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RESEARCH ARTICLE

Anti-virulence activity of novel (1-heteroaryloxy-2-hydroxypropyl)- phenylpiperazine derivatives against both wild-type and clinical drug-resistant *Candida albicans* strains

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Abstract

Candida albicans is an important human fungal pathogen. Our previous study disclosed that aryloxy-phenylpiperazine skeleton was a promising molecule to suppress *C. albicans* virulence by inhibiting hypha formation and biofilm formation. In order to deeply understand the efficacy and mechanism of action of phenylpiperazine compounds, and obtain new derivatives with excellent activity against *C. albicans*, hence, we synthesized three series of (1-heteroaryloxy-2-hydroxypropyl)-phenylpiperazines and evaluated their inhibitory activity against *C. albicans* both in vitro and in vivo in this study. Compared with previously reported aryloxy-phenylpiperazines, part of these heteroaryloxy derivatives improved their activities by strongly suppressing hypha formation and biofilm formation in *C. albicans* SC5314. Especially, (9H-carbazol-4-yl)oxy derivatives **25**, **26**, **27** and **28** exhibited strong activity in reducing *C. albicans* virulence in both human cell lines in vitro and mouse infection models in vivo. The compound **27** attenuated the virulence of various clinical *C. albicans* strains, including clinical drug-resistant *C. albicans* strains. Moreover, additive effects of the compound **27** with antifungal drugs against drug-resistant *C. albicans* strains were also discussed. Furthermore, the compound **27** significantly improved the composition and richness of the faecal microbiota in mice infected by *C. albicans*. These findings indicate that these piperazine compounds have great potential to be developed as new therapeutic drugs against *C. albicans* infection.

Junjun Huang and Shihao Song contributed equally to this work.

INTRODUCTION

Candida albicans is the most common fungal pathogen of humans and usually causes a mucosal disease known as candidiasis (Kim & Sudbery, 2011). Normally, *C. albicans* is harmless to the human body, but it may become invasive, infectious and even lethal by targeting many organ systems, as observed in patients with AIDS or those treated with cancer chemotherapy or transplantation procedures (MacCallum, 2010; Perlroth et al., 2007). Candidiasis is the third to fourth most common nosocomial infection in hospitals worldwide (Brown et al., 2012; Pfaller & Diekema, 2007; Wisplinghoff et al., 2004). It is estimated that the cost of treatment for *C. albicans* infections exceeds one billion dollars per year (Pappas et al., 2003). Although many factors affect the pathogenicity of *C. albicans*, cell adhesion and biofilm formation of *C. albicans* cells on biological surfaces (host tissues and abiotic matrices, such as catheters, implants and dentures) are important factors.

Previous studies have demonstrated that the dramatic and frequent transformation of *C. albicans* from spheroidal yeast to filamentous pseudomycelium and mycelial growth is necessary for the infection process (Kumamoto & Vinces, 2005). The transformation of *C. albicans* from the yeast state to mycelial growth is induced by many specific conditions, such as low cell density, alkaline pH, high temperature, carbon-poor conditions and the serum environment (Hornby et al., 2001; Zheng et al., 2004). In addition to these environmental factors, the quorum-sensing (QS) molecules farnesol and tyrosol can also regulate the morphology of *C. albicans*, including the yeast-to-hypha transition and biofilm formation (Chen et al., 2004; Patrícia & Arturo, 2012; Wongsuk et al., 2016). Increasing evidence suggests that, as bacteria and fungi usually coexist in the body, the QS signals produced by bacteria could also mediate the communication between bacterial cells and fungal cells (Tarkka et al., 2009). It was demonstrated that the QS molecule *cis*-2-dodecenoic acid (BDSF) produced by the conditional pathogen *Burkholderia cepacia*, inhibits the formation of *C. albicans* hyphae (Boon et al., 2008; Deng et al., 2010; Tian et al., 2013; Zhang et al., 2011).

Environmental cues and QS molecules modulate the morphological transition of *C. albicans* by affecting a set of transcription factors through various upstream pathways, including cyclic adenosine monophosphate (cAMP)-dependent pathways and mitogen-activated protein kinase (MAPK) pathways (Biswas et al., 2007; de Dios et al., 2010; Leberer et al., 2001; Martin et al., 2005; Ramage et al., 2002; Staib, 2002). In view of the important role of hyphal formation and biofilm formation in the pathogenic process of *C. albicans*, it is a general trend to prevent and treat *C. albicans* infection by inhibiting the pathogenic factors of *C. albicans* without affecting its growth.

Heteroaryloxy group was a commonly used pharmacophore for drug candidates within various biological activities, such as antimicrobial or anti-HIV activities (Roughley & Jordan, 2011). Our previous study demonstrated that aryloxy phenylpiperazine might be a new molecule chemotype, which exhibited moderate antifungal activity against *C. albicans* by inhibiting hypha formation and biofilm formation in vitro (Zhao et al., 2018), however, without in vivo study and further investigation. In this study, aryloxy moiety was changed to heteroaryloxy substitutions, that is, three series of phenylpiperazine derivatives with indole, 9H-carbazole and acetophenone moieties (Figure 1) were synthesized and their antifungal activities against *C. albicans* were evaluated both in vitro and in vivo. Three mouse infection models, that is, the systemic infection model, the oropharyngeal Candidiasis (OPC) infection model and the faecal microbiota analysis of infection model were used. Among these compounds, the compound **27**, (9H-carbazol-4-yl)oxy derivative, showed significant efficacy against both the wild-type and clinical *C. albicans* strains, including clinical drug-resistant *C. albicans* strains. Intriguingly, the compound **27** also showed additive effects with fluconazole in the treatment of infections caused by the clinical drug-resistant *C. albicans* strain HCH60 in mouse models. Our results suggested that the compound **27** might be a promising candidate antifungal agent for the treatment of infections caused by clinical *C. albicans* strains.

