2-propanol, reflux.

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The inhibitory activity of piperazine derivatives on C. albicans hyphae formation, biofilm formation and virulence is dose dependent

To determine whether the effects of piperazine derivatives on C. albicans are related to their dosage, different concentrations of compounds 1c and 28e were assessed for their inhibitory activity during C. albicans morphological transition, biofilm formation and virulence (Fig. 5A). We also examined the effects of different concentrations of fluconazole, which is always used for clinical treatment. Both compounds 1c and 28e exhibited dose-dependent activity in which they reduced C. albicans cytotoxicity by more than 50% at a final concentration of 50 μM but did not affect the growth rate of pathogenic cells (Fig. 5B and C). As a control,
fluconazole severely inhibited the growth rate of C. albicans cells, suggesting that compounds 1c and 28e might be good candidates for development as novel anti-virulence agents against C. albicans infections. The addition of compounds 1c and 28e at final concentrations of 6.75–100 μM decreased C. albicans biofilm formation by 10% to 50% and 13% to 85%, respectively, but the addition of fluconazole had no detectable effect (Fig. 5D). Additionally, compounds 1c and 28e inhibited hyphae formation by 20% to 78% and 16% to 65%, respectively (Fig. 5E), while most C. albicans cells formed short stick mycelia that were considered hyphae after the addition of fluconazole.

Piperazine derivatives interfere with the morphological transition of C. albicans through multiple pathways

Besides the statistics of hyphae formation per cent, the photograph of hyphae formation was also provided to reflect the effect of the candidate compounds. After adding compounds 1c and 28e, the count of hyphae declined obviously (Fig. 6A). In good agreement with the inhibition of hyphae formation, compound 28e also obviously affected the colony morphology of C. albicans (Fig. 6B). After addition of compound 28e, the colonies were changed from wrinkled to slippery (Fig. 6B). To explore the working model of piperazine derivatives on the morphological transition of C. albicans, we continued to test whether these compounds interfered with the signalling pathways of the hyphae development process (Sudbery et al., 2004; Lu et al., 2011; Shareck and Belhumeur, 2011; Nobile et al., 2012). Real-time PCR analysis showed that exogenous addition of the leading compound 28e inhibited the expression levels of CDC35, EFG1, TEC1 and HWP1, which are regulators involved in the cAMP-PKA pathways (Fig. 6C and D) (Sudbery, 2011). In addition, some regulators of the MAPK cascade (Zhao et al., 2013), such as HST7, CEK1 and CPH1, were also downregulated by the exogenous addition of compound 28e (Fig. 6C and D).

Fig. 4. Effects of piperazine derivatives on Candida albicans virulence using a cell line.
A. Analysis of the toxicity of compounds to A549 cells. Compounds were dissolved in DMSO, and the same amount of DMSO as used as the solvent for compounds was used as control.
B. Analysis of the effect of compounds on the cytotoxicity of C. albicans to A549 cells. CA: C. albicans. Cell cytotoxicity was detected and measured as LDH release. The LDH released by A549 cells in (B) after inoculation with C. albicans in the absence of compounds was defined as 100% and used to normalize the LDH release ratios of other treatments. Data are the mean ± standard deviation of three independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired t test).
Collectively, these results demonstrated that compound 28e influenced complex signal transduction pathways to interfere with the *C. albicans* filamentation process.

**Discussion**

Our findings demonstrated for the first time that (1-aryloxy-2-hydroxypropyl)-phenylpiperazine compounds could be developed as potential antifungal agents to attenuate *C. albicans* virulence by targeting the filamentation process. The yeast-to-hyphae transition is always thought to be an indispensable step for invasion, and *C. albicans* mutants defective in morphological transitions are avirulent during infection (Braun et al., 2000; Saville et al., 2003; Zheng et al., 2003). The current clinical treatments against candidiasis caused by *C. albicans* almost always rely on limited antifungal agents, which are compromised by the limitation of drug development and the rapid emergence of drug-resistant pathogens. Developing new strategies to treat fungal pathogens is a key research area for these reasons. As morphological transitions between yeast cells and filamentous forms play a vital role in pathogenesis, recent studies have focused on determining how to inhibit hyphae formation in fungal pathogens. Interestingly, some cell–cell communication signals, including farnesol, a quorum sensing signal produced by *C. albicans* and a BDSF quorum sensing signal from *Burkholderia cenocepacia*, show an excellent ability to inhibit *C. albicans* hyphae formation (Boon et al., 2008; Shirtliff et al., 2009). Our study here provided additional evidence that designing antifungal drugs using a function-based approach that relies on the inhibition of hyphae formation but not the direct killing of pathogens is a feasible strategy.

