Inhibition of yeast-to-hypha transition and virulence of *Candida albicans* by 2-alkylaminoquinoline derivatives

Lili Meng[1,2#], He Zhao[3#], Shuo Zhao[1,2#], Xiuyun Sun[1,2], Min Zhang[3]*, Yinyue Deng[1,2]*

[1]*Integrative Microbiology Research Centre, College of Agriculture, South China Agricultural University, Guangzhou 510642, China*

[2]*School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Guangzhou 510275, China*

[3]*Key Laboratory of Functional Molecular Engineering of Guangdong Province, School of Chemistry and Chemical Engineering, South China University of Technology, Guangzhou 510641, China*

[#]These authors have contributed equally to this work.

***Corresponding authors:**

Yinyue Deng: yinyue_deng@hotmail.com

Min Zhang: minzhang@scut.edu.cn

Tel: (+86)2085286471; Fax: (+86)2085280203

Running title: 2-Alkylaminoquinolines inhibit *C. albicans* virulence

ABSTRACT

A rapid increase in *Candida albicans* infection and drug resistance has caused an emergent need for new clinical strategies against this fungal pathogen. In this study, we evaluated the inhibitory activity of a series of 2-alkylaminoquinoline derivatives against *C. albicans* isolates. A total of 28 compounds were assessed for their efficacy in inhibiting the yeast-to-hypha transition, which is considered one of the key virulence factors in *C. albicans*. Several compounds showed strong activity to decrease the morphological transition and virulence of *C. albicans* cells. The two leading compounds, compound 1 (2-[piperidin-1-yl]quinolone) and compound 12 (6-methyl-2-[piperidin-1-yl]quinoline), remarkably attenuated *C. albicans* hyphal formation and cytotoxicity in a dose-dependent manner, but they showed no toxicity to either *C. albicans* cells or human cells. Intriguingly, compound 12 showed an excellent ability to inhibit *C. albicans* infection in the mouse oral mucosal infection model. This leading compound also interfered with the expression levels of hypha-specific genes in the cAMP-PKA and MAPK signaling pathways. Our findings suggest that 2-alkylaminoquinoline derivatives could potentially be developed as novel therapeutic agents against *C. albicans* infection due to their interference with the yeast-to-hypha transition.

KEYWORDS: *Candida albicans*; yeast-to-hypha transition; virulence; 2-alkylaminoquinoline derivatives; oral mucosal infection model

INTRODUCTION

Candida albicans is known as an opportunistic pathogen of humans that may lead to serious and even life-threatening diseases in immunocompromised patients, resulting in an approximately 40% mortality rate (1). Normally, *C. albicans* cells first adhere to the tissue surface of the host, and the yeast form of *C. albicans* cells has been shown to play an important role in tissue surface adhesion (2). Morphological transitions from yeast to filamentous forms are the major contributor to the pathogenicity of *C. albicans* (3). These conversions depend on environmental cues, including living conditions, nutrient substances and several signaling metabolites (4,5). Farnesol, the first quorum-sensing system identified in eukaryotes, can mediate *C. albicans* dimorphism (6). In contrast, tyrosol can accelerate *C. albicans* growth and germ tube formation (7).

Previous studies have indicated that morphological changes in *C. albicans* depend on a network including multiple signaling pathways. The two best-studied pathways are the cAMP-protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways (8). Overexpression of *EFG1*, which encodes an essential transcription factor that activates the PKA pathway, was shown to enhance the filamentous form of *C. albicans* and stimulate the expression of hypha-specific genes (9-11). CPH1 is a transcription factor in the MAPK pathway, and the *cph1/cph1/efg1/efg1* double mutant was restricted to the yeast form, while either the *cph1/cph1* mutant or the *efg1/efg1* mutant retained some ability to switch from the yeast to the filamentous form, suggesting that morphogenesis is mostly controlled by these two pathways (12).

Some antifungal agents have been successfully used in therapeutic treatments against *C. albicans*. Triazoles, such as voriconazole and fluconazole, have been widely used to treat the infections caused by *Candida* spp. Under anaerobic and aerobic conditions, fluconazole inhibits *C. albicans* cells by 99% and 90%, respectively (13). Amphotericin B is an important antifungal agent against deep-seated fungal infection despite the side effects (14,15). Drug combination is also

used as a strategy to combat candidiasis. It was reported that lovastatin has synergistic effect with itraconazole to inhibit biofilms of *C. albicans*; and curcumin takes synergistic action with fluconazole to treat clinical isolates of *C. albicans* (16,17). However, serious drug resistance has arisen rapidly in recent years, compromising the use of these antifungal agents. Therefore, there is an emergent need to develop new strategies and novel drugs to combat candidiasis.