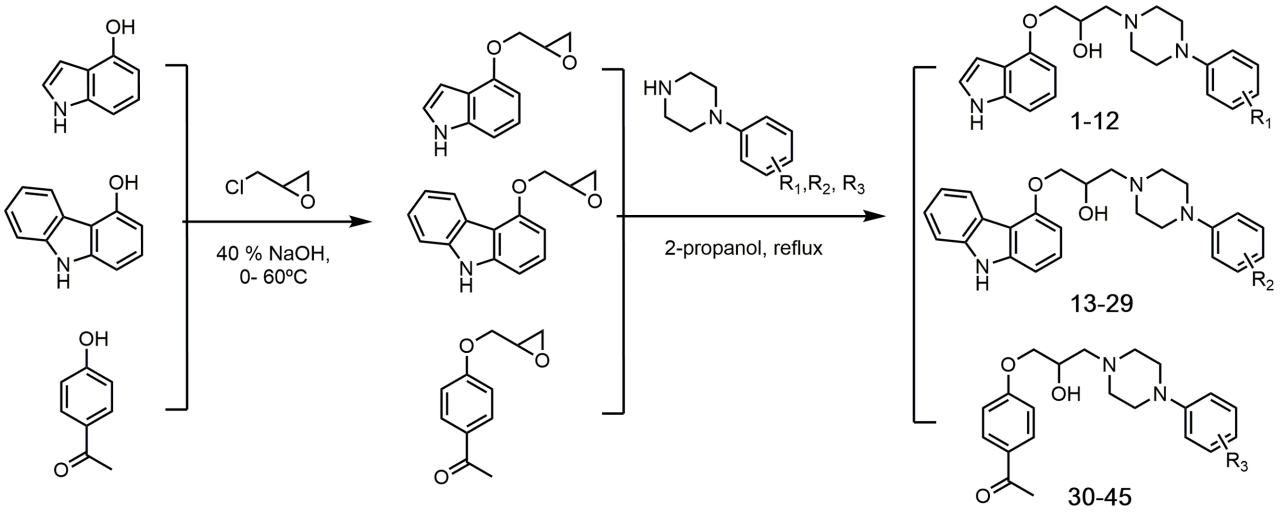
EXPERIMENTAL PROCEDURES

Chemistry

Melting points were determined with an X-4 apparatus, and the temperature was uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ or DMSO-d_6 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 Liquid Chromatograph Mass Spectrometer (LC/MS) system. Thin-layer chromatography (TLC) was performed on an aluminium plate precoated with silica gel and a fluorescence indicator (Merck). Detection of the compounds on the TLC plate was conducted by ultraviolet (UV, 254 nm) illumination. Target compounds were prepared according to reported protocols (Zhao et al., 2018) with moderate yield. All the other reagents and chemicals were obtained from commercial sources and used as received unless otherwise stated.

Strains, culture and agents

The *C. albicans* strains used in this study were *C. albicans* SC5314 (ATCC MYA-2876TM) and clinical



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2: R_1= 4-OCH_3
3: R_1= 2, 4-OCH_3
4: R_1= 3, 4-OCH_3
5: R_1= 2, 5-OCH_2CH_3
6: R_1= 2, 4-OCH_3-5-Cl
7: R_1= 4-CH_3
8: R_1= 2, 4-CH_3
9: R_1= 2-Cl
10: R_1= 2-Br
11: R_1= 4-Br
12: R_1= 3, 4-Cl

13: R_2= 2-OCH_3
14: R_2= 4-OCH_3
15: R_2= 2, 4-OCH_3
16: R_2= 2, 5-OCH_3
17: R_2= 3, 4-OCH_3
18: R_2= 2-OCH_2CH_3
19: R_2= 2,5-OCH_2CH_3
20: R_2= 2,4-OCH_3-5-Cl
21: R_2= 4-CH_3
22: R_2= 2,3-CH_3
23: R_2= 2,4-CH_3
24: R_2= 2-CH_3-6-CH_2CH_3
25: R_2= 4-CF_3
26: R_2= 2-Cl
27: R_2= 2-Br
28: R_2= 4-Br
29: R_2= 3,4-Cl

30: R_3= 2-OCH_3
31: R_3= 4-OCH_3
32: R_3= 2, 4-OCH_3
33: R_3= 2, 5-OCH_3
34: R_3= 3, 4-OCH_3
35: R_3= 2-OCH_2CH_3
36: R_3= 2, 5-OCH_2CH_3
37: R_3= 2, 4-OCH_3-5-Cl
38: R_3= 4-CH_3
39: R_3= 2, 3-CH_3
40: R_3= 2-CH_3-6-CH_2CH_3
41: R_3= 4-CF_3
42: R_3= 2-Cl
43: R_3= 2-Br
44: R_3= 4-Br
45: R_3= 3,4-Cl

FIGURE 1 Chemical synthesis of piperazine compounds **1–45**. Twenty compounds highlighted in red colour are new structures.

C. albicans strains. Clinical *C. albicans* strains were obtained from Hunan Child's Hospital of China from the oral cavity. The fungal cells were grown in 6.7 g/L yeast nitrogen broth lacking amino acids (YNB) supplemented with 2% glucose at 30°C with shaking at 220 rpm (Meng et al., 2019; Song et al., 2021). Human lung epithelial A549 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) at 37°C in 95% air/5% CO_2 (Meng et al., 2019; Song et al., 2021).

Mouse mortality and oral mucosal infection

All animal experimental procedures were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals and were affirmed by the Animal Care and Use Committee of South China Agricultural University. The mouse systemic infection experiment was performed according to a previously described experimental scheme (Barchiesi et al., 2000). In brief, *C. albicans* cells were collected and washed three times with cold sterile phosphate buffered saline (PBS, pH = 7.4). Next, the suspension was diluted with PBS to an OD_{600} of 0.5. The *C. albicans* cell suspension was infused into male BALB/C mice (n = 8) aged 6–8 weeks through the tail vein, with a final volume of 200 μl. Four hours after infection, mice in the treatment groups were infused with 200 μl of the compounds at a concentration of 100 μM. The control group was treated with the same protocol but injected with only PBS. Mouse mortality was recorded to determine the survival curve, and the data were analysed with GraphPad Prism 8.