Biofilms formed by *C. albicans* cells usually increase resistance to commonly used antifungal drugs, such as azoles and amphotericin B (Walker et al., 2010; Fiori et al., 2011). It is important to develop new antifungal drugs that efficiently control biofilm formation. Our results indicated that some piperazine derivatives showed a strong ability to inhibit *C. albicans* biofilm formation (Fig. 3). This ability was also consistent with their inhibition of hyphae formation (Fig. 2). As hyphae formation plays a vital role in *C. albicans* biofilm formation (Lu et al., 2014; Gulati and Nobile, 2016), the strategies to target morphological transition and biofilm development may be closely interlinked.

*P* < 0.05; **P < 0.01; ***P < 0.001 (unpaired *t* test).
Piperazine is an important scaffold associated with several biological activities (Shaquiquzzaman et al., 2015; Singh et al., 2015). Many synthetic piperazine derivatives have been reported to exhibit promising antifungal activity by causing the death of fungal cells (Moraca et al., 2014; Thamban et al., 2017). However, our study verified for the first time that (1-aryloxy-2-hydroxypropyl)-phenylpiperazine scaffolds could be developed as antifungal agents against C. albicans using an anti-virulence strategy. Another interesting characteristic of piperazine derivatives is that substitution on the benzene ring notably influences their activity (Figs 1–4). We found that 2,4-dichlorophenol derivatives showed strong anti-biofilm activity but exhibited high toxicity against cell lines (Figs 3 and 4). Conversely, 4-hydroxybiphenyl derivatives had high anti-biofilm activity and low cytotoxicity, suggesting that they are appropriate candidates for further development. The novel compound 28e containing 4-hydroxybiphenyl and the 4-OCH3CH3 moiety might be a promising potential agent against C. albicans and other clinical isolated Candida species given its excellent efficacy (Figs 5 and 6, S1). In general, our findings demonstrated that the high efficacy of (1-aryloxy-2-hydroxypropyl)-phenylpiperazine compounds in terms of both hyphae formation and biofilm formation by C. albicans increases their advantages for their development as new antifungal drugs.

Fig. 6. Influence of piperazine derivatives on Candida albicans morphology and hyphae formation. A. Effects of compounds 1c and 28e on hyphae formation of C. albicans. C. albicans cells were grown under non-induction conditions (30°C) (a), or under induction conditions (37°C) (b). In (c and d), the cells were grown under the same condition as in (b) but treated with 100 μM of compound 1c and compound 28e respectively. The photographs were taken 4 h after induction. B. Effects of compounds 1c and 28e (100 μM) on colony morphology of C. albicans. C. Comparison of relative fold changes of regulator encoding genes between C. albicans cells with and without addition of compound 28e. qRT-PCR results were normalized using the Ct values obtained for the GSP1 amplifications run in the same plate. The relative levels of gene transcripts were determined from standard curves. Data are the mean ± standard deviation of three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001 (unpaired t-test).

C. albicans affected by compound 28e.
Experimental procedures

Chemistry

$^1$H NMR (500 MHz) spectra were recorded in CDCl$_3$ or DMSO on a Bruker Avance spectrometer using tetramethylsilane (TMS) as an internal standard. Electrospray ionization (ESI) mass spectra (electron ionization (EI), 70 eV) were recorded on an Agilent 6330 ion trap LC/MS system. HRESI-MS spectra were recorded using a Shimadzu LCMS-IT-TOF mass spectrometer. Thin-layer chromatography (TLC) was performed on an aluminium plate pre-coated with silica gel and a fluorescence indicator (Merck, Darmstadt, Germany). The compounds were detected on the TLC plates using UV light (254 nm). All other reagents and chemicals were obtained from commercial sources and used as received unless otherwise stated.