2-Alkylaminoquinolines, which are widely used for their pharmaceutical and biological activities, have been attracting increasing attention in physiological and pathophysiological studies. They were confirmed as the antagonist that modulated native TRPC4/C5 ion channels in various cells and tissues (18,19). In our previous report, we modified the synthesis of 2-alkylaminoquinolines by copper-catalyzed dehydrogenative α-C(sp^3)−H amination of tetrahydroquinolines with *O*–benzoyl hydroxylamines under mild conditions (20). In this study, we demonstrate the screening and evaluation of a series of 2-alkylaminoquinoline derivatives in the inhibition of *C. albicans* yeast-to-hypha transition and virulence. The two leading derivatives showed excellent efficacy in blocking the morphological transition and virulence but did not obviously influence the growth rate of *C. albicans* cells. Overall, our methods focus on evaluating pathogenesis-related functions using both *in vitro* and *in vivo* models to promote the development of novel antifungal therapeutics against *C. albicans* infection.

RESULTS

2-Alkylaminoquinoline derivatives inhibit hyphal formation in *C. albicans*

The morphological transition is important for *C. albicans* to infect humans and cause disease (12; 21-23). Thus, the influences of the 2-alkylaminoquinoline derivatives on the *C. albicans* yeast-to-hypha transition were evaluated *in vitro* under hyphal induction conditions at 37°C. After 6 h induction, the majority of *C. albicans* cells in the control group had formed germ tubes, while hyphal formation was obviously inhibited by the addition of many of the 2-alkylaminoquinoline derivatives (Fig. S1). At least 9

compounds (Nos. 1, 2, 5, 6, 7, 11, 12, 13, 16) reduced hyphal formation in *C. albicans* cells by more than 70% when present at a final concentration of 100 μM (Fig. 1). Among them, compounds 7 and 11 inhibited hyphal formation by approximately 95% (Fig. 1).

2-Alkylaminoquinoline derivatives attenuate *C. albicans* virulence

To evaluate the effects of 2-alkylaminoquinoline derivatives on the pathogenicity of *C. albicans*, we then investigated whether these compounds influenced *C. albicans* virulence in a human cell line. Cytotoxicity was measured by quantifying the release of lactate dehydrogenase (LDH) into the supernatants of cultured A549 cells. Many derivatives, such as compounds 1, 9, 11, 12, 14, 18, and 21-28, showed no toxic effects on A549 cells at a final concentration of 100 μM (Fig. 2A). In addition, the exogenous addition of some 2-alkylaminoquinoline derivatives led to a significant reduction in *C. albicans* cytotoxicity to A549 cells (Fig. 2B). Compounds 1, 5, 6, 7, 9 and 12 were highly effective at attenuating *C. albicans* cytotoxicity by more than 80% when present at a final concentration of 100 μM, and compounds 1, 9 and 12 also exerted no toxic effects on the cell line at 8 h postinoculation (Fig. 2A, 2B). Given that compound 9 did not efficiently inhibit hyphal formation in *C. albicans* (Fig. 1), compounds 1 and 12 were then selected for further investigation.

Compounds 1 and 12 do not obviously affect *C. albicans* growth rate but alter its morphology

Both compounds 1 (2-[piperidin-1-yl]quinolone) and 12 (6-methyl-2-[piperidin-1-yl]quinoline) (Fig. 3A, S2) exhibited high capacity to reduce *C. albicans* cytotoxicity by more than 80% at a final concentration of 100 μM (equivalent to 21.2 μg/mL and 22.6 μg/mL, respectively) (Fig. 2B) but did not obviously affect the growth rate of the pathogenic cells (Fig. 3B). Given that compounds 1 and 12 showed excellent inhibition of hyphal formation in *C. albicans* (Fig. 1, 3C), these compounds might be good candidates for development as novel antivirulence agents against *C. albicans* infection. We then further investigated the effects of compounds 1 and 12 on

C. albicans morphology; in good agreement with their inhibition of hyphal formation, compounds 1 and 12 also obviously affected the colony morphology of *C. albicans*. After the addition of compound 1 or 12, the colonies changed from wrinkled to slippery (Fig. 3D).