The mouse model OPC experiment was based on a published study (Barchiesi et al., 2000; Solis & Filler, 2012). Mice were subcutaneously injected with 225 mg/kg cortisone acetate one day before and after

inoculation for immunosuppression. *C. albicans* cells were collected and washed three times with cold sterile PBS (pH = 7.4). Then, the suspension was diluted with PBS to 3×10^6 cells/ml. Mice were anaesthetized with 40 mg/kg pentobarbital sodium before inoculation, placed on a constant-temperature pad at 37°C, and sublingually inoculated with a swab soaked with cold *C. albicans* suspension for 75 min. One day later, the mice were administered 400 μl of the compounds (100 μM) or the same volume of PBS by gavage once a day. Five days later, the mice were euthanized, and their tongues were separated vertically. One half of each tongue was utilized for colony-forming unit (CFU) examination, and the other half was utilized for histological examination.

Microbiota analysis

Five-week-old female Kunming mice were cohoused in a large cage. Then, some of the mice were inoculated orally with 5×10^7 *C. albicans* cells in a 25 μl volume (Fan et al., 2015). All of the inoculated mice described in this study became colonized with *C. albicans* following a single inoculation. All the mice were transferred from the large cage to standard-sized cages, and three mice were housed per cage. After two days of *C. albicans* colonization, the mice were orally inoculated with 200 μl of the compounds at a concentration of 100 μM or with 200 μl of fluconazole at a concentration of 10 μg/ml once every 2 days, three times in total. Next, mouse faeces were collected, and microbial DNA was extracted. The V1-V9 region of the bacterial 16S ribosomal RNA gene was amplified by PCR using the primers 27F 5'-AGRGTTYGATYMTGGCTCAG-3′ and 1492R 5'-RGYTACCTTGTTACGACTT-3′, where the barcode is an eight-base sequence unique to each sample. The fungal ITS region was amplified by PCR using the primers ITS1 5'-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5'-TCCTCCGCTTATTGATATGC-3′. SMRTbell libraries were prepared from the amplified DNA by blunt ligation according to the manufacturer's instructions (Pacific Biosciences). Purified SMRTbell libraries from the Zymo and HMP mock communities were sequenced on dedicated PacBio Sequel II 8 M cells using Sequencing Kit 2.0 chemistry. Purified SMRTbell libraries from the pooled and barcoded samples were sequenced on a single PacBio Sequel II cell. All amplicon sequencing was performed by Shanghai Biozeron Biotechnology Co., Ltd. PacBio raw reads were processed using SMRT Link Analysis software version 9.0 to obtain demultiplexed circular consensus sequence (CCS) reads with the following settings: minimum number of passes = 3, minimum predicted accuracy = 0.99. Raw reads were processed through the SMRT Portal to filter sequences for length (<800 or >2500 bp) and quality. Sequences were further filtered by removing barcodes, primer sequences, chimeras and sequences that contained ten consecutive identical bases. OTUs were clustered with a 98.65% similarity cut-off using UPARSE (version 7.1 http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S or ITS rRNA gene sequence was analysed by RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU132)16S or ITS rRNA database using a confidence threshold of 70% (Amato et al., 2013).

Statistics

All data are presented as the mean ± standard deviations from three independent experiments. Statistical analysis was performed with GraphPad Prism 8. Statistical significance is indicated as follows: $*p<0.05$, $**p<0.01$ or $***p<0.001$ (one-way ANOVA or two-way ANOVA).

RESULTS

Chemical synthesis

The compounds **1–45** were synthesized through the routes outlined in Figure 1. 4-Hydroxyindole, 9H-carbazol-4-ol and 4-hydroxyacetophenone were the starting materials, which were converted to epoxides through nucleophilic substitution with epichlorohydrin, followed by substitution with different piperazines in 2-propanol to yield the target compounds **1–45**. Among all the 45 structures, 20 compounds highlighted in red colour are new structures (Figure 1). The derivatives with novel structures were characterized on the basis of HRESI-MS and ^{1}H-NMR spectral data (Supporting Information), which were fully consistent with the depicted structures.

Heteroaryloxy phenylpiperazines inhibited hypha formation, biofilm formation and virulence in *C. albicans*

Both biofilms and mycelia are important virulence factors of *C. albicans*. Therefore, the inhibitory effects of all 45 compounds on hypha formation and biofilm formation in *C. albicans* were tested. *C. albicans* cells were evaluated in vitro under hyphal induction conditions at 37°C for 6 h. In the control group, most of the *C. albicans* cells formed germ tubes, while the addition of some piperazine compounds significantly inhibited hypha formation. Thirteen derivatives within 100 μM reduced hypha formation in *C. albicans* cells by more than 50%, especially, the compounds **22, 23, 24, 25** and **26** showed an inhibitory effect of more than 70% (Figure 2A). Effects on biofilm formation in *C. albicans* were also measured. Twenty-nine compounds reduced biofilm formation in *C. albicans* cells by more than 50% (Figure 2B). Among