Strains, culture and agents

The C. albicans strain used in this study was the standard wild-type strain SC5314 (ATCC® MYA-2876TM), which was grown either in 6.7 g l$^{-1}$ yeast nitrogen broth without amino acids (YNB) with 2% glucose or in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) at 30°C (non-hyphae induction conditions) or 37°C (hyphae induction conditions). Human lung epithelial A549 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

Biofilm formation assays

Candida albicans SC5314 strain was cultured overnight in YNB + 2% glucose media at 30°C and inoculated in the same medium to an OD$_{600}$ of 0.5 in the presence of compounds at different concentrations, as indicated. One hundred microlitres of inoculated culture was grown in each well of a 96-well polystyrene plate at 37°C for 4 h without shaking. The cultures were removed, and 0.02% crystal violet was added for 45 min. The plate was rinsed six to eight times with iced distilled water and decolorized with 200 µl of 75% ethanol. The quantity of crystal violet was determined by measuring the absorbance at 590 nm.

Hyphal formation assays

An overnight culture of C. albicans SC5314 grown at 30°C was diluted in fresh YNB + 2% glucose to an OD$_{600}$ of 0.1. Compounds were then added separately as indicated, and the cells were induced for 6 h at 37°C. Cells were centrifuged for 10 min at 5000 rpm, and the pellet was resuspended in 100 µl of fresh medium.

Images of cells were captured using a Leica inverted fluorescence microscope with 20× magnification.

Colony morphology

Spider medium agar plates (1% peptone, 1% mannitol, 0.2% K$_2$HPO$_4$ and 1.5% agar) were supplemented with different concentrations of compounds as indicated. C. albicans SC5314 cells were grown in the plates at 37°C for 24–30 h. Images of the colonies were obtained using a Leica DMI8 microscope and a Nikon Coolpix digital camera.

Cell growth analysis

For the cell growth assay, C. albicans cells were cultured in YNB + 0.2% glucose and inoculated in the same medium to an OD$_{600}$ of 0.05 in the absence or presence of compounds at final concentrations as indicated. Three hundred microlitres of inoculated culture was grown in each well at 30°C in a low-intensity shaking model using a Bioscreen-C Automated Growth Curves Analysis System (OY Growth Curves AB, Finland).

Cytotoxicity assays

Cytotoxicity was assessed by measuring the release of LDH from A549 cells. The A549 cells were routinely grown in DMEM medium supplemented with 10% FBS in a 96-well tissue culture plate with $1.5 \times 10^4$ cells/well. Confluent A549 cells were washed and incubated with DMEM containing 1% FBS before infection. Overnight C. albicans cells were diluted to an OD$_{600}$ of 0.1 with DMEM containing 1% FBS in the absence or presence of compounds at a final concentration as indicated. A549 cells were infected with fungal cells for 8 h. LDH in the supernatant was measured, and cytotoxicity was calculated relative to that of the uninfected control. Different strains and compounds were tested as the same way.

Quantitative real-time PCR

Real-time PCR assays were conducted as described previously (Li et al., 2013, 2014). Overnight cultures of C. albicans cells grown in YNB + 0.2% glucose at 30°C were inoculated in the same medium to an OD$_{600}$ of 0.1 in the absence or presence of compounds at a final concentration of 100 µM. After incubation for 6 h at 37°C, the cell samples were collected and washed with PBS. Total RNA was extracted using TRizol (Invitrogen, Carlsbad, California, America) and quantified. cDNA was obtained through a reverse transcription reaction using a reverse transcription kit (TaKaRa Biotechnology) with the...
primers shown in Table S2, and real-time PCR was performed with a 7300 plus real-time PCR system (Applied Biosystems, America). The expression level of each gene was normalized to that of GSP1, which is a housekeeping gene in C. albicans cells (Clément et al., 2000). The relative expression levels of the target genes were calculated using the quantitation-comparative CT(ΔΔCt) method.

Acknowledgements

This work was supported financially by grants from the Guangdong Natural Science Funds for Distinguished Young Scholars (No. 2014A030306015), the National Key Project for Basic Research of China (973 Project, 2015CB150600), the National Natural Science Foundation of China (No. 31571969) and the Introduction of Innovative R&D Team Program of Guangdong Province (No. 2013S034), China.

Conflict of interests

The authors declare no conflict of interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Analysis of chemical structures of piperazine derivatives.

Table S2. PCR primers used in this study.

Fig. S1. Effects of piperazine derivatives on different clinic isolated C. albicans strains and other Candida species virulence using a cell line.

Fig. S2. Influences of different piperazine compounds on C. albicans SC5314 virulence using a cell line.