2-Alkylaminoquinoline derivatives inhibit *C. albicans* hyphal formation and virulence in a dose-dependent manner

To determine whether the effects of 2-alkylaminoquinoline derivatives on *C. albicans* are related to their dosage, different concentrations of compounds 1 and 12 were assessed for their inhibitory activity on the *C. albicans* morphological transition and virulence (Fig. 4). Both compounds 1 and 12 exhibited dose-dependent activity, in which they reduced *C. albicans* hyphae formation by more than 70% at a final concentration of 50 μM (equivalent to 10.6 μg/mL and 11.3 μg/mL, respectively) (Fig. 4A). The addition of compounds 1 and 12 at a final concentration of 50 μM decreased *C. albicans* virulence by approximately 40% and 60%, respectively (Fig. 4B). Compound 12 inhibited *C. albicans* virulence by more than 49% when present at a final concentration of 25 μM (equivalent to 5.65 μg/mL) (Fig. 4B).

Compound 12 inhibits *C. albicans* infection in the oral mucosal infection model

In addition to the assays *in vitro*, we continued to test whether the 2-alkylaminoquinoline derivatives have antifungal activity against *C. albicans in vivo* by using the mouse oral mucosal infection model. In the presence of compound 12, the number of *C. albicans* cells aggregated in the pathological tissues was much less than that in the tissues infected with only *C. albicans,* and addition of compound 12 restored the tissues to a state similar to that of the uninfected group (Fig. 5).

Compound 12 inhibits the *C. albicans* morphological transition by interfering with the cAMP-PKA and MAPK pathways

To establish a putative model of the effects of 2-alkylaminoquinoline derivatives on the *C. albicans* morphological transition, we continued to investigate whether compound

12 interfered with the signaling pathways involved in hyphal development. Hyphal formation in *C. albicans* is associated with two established signaling pathways: cAMP-PKA (cyclic adenosine monophosphate/protein kinase A) and MAPK (mitogen-activated protein kinase cascade) pathways. We then used real-time PCR analysis to analyze the effects of the compounds on the hypha-specific genes. Exogenous addition of compound 12 inhibited the expression of PDE2, CDC35 and TEC1, which are regulators involved in the cAMP-PKA pathway (Fig. 6A, 6B) (8, 24). In addition, some regulators of the MAPK cascade, such as HST7 and CPH1 (8), were downregulated by the exogenous addition of compound 12 (Fig. 6A, 6B). The expression of ALS3, which plays a crucial role in adhesion (25), was also obviously repressed (Fig. 6A). In addition, the expression level of HWP1, a glucan-linked protein with serine/threonine-rich regions that were forecast to function in extending a ligand-binding domain into the extracellular space (26), was reduced dramatically (Fig. 6A). Taken together, these results demonstrated that compound 12 influenced complex signal transduction pathways to interfere with the *C. albicans* filamentation process.

Compound 12 inhibits hyphal formation and cytotoxicity in various clinical *Candida* species

To determine whether the efficacy of 2-alkylaminoquinoline derivatives on *C. albicans* is widely conserved, we collected several different clinical *Candida* species, including *C. albicans* ATCC 90028, *C. albicans* ATCC 10231, *C. albicans* ATCC 14053, *C. tropicalis* ATCC 750 and *C. glabrata* ATCC 2001, and investigated the effects of compound 12 on their morphological transition and virulence. Intriguingly, only the cells of *C. albicans* ATCC 9008 and *C. albicans* ATCC 10231 formed hyphae under this conditions, exogenous addition of compound 12 exerted strong inhibition on hyphal formation in both *C. albicans* ATCC 9008 and *C. albicans* ATCC 10231 (Fig. 7A). Addition of 100 μM compound 12 (equivalent to 22.6 μg/mL) caused a reduction in hyphal formation in *C. albicans* ATCC 90028 and *C. albicans* ATCC 10231 to

approximately 30% and 50% of that untreated group, respectively (Fig. 7B), while it reduced the cytotoxicity of *C. albicans* ATCC 90028, *C. albicans* ATCC 10231, *C. albicans* ATCC 14053, *C. tropicalis* ATCC 750 and *C. glabrata* ATCC 2001 on A549 cells to 45%, 37%, 12%, 25% and 30% of that untreated group, respectively (Fig. 7C).