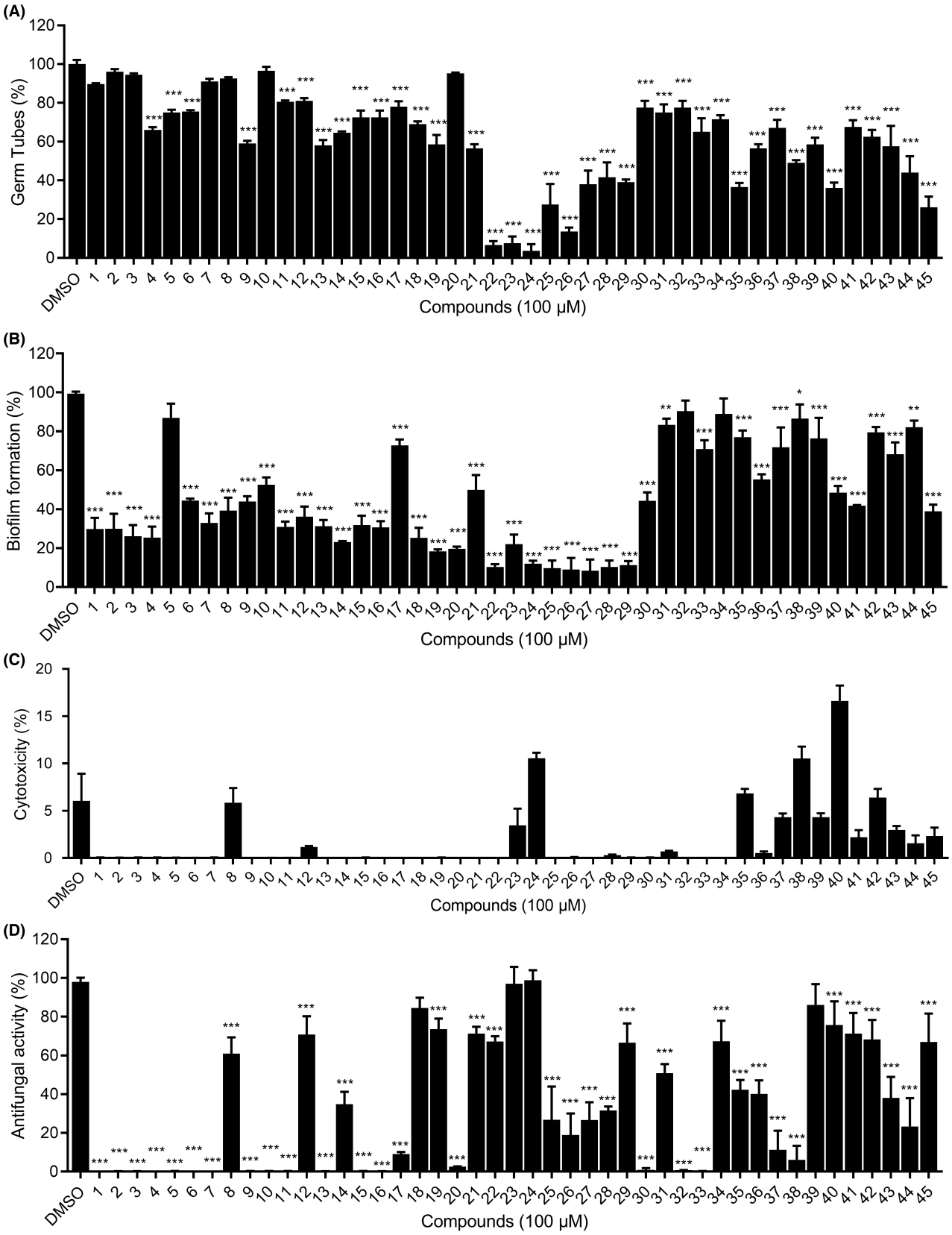

FIGURE 2 Influence of the piperazine compounds on *C. albicans*. Effects of the compounds (100 μM) on *C. albicans* hypha formation (A) and biofilm formation (B). (C) Analysis of the toxicity of the compounds (100 μM) to A549 cells. (D) Analysis of the effects of the compounds (100 μM) on the antifungal activity of *C. albicans* to A549 cells. *C. albicans* cells were grown under induction conditions (37°C). The photographs were taken 6 h after induction. The data are the mean ± standard deviation of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (one-way ANOVA).

them, the compounds **19, 20, 22, 24, 25, 26, 27, 28** and **29** had a stronger inhibitory effect by more than 80%.

We continued to test the effect of these compounds on *C. albicans* virulence in the A549 human cell line. Most of the compounds showed no or weak cytotoxicity to A549 cells at 100 μM (Figure 2C). The compounds **1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 13, 15, 16, 17, 20, 26, 27, 28, 30, 32, 33, 37** and **38** showed strong inhibition of the antifungal activity of *C. albicans* (Figure 2D). As the compounds **25, 26, 27** and **28** also effectively inhibited both hypha formation and biofilm formation in *C. albicans* SC5314 (Figure 2), these four compounds were selected for further study.

Heteroaryloxy phenylpiperazines inhibited hypha formation, biofilm formation and virulence in *C. albicans* in a dose-dependent manner

To determine whether the effects of the piperazine compounds on *C. albicans* were dose-dependent, we continued to measure the effects of the selected compounds on *C. albicans* at different final concentrations. All four compounds exhibited dose-dependent effects on biological functions (Figure 3), but they did not inhibit the growth of *C. albicans* SC5314 under these conditions at a final concentration of 100 μM (Figure S2A). Evans blue staining further revealed that the mortality of *C. albicans* cells treated with the compound **27** (100 μM) was only 7.41% (Figure S1). When the compounds **25, 26, 27** and **28** were administrated at a final concentration of 100 μM, they inhibited *C. albicans* hypha formation by more than 50% (Figure 3A; Figure S2). Among them, the compound **26** reduced hypha formation by approximately 80% (Figure 3A). These compounds also had a marked inhibitory effect on the formation of *C. albicans* biofilms even at a low concentration of 12.5 μM (Figure 3B). They inhibited *C. albicans* virulence by more than 60% at a final concentration of 100 μM (Figure 3C). The biofilms formed in vitro were also analysed by scanning electron microscopy (SEM), in which we observed the formation

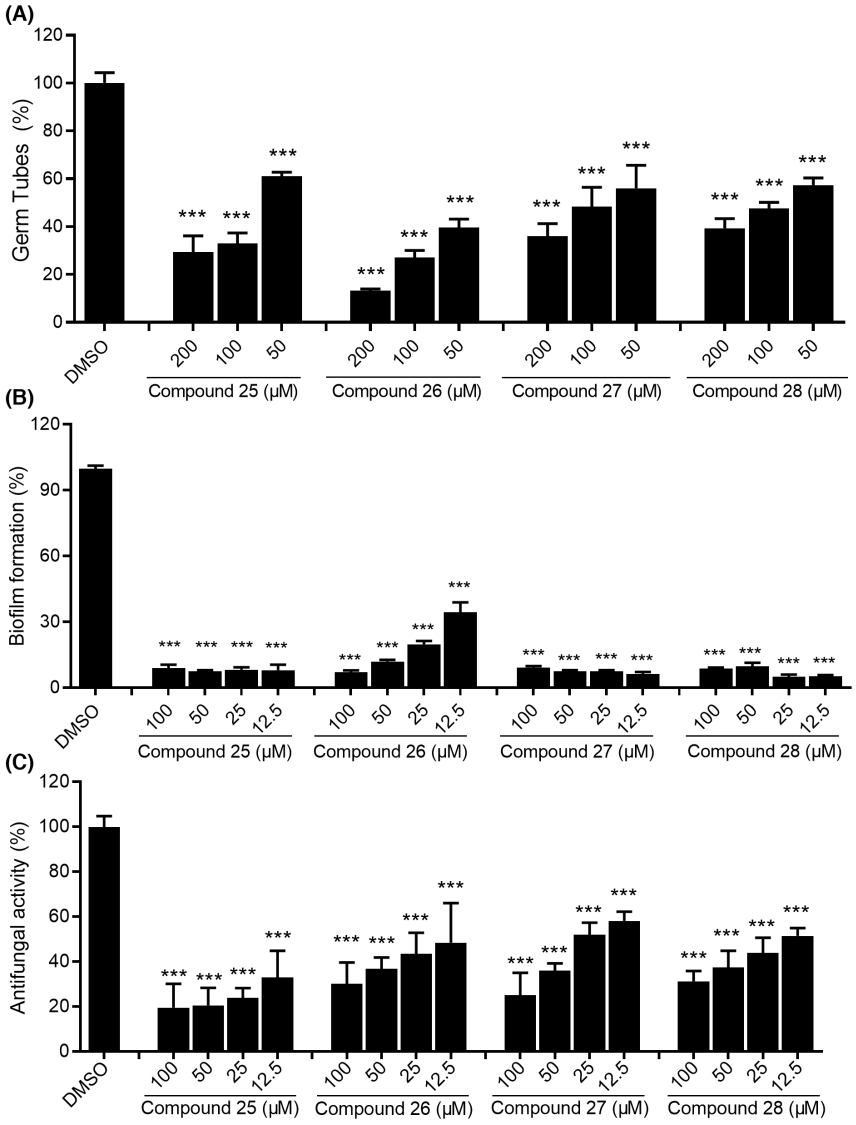

FIGURE 3 Effects of the compounds **25, 26, 27,** and **28** (12.5, 25, 50, 100, 200 μM) on *C. albicans* hypha formation (A), biofilm formation (B) and antifungal activity (C). The data are the mean ± standard deviation of three independent experiments. ***$p < 0.001$ (two-way ANOVA).

of a mature biofilm on acrylic resin discs after 48 h of incubation. Apparently, the biofilms formed by *C. albicans* treated with DMSO showed a thick and dense hyphal structure, however, the biofilm and hyphae of *C. albicans* treated with the compound **27** decreased significantly (Figure S3).

Heteroaryloxy phenylpiperazines attenuated the pathogenicity of *C. albicans* SC5314 in mouse infection models

To further investigate whether these compounds attenuated *C. albicans* pathogenicity in vivo, an oropharyngeal Candidiasis (OPC) experiment was conducted in a murine model. All four compounds reduced the lethality of *C. albicans* in mice. Among them, the compound **27** yielded the maximum efficiency in terms of the survival percentage of mice infected by *C. albicans* (Figure 4A). Therefore, we further studied the inhibitory effect of the compound **27** on the infection of mouse tongue by *C. albicans*. The tongue is one of the most important niches for *C. albicans*. In the presence of the compound **27**, the level of *C. albicans* infection in the mouse tongue decreased significantly (Figure 4B), and pathological sections of the mouse tongue also showed that there were almost no *C. albicans* cells present after treatment with the compound **27** (Figure 4C). It is worth noting that the compounds showed a better efficacy in the OPC infection model than in the systemic infection model.

Compound 27 inhibited *C. albicans* SC5314 hypha formation by interfering with the cAMP-PKA and MAPK pathways

To study the mechanism by which the compound **27** inhibits hypha formation in *C. albicans*, we analysed and compared the transcriptomic profiles of *C. albicans* SC5314 cultured in the presence or absence of compound **27** by using RNA sequencing (RNA-seq). Differential gene expression analysis showed that upon the addition of the compound **27**, 170 genes were upregulated and 123 genes were downregulated (log double change ≥1.0) (Figure S4A, Table S2). Quantitative RT–PCR analysis of selected genes confirmed the results of RNA-seq (Figure S4B). These differentially expressed genes were associated with a range of biological functions, including heat shock proteins, cell wall proteins, transcription factors, filamentous growth proteins, and transporters (Figure S4C, Table S2). These genes include cAMP-PKA and MAPK pathway genes (PDE2, EFG1, TEC1, HWP1, HST7 and CEK1). In the pathway, PDE2 is a 3′,5′-cyclic-nucleotide phosphodiesterase. EFG1 is a transcription factor that is activated by the PKA pathway and directly interacts with hypha-specific promoters during filament development. TEC1 is regulated by EFG1 and also regulates filamentous formation. HWP1 is a cell wall protein critical for cellular adhesion and filamentous formation. Upon further conducting a Western blot analysis, we found that the compound **27** significantly inhibited the expression of EFG1 and TEC1 (Figure S4D). Many filamentous