DISCUSSION

Numerous studies have suggested that morphogenesis is an essential factor for the pathogenicity of dimorphic fungi. In this study, 2-alkylaminoquinoline-derived compounds were first evaluated for their inhibitory activity against *C. albicans* morphogenesis. Our results indicated that some 2-alkylaminoquinoline compounds are excellent agents against the yeast-to-hypha transition in *C. albicans* (Fig. 1, 3C). Given the interaction between morphological transition and virulence in *C. albicans*, our results also demonstrated the notable ability of 2-alkylaminoquinoline derivatives to attenuate *C. albicans* virulence (Fig. 2B, 4B, 5). Moreover, these compounds showed low toxicity to human cell line A549 (Fig. 2A). We also confirmed the efficacy of the leading compound 12 against other *Candida*. spp on the hyphal formation and cytotoxicity. These results suggested that 2-alkylaminoquinoline derivatives might be good candidates for the development of new antifungal agents that block hyphal formation.

The current clinical treatments for candidiasis caused by *C. albicans* or other *Candida* spp. rely almost entirely on limited conventional antifungal agents, such as polyenes, which usually kill the pathogenic cells directly, and azoles that inhibit 14α-demethylation of lanosterol in ergosterol biosynthetic pathway (27, 28). However, the limitations of drug development has compromised the strategies currently used in clinical treatment. Therefore, the development of new strategies and novel drugs to treat *Candida* spp. pathogens is urgently required. As morphological transitions between yeast cells and filamentous forms play a vital role in pathogenesis, some recent studies have already focused on the inhibition of hyphal formation in *C.*

albicans (29, 30). Our results showed that compound 12 obviously interfered with the cAMP-PKA and MAPK pathways, which are widely employed by fungal pathogens to control the morphological transition. In addition, compound 12 also inhibits the expression of HWP1 and ALS3 (Fig. 6). HWP1 is a membrane-anchored protein that plays an important role in biofilm formation, while ALS3 is an invasin of *C. albicans* (8). Our study here provides an additional option for the design of antifungal drugs using a functional approach. Given that compound 12 showed an excellent ability to inhibit *C. albicans* infection in the mouse oral mucosal infection model (Fig. 5), compound 12 appeared to be highly promising in preventing *C. albicans* pathogenicity via inhibition of hyphal formation rather than direct killing of pathogenic cells.

2-Alkylaminoquinolines were previously reported to exhibit extensive biological functions and pharmacological activities, which inspired us to further exploration of the pharmacological activity of 2-alkylaminoquinoline-derived compounds. In this study, we report 28 2-alkylaminoquinoline derivatives for the first time and assess their ability to inhibit the morphological transition and virulence in *C. albicans*. Some of the derived compounds showed excellent efficacy in preventing the yeast-to-hypha transition and reducing virulence *in vitro* and *in vivo* but did not obviously interfere with the growth rate of *C. albicans* cells (Fig. 1, 2B, 3B, 5). Intriguingly, these compounds were nontoxic or only slightly toxic to human cells (Fig. 2A). For these compounds, we would like to continue to modify the structures and perform more assays on the animal models. We also found that the compounds have a significantly synergistic effect with fluconazole against the antifungal resistant isolate FLU-R in cell line model (Table S1, Fig. S3), we would also continue to test the synergistic effect of these compounds with different conventional antifungal agents on the treatment of *C. albicans* infection. Overall, our findings focus on targeting the morphological transition instead of killing pathogenic fungal cells to promote the development of a novel strategy against *C. albicans* infection.

MATERIALS AND METHODS

Strains and growth conditions

Candida albicans SC5314 (ATCC® MYA-2876TM), *C. albicans* ATCC 90028, *C. albicans* ATCC 10231, *C. albicans* ATCC 14053, *C. tropicalis* ATCC 750 and *C. glabrata* ATCC 2001 used in this study were maintained at 30°C in yeast peptone dextrose (YPD, 2% peptone, 2% glucose, 1% yeast extract and 2% agar) agar plates. Before the following assays, *C. albicans* was incubated at 30°C with shaking at 200 rpm in GMM (glucose minimal medium; 6.7 g Bacto yeast nitrogen base and 0.2% glucose per liter) overnight. Human lung epithelial A549 cells were incubated in DMEM (Dulbecco's modified Eagle's medium) with 10% FBS (fetal bovine serum) at 37°C with 5% CO_2.