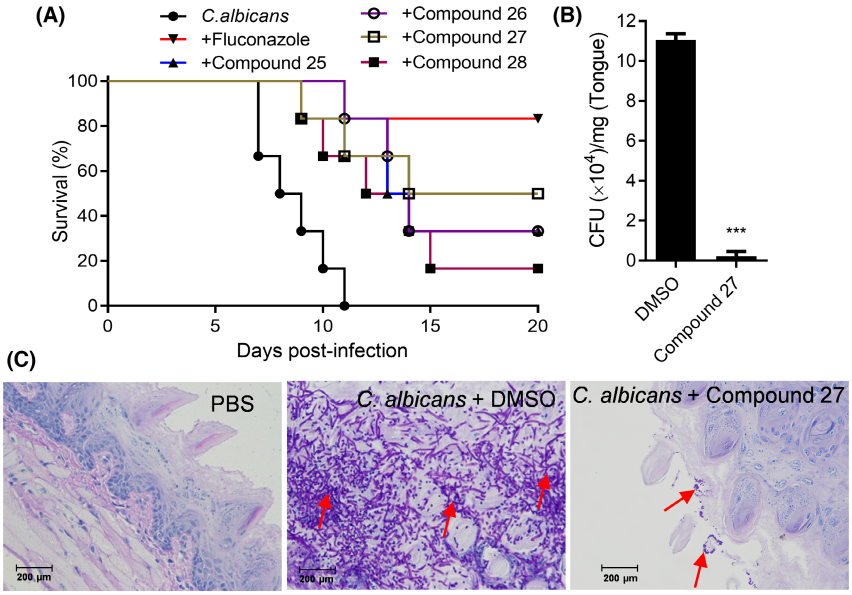

FIGURE 4 Efficacy of the piperazine compounds (100 μM) against *C. albicans* SC5314 in the mouse infection models. (A) The compounds (100 μM) reduced the mortality of mice infected with *C. albicans* SC5314. (B) The compound **27** (100 μM) reduced the in vivo pathogen cell number in mouse tongue infected by *C. albicans* SC5314 in the presence or absence of compound **27**. (C) Pathological sections of mouse tongues infected by *C. albicans* SC5314 in the presence or absence of compound **27**. The red arrows show the *C. albicans* SC5314 infection sites in the mouse tongues. The data are the means ± standard deviations from three independent experiments. ***$p < 0.001$ (one-way ANOVA).

growth genes, such as TYE7, RNR1, ERG3, GIN4, ATP20, PMT4, NIP7, RNH1, SHA3 and CSR1, were downregulated in *C. albicans* SC5314 in the presence of compound **27**. In summary, the intervention of the compound **27** on pathogenicity of *C. albicans* such as morphological transformation, is achieved by affecting a variety of signalling pathways (Figure S4E).

Compound 27 inhibited virulence in various clinical *C. albicans* strains

To determine whether the compound **27** exhibits efficacy against clinical *C. albicans* strains, we chose ten different clinical *C. albicans* strains from the oral cavity collected from Hunan Child's Hospital of China and studied the effects of the compound **27** on the virulence of these strains. Intriguingly, the compound **27** strongly inhibited the virulence of these clinical *C. albicans* strains at 100 μM (Figure S5). Among these clinical *C. albicans* strains, the compound **27** inhibited the virulence of clinical *C. albicans* strains HCH 52, 54, 55, 56, 57 and 61 by more than 50% at a final concentration of 100 μM (Figure S5). We also tested the minimum inhibitory concentrations (MICs) of fluconazole against these clinical *C. albicans* strains. The results showed that two of the clinical *C. albicans* strains, clinical strain HCH 53 and clinical strain HCH 60, were fluconazole resistant (Table 1). The compound **27** could also inhibit the virulence of the clinical fluconazole-resistant strains HCH53 and HCH60 (Figure S5).

Compound 27 decreased the pathogenicity of clinical drug-resistant *C. albicans* strains

Combination treatment is commonly used to improve curative effects or to overcome bacterial or fungal drug resistance. As the compound **27** exhibited excellent activity against the biological function and virulence of *C. albicans*, we continued to investigate whether the compound **27** has additive activity with antifungal agents against drug-resistant *C. albicans* strains in addition to its antifungal activity. The results showed that, compared to fluconazole, which showed no antifungal activity against the clinical fluconazole-resistant *C. albicans* strain HCH60, the compound **27** still effectively inhibited the virulence of strain HCH60 (Figure 5A). Interestingly, the combination of the compound **27** with fluconazole showed a stronger inhibitory effect on the virulence of the fluconazole-resistant *C. albicans* strain HCH60 than the addition of compound **27** alone. As shown in Figure 5A, the virulence of the fluconazole-resistant *C. albicans* strain HCH60 to A549 cells was reduced to less than 40% after combined treatment with 4, 8 or 16 μg/ml fluconazole and compound **27** at 100 μM. Consistent with this, in the presence of compound **27**, the level of clinical fluconazole-resistant *C. albicans* strain HCH60 infection in the mouse tongue decreased significantly (Figure 5B), and pathological sections of the mouse tongue also showed that there was almost no clinical fluconazole-resistant *C. albicans* strain HCH60 infection in the presence of compound **27** (Figure 5C). These results showed that the compound **27** not only had a good therapeutic effect on the infection caused by a clinical drug-resistant *C. albicans* strain in mice but also had an excellent additive effect with antifungal agents.

TABLE 1 Minimum inhibitory concentrations (MICs) of the compound **27** and fluconazole against the *C. albicans* strain SC5314 and clinical *C. albicans* strains used in this study.

C. albicans strain	Fluconazole MIC (μg/ml)	Compound 27 (μM)
SC5314 (wild type)	4	>200
Clinical strain HCH 52	4	>200
Clinical strain HCH 53	128	>200
Clinical strain HCH 54	1	>200
Clinical strain HCH 55	0.25	>200
Clinical strain HCH 56	0.5	>200
Clinical strain HCH 57	0.5	>200
Clinical strain HCH 58	8	>200
Clinical strain HCH 59	1	>200
Clinical strain HCH 60	128	>200
Clinical strain HCH 61	0.5	>200

Compound 27 restored the faecal microbiota analysis of mice infected by *C. albicans*

The intestinal microbiota is very important for the host. Approximately 10 trillion bacteria reside in the human intestine. The intestinal microbiota can synthesize various nutrients needed for human growth and development. It is also involved in the metabolism and absorption of a variety of nutrients (Kunz et al., 2009). *C. albicans* is a normal resident of the human gut microbiota and causes opportunistic disseminated infections in individuals with weakened immune functions (Heng et al., 2021). Therefore, we studied the effects of *C. albicans* infection and treatment with fluconazole or compound **27** on the intestinal microbiota in mice by sequencing 16 S rRNA (bacteria) and internal transcribed sequence (ITS) region (fungi) genes. We found that the gut microbiome composition in mice changed significantly after *C. albicans* infection and fluconazole treatment (Figure 6). More than 50% of the bacterial flora abundance changed. In conclusion, compared with those of the control group, the composition and abundance of the gut microbiota in the *C. albicans* infection