Chemical synthesis of 2-alkylaminoquinolines

Briefly, under oxygen atmosphere, a mixture of 1,2,3,4-tetrahydroquinoline (0.2 mmol), 1-benzoyloxy-piperidine (0.6 mmol), CuI (0.08 mmol), KOH (0.3 mmol), K_2CO_3 (0.3 mmol), BHT (0.45 mol %) and 3 mL distill THF was stirred at 80 ^{o}C for 18 h. The mixture was extracted with EtOAc three times, and the combined organic extracts were washed with aq. NaCl three times and dried over Na_2SO_4 and concentrated in vacuo. All compounds (Fig. S1B) were dissolved in dimethyl sulfoxide (DMSO) at an original concentration of 20 mM.

Hyphal formation assays

The overnight-cultured *C. albicans* grown at 30°C was diluted to an optical density (OD_{600}) of 0.1 using fresh GMM. The yeast cells were incubated at 37°C for 6 h with compounds at the concentration of 100 μM, and the same volume of DMSO was used as a control. Cells were harvested by centrifugation at 5000 rpm for 10 min. Cell suspensions were visualized directly under a Leica inverted fluorescence microscope with 100× magnification. All the strains were treated following the same methods.

Cytotoxicity assays

Cytotoxicity was assessed by measuring the release of LDH from A549 cells. The

A549 cells were routinely grown in DMEM supplemented with 10% FBS in a 96-well tissue culture plate with 1.5×10^4 cells/well. Confluent A549 cells were washed and incubated with DMEM containing 1% FBS before infection. Overnight-cultured *C. albicans* cells were diluted to an OD_{600} of 0.1 with DMEM containing 1% FBS in the absence or presence of the tested compounds at the final concentrations indicated. A549 cells were infected with fungal cells for 8 h. The LDH level in the supernatant was measured, and cytotoxicity was calculated by comparing the LDH level to that of the uninfected control. Different strains and compounds were tested using the same method.

Colony morphology. Spider medium agar plates (1% peptone, 1% mannitol, 0.2% K_2HPO_4 and 1.5% agar) were supplemented with different concentrations of compounds as indicated. *C. albicans* SC5314 cells were grown in these 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 diluted in the same medium to an OD_{600} of 0.05 in the absence or presence of the tested compounds at the final concentrations indicated for 48h at least. Three hundred microliters of inoculated culture were 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).

Mouse oral mucosal infection model

The protocol of the mouse oral mucosal infection was based on a published study with minor modifications (31, 32). In this experiment, 20-22 g male BaLB/c mice (3 mice per group) were subcutaneously injected with hydrocortisone (225 mg/kg) dissolved in PBS (phosphate-buffered saline) containing 0.5% Tween-20 on the first day. The next day, the overnight-cultivated cells were washed with Hank's Balanced Salt Solution (Biohao Biotechnology Co., Ltd, Wuhan, China) and then twice with PBS. The cells

were resuspended in PBS to an OD_{600} of 0.1 in the absence or presence of compound 12 at a final concentration of 100 μM (equivalent to 22.6 μg/mL). Then, 10% chloral hydrate (Yuanye Biotech, Shanghai, China) was injected into mice, the anesthetized mice were placed on an isothermal mat maintained at 37°C, and cotton balls soaked with pathogenic cells were placed under their tongues for 75 min. Mice were sacrificed on the fifth day, and their tongues were dissected for further analysis using pathological sections and scanning electron microscope observation.

Quantitative real-time PCR

Overnight cultures of *C. albicans* cells grown in YNB + 0.2% glucose at 30°C were diluted in the same medium to an OD_{600} of 0.1 in the absence or presence of the tested 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, California, America) and quantified. cDNA was obtained through a reverse transcription reaction using a reverse transcription kit (TaKaRa Biotechnology, Dalian, China) with the primers shown in Table S2, and real-time PCR was performed with a 7300Plus 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 (33). The relative expression levels of the target genes were calculated using the comparative CT (ΔΔCT) method.