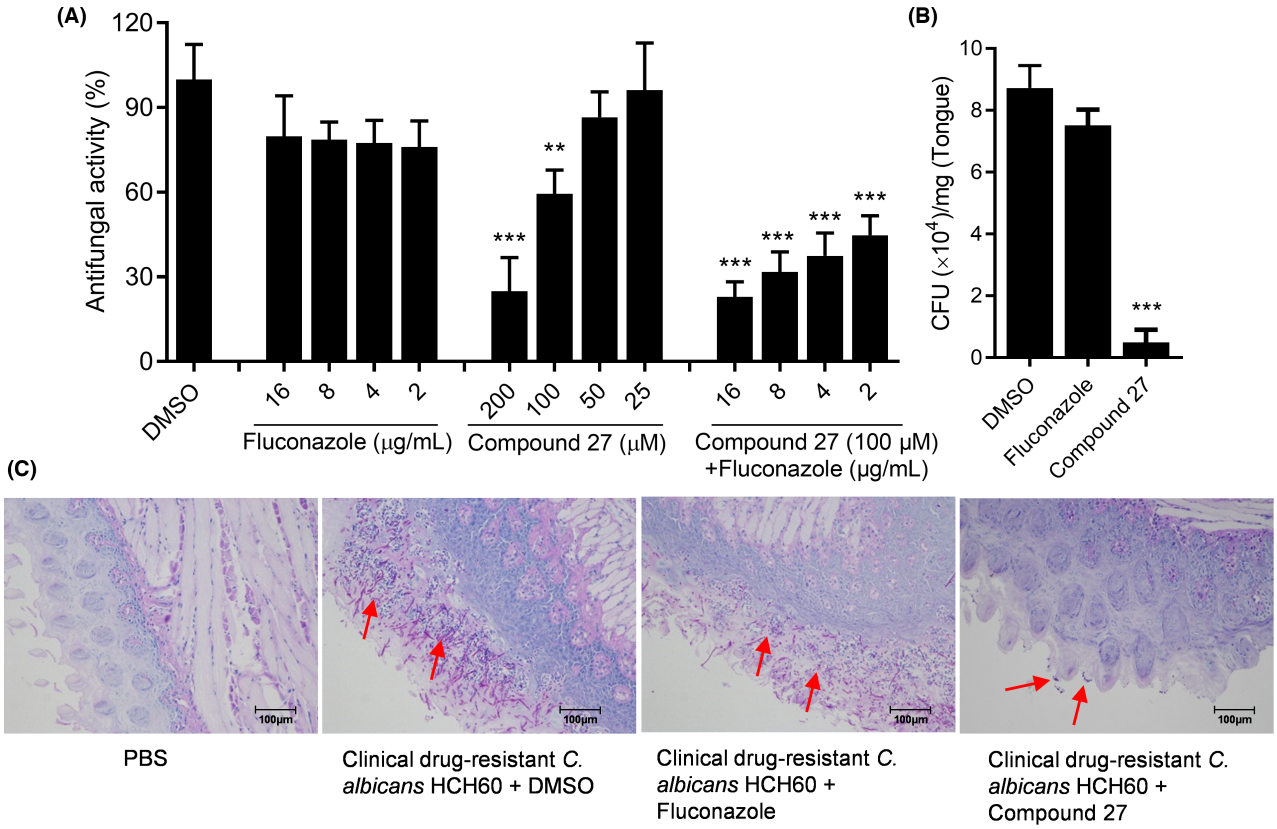

FIGURE 5 Effects of the compound **27** on the clinical fluconazole-resistant *C. albicans* strain. (A) Effects of the compound **27** and its additive activity with fluconazole on the virulence of the clinical fluconazole-resistant *C. albicans* strain. (B) In vivo pathogen cell number in mouse tongue infected with the clinical fluconazole-resistant *C. albicans* strain treated with or without the compound **27** (100 μM). (C) Pathological sections of mouse tongues infected with the clinical fluconazole-resistant *C. albicans* strain treated with or without the compound **27** (100 μM). The red arrows show the infection sites of the clinical fluconazole-resistant *C. albicans* strain in the mouse tongues. The data are the means ± standard deviations from three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$ (A, two-way ANOVA; B, one-way ANOVA).

group and fluconazole treatment group changed significantly. Interestingly, the composition and richness of the faecal microbiota treated with the compound **27** were clearly restored to those under normal conditions, which was better than fluconazole ingesting, and tended to be more normal faecal microbiota ecology (Figure 6).

DISCUSSION

Piperazine derivatives have been reported to show promising antifungal activity by causing fungal cell death (Moraca et al., 2014; Thamban et al., 2017). Differently, our previous study showed for the first time that compound 1-(4-ethoxyphenyl)-4-(1-biphenylol-2-hydroxypropyl)-piperazine suppresses the virulence of *C. albicans* by inhibiting the formation of hyphae and biofilms (Zhao et al., 2018), instead of inducing fungal death. Encouraged by this, we keep on studying piperazine skeleton compounds by introducing different heteroaryloxy moieties (Figure 1) in the present study, and deeply evaluated their effects on the formation of mycelia and biofilms in *C. albicans* (Figure 2A,B; Figures S2 and S3).

Indole and acetophenone derivatives exhibited weak activity on hypha formation and biofilm formation compared with 9H-carbazole derivatives (compounds **13**–**39**), probably because the bulky size of hetero-aryloxy group might benefit for the inhibitory activities, which is consistent with previous SAR. 9H-carbazole derivatives also exhibited best anti-virulence activities induced by *C. albicans* (Figure 2). Moreover, different substitutions on phenylpiperazine group exhibited different activities. It was shown that halogen substitutions compounds (**25, 26, 27** and **28**) are better than alkyl or alkoxy substitutions against *C. albicans*. Similar with previous results (Moraca et al., 2014; Thamban et al., 2017; Zhao et al., 2018), these compounds affected *C. albicans* physiology and pathogenicity but have no effect on the growth of *C. albicans* (Figure 3; Figure S1). These results provide additional evidence that piperazine compounds might be promising candidates for the development of novel drugs against *C. albicans* infection.

Candida albicans is a polymorphic fungus that grows as yeast, pseudohyphae and hyphae. The transition

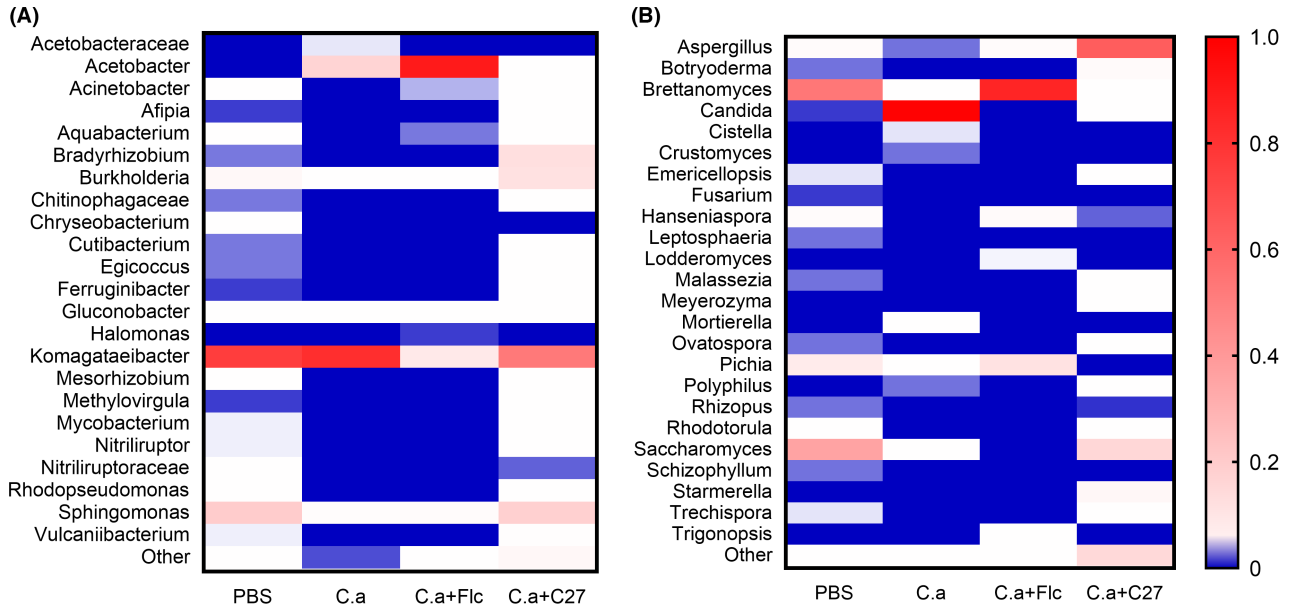

FIGURE 6 Mouse faecal microbiota analysis profiling. Faecal microbiota heatmap for mice in each of the four groups at the end of the experimental period. Composition of the murine faecal bacteriome (A) and mycobiome (B) with or without fluconazole treatment or compound **27** treatment at the genus level. A colour key with the correlation coefficient is shown to the right of the heatmap; red represents a positive correlation, and blue represents a negative correlation. C.a, *C. albicans*; C27, Compound **27**; Flc, Fluconazole.

from yeast state to mycelial state is a virulence characteristic of *C. albicans* (Leberer et al., 2001; Zheng et al., 2004). The hyphae of *C. albicans* play important roles in tissue invasion, cell damage, and immune escape (d'Enfert et al., 2021). Furthermore, the ability of *C. albicans* to form hyphae is critical for proper biofilm growth and maintenance (Sudbery, 2011). The formation of *C. albicans* biofilm is closely related to drug resistance (Colosi et al., 2009). Some studies have found that indole derivatives may inhibit mycelium growth and biofilm formation through the Ras-cAMP-PKA pathway (Ma et al., 2022). And our previous study also showed (1-aryloxy-2-hydroxypropyl)-phenylpiperazine compounds disrupt the hypha formation and biofilm formation of *C. albicans* mostly by interfering with the cAMP-PKA and MAPK pathways (Zhao et al., 2018). In this study, we also found that the compound **27** has a similar pathway to mechanism of action through an analysis of the results of RNA-Seq, quantitative reverse transcription PCR (RT-qPCR) and Western blot (Figures S4).

At present, the abuse of antibiotics and the emergence of drug-resistant *C. albicans* have become serious problems worldwide (Mendelson et al., 2017). New strategies for the treatment of *C. albicans* infections is therefore a key research area. Our present study showed that (1-heteroaryloxy-2-hydroxypropyl)-phenylpiperazines possessed high application potential in preventing the pathogenicity of *C. albicans* by inhibiting the formation of mycelia and biofilms, rather than directly killing pathogenic cells to prevent the emergence of drug resistance. We also found that the compounds **25, 26, 27** and **28** showed excellent anti-virulence activity in both in vitro experiments and in vivo mouse infection models (Figures 2–4). Intriguingly, the lead compound **27** strongly inhibited the virulence of the 10 clinical *C. albicans* strains at 100 μM, including two clinical drug-resistant *C. albicans* strains (Figure S5). This result indicated that the compound **27** had good prospects for attenuating the virulence of clinical *C. albicans* strains and clinical drug-resistant *C. albicans* strains (Figure 5; Figure S5).

We also tested the cytotoxicity of compound **27** to A549 cells at the final concentration of 200, 300, 400, 500 μM (96.2, 144.3, 192.4, 240.5 μg/ml). The result showed that the compound **27** exhibited low cytotoxicity only at the final concentration of 500 μM (Figure S6). At the same time, the compound **27** and fluconazole were found to have additive effects on the clinical fluconazole-resistant strain (Figure 5A). These results suggested that the compound **27** showed good efficacy against both the wild-type and clinical *C. albicans* strains, especially clinical drug-resistant *C. albicans* strains, providing a potential solution for overcoming the current clinical challenge. The importance of these findings was enhanced by the fact that the compound **27** could significantly reduce the content of *Candida* in the mouse faecal microbiota and restore the richness of the mouse faecal microbiota (Figure 6). In general, these results suggested that the new compound **27** may be a promising antifungal drug candidate or adjuvant for use against infections caused by *C. albicans* strains, including clinical drug-resistant *C. albicans* strains.

AUTHOR CONTRIBUTIONS

J.H., and S.S. contributed equally to this work. Y.D., J.H., and S.S. designed the research. J.H., S.S., S.Z., X.S., Z.W., X.H., and Q.X. performed the research. J.H., S.S., S.Z., X.S. and Y.D. analysed the data. J.H., S.S. and Y.D. wrote the paper.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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