STATISTICAL ANALYSIS

For statistical analysis, the Excel data analysis package was used to calculate the means and the standard deviation of the means. The data were analyzed using the GraphPad Instate software package (version 7.0) according to the Tukey-Kramer multiple comparison test at a $p<0.05$ or $p<0.01$ level of significance. All results were calculated from the means of three separate experiments. The results were expressed as the means ± standard deviation.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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) and the National Natural Science Foundation of China (No. 31571969).

REFERENCES

1. Ford CB, Funt JM, Abbey D, Issi L, Guiducci C, Martinez DA, Delorey T, Li BY, White TC, Cuomo C, Rao RP, Berman J, Thompson DA, Regev, A. 2015. The evolution of drug resistance in clinical isolates of *Candida albicans*. eLIFE 4: e00662. https://doi.org/10.7554/eLife.00662.001.
2. Bohm L, Torsin S, Tint SH, Eckstein MT, Ludwig T, Perez JC. 2017. The yeast form of the fungus *Candida albicans* promotes persistence in the gut of gnotobiotic mice. PLoS Pathog 13: 1-26. https://doi.org/10.1371/journal.ppat.1006699.
3. Murad A M, Leng P, Straffon M, Wishart J, Macaskill S, MacCallum D, Schnell N, Talibi D, Marechal D, Tekaia F, d'Enfert C, Gaillardin C, Odds FC, Brown, AJ. 2001. NRG1 represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. EMBO J 20: 4742-4752. https://doi.org/10.1093/emboj/20.17.4742.
4. Nickerson KW, Atkin AL, Hornby JM. 2006. Quorum Sensing in Dimorphic Fungi: Farnesol and Beyond. Appl Environ Microbiol 72: 3805-3813. https://doi.org/10.1128/AEM.02765-05.
5. Mattia E, Cassone A. 1979. Inducibility of germ-tube formation in *Candida albicans* at different phases of yeast growth. J Gen Microbiol 113: 439-442. https://doi.org/10.1099/00221287-113-2-439.
6. Jacob M. Hornby, Ellen C. Jensen, Amber D. Lisec, Joseph J. Tasto, Brandon

389 Jahnke, Richard Shoemaker, Patrick Dussault, Kenneth W. Nickerson. 2001.
390 Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by
391 farnesol. Appl Environ Microbiol 67: 2982-2992.
392 https://doi.org/10.1128/AEM.67.7.2982–2992.2001.
393 7. Chen H, Fujita M, Feng Q, Clardy J, Fink GR. 2004. Tyrosol is a quorum-sensing
394 molecule in *Candida albicans*. Proc Natl Acad Sci USA 101: 5048-5052.
395 https://doi.org/10.1073/pnas.0401416101.
396 8. Han TL, Cannon RD, Villas-Bôas SG. 2011. The metabolic basis of *Candida*
397 *albicans* morphogenesis and quorum sensing. Fungal Genet Biol 48: 747-763.
398 https://doi.org/10.1016/j.fgb.2011.04.002.
399 9. Stoldt, VR, Sonneborn A, Leuker CE, Ernst, JF. 1997. Efg1p, an essential
400 regulator of morphogenesis of the human pathogen *Candida albicans*, is a
401 member of a conserved class of bHLH proteins regulating morphogenetic
402 processes in fungi. EMBO J 16: 1982-1991.
403 https://doi.org/10.1093/emboj/16.8.1982.
404 10. Sharkey LL, McNemar MD, Saporito-Irwin SM, Sypherd PS, Fonzi WA. 1999.
405 *HWP1* functions in the morphological development of *Candida albicans*
406 downstream of *EFG1*, *TUP1*, and *RBF1*. J Bacteriol 181: 5273-5279.
407 11. Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan QT, Edwards JE, Filler SG,
408 Mitchell AP. 2006. Critical role of Bcr1-dependent adhesins in *Candida albicans*
409 biofilm formation in vitro and in vivo. PLoS Pathog 44: 61-72.
410 https://doi.org/10.1371/journal.ppat.0020063.
411 12. Lo HJ, Köhler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR. 1997.
412 Nonfilamentous *Candida albicans* mutants are avirulent. Cell 90: 939-949.
413 https://doi.org/10.1016/S0092-8674(00)80358-X.
414 13. Zimmermann K, Bernhardt J, Knoke M, Bernhardt H. 2002. Influence of
415 voriconazole and fluconazole on *Candida albicans* in long-time continuous flow
416 culture. Mycoses 45: 41-46. https://doi.org/10.1111/j.1439-0507.2002.tb04545.x.
417 14. Burgess DS, Hastings RW, Lewis JS II. 2000. A time-kill evaluation of fluconazole
418 and amphotericin B against *Candida* isolates. J Pharm Tech 16: 102-106.

https://doi.org/10.1177/875512250001600307.

15. Louie A, Liu W, Miller DA, Sucke AC, Liu QF, Drusano GL, Mayers M, Miller MH. 1999. Efficacies of high-dose fluconazole plus amphotericin B and high-dose fluconazole plus 5-fluorocytosine versus amphotericin B, fluconazole, and 5-fluorocytosine monotherapies in treatment of experimental endocarditis, endophthalmitis, and pyelonephritis due to *Candida albicans*. Antimicrob Agents Chemother 12: 2831-40.
16. Zhou Y, Yang H, Zhou X, Luo H, Tang F, Yang J, Alterovitz G, Cheng L, Ren B. 2018. Lovastatin synergizes with itraconazole against planktonic cells and biofilms of *Candida albicans* through the regulation on ergosterol biosynthesis pathway. Appl Microbiol Biotechnol 102: 5255-5264. https://doi.org/10.1007/s00253-018-8959-8.
17. Garcia-Gomes AS, Curvelo JA, Soares RM, Ferreira-Pereira A. 2012. Curcumin acts synergistically with fluconazole to sensitize a clinical isolate of *Candida albicans* showing a MDR phenotype. Med Mycol 1: 26-32. https://doi.org/10.3109/13693786.2011.578156.
18. Miller M, Shi J, Zhu Y, Kustov M, Tian JB, Stevens A, Wu M, Xu J, Long S, Yang P, Zholos AV, Salovich JM, Weaver CD, Hopkins CR, Lindsley CW, McManus O, Li M, Zhu MX. 2001. Identification of ML204, a novel potent antagonist that selectively modulates native TRPC4/C5 ion channels. J Biol Chem 286: 33436-33446. https://doi.org/10.1074/jbc.M111.274167.
19. Clark DE, Higgs C, Wren SP, Dyke HJ, Wong M, Norman D, Lockey PM, Roach AG. 2004. A virtual screening approach to finding novel and potent antagonists at the melanin-concentrating hormone 1 receptor. J Med Chem 47: 3962-3971. https://doi.org/10.1021/jm040762v.
20. Zhao H, Chen XW, Jiang HF, Zhang M. 2017. Copper-catalysed dehydrogenative α-C(sp^3)–H amination of tetrahydroquinolines with O-benzoyl hydroxylamines. Org Chem Front 5: 539-543. https://doi.org/10.1039/c7qo00794a.
21. Sudbery P, Gow N, Berman J. 2004. The distinct morphogenic states of *Candida albicans*. Trends Microbiol 12:317-324. https://doi.org/10.1016/j.tim.2004.05.008.

22. Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL. 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot Cell 2:1053–1060. https://doi.org/10.1128/EC.2.5.1053-1060.2003.

23. Finkel JS, Mitchell AP. 2011. Genetic control of *Candida albicans* biofilm development. Nat Rev Microbiol 9:109–118. https://doi.org/10.1038/nrmicro2475.

24. Sudbery PE. 2011. Growth of Candida albicans hyphae. Nat Rev Microbiol 9:737–748. https://doi.org/10.1038/nrmicro2636.

25. Hoyer, LL. 2001. The *ALS* gene family of *Candida albicans*. Trends Microbiol 9: 176-180. https://doi.org/10.1016/S0966-842X(01)01984-9.

26. Staab JF, Sundstrom P. 1998. Genetic organization and sequence analysis of the hypha-specific cell wall protein gene *HWP1* of *Candida albicans*. Yeast 14: 681-686. https://doi.org/10.1002/(SICI)1097-0061(199805)14:7<681::AID-YEA256>3.0.CO;2-8.

27. Odds FC, Brown AJP, Gow NAR. 2003. Antifungal agents: mechanisms of action. Trends Microbiol 11:272-279. https://doi.org/10.1016/S0966-842X(03)00117-3.

28. Pierce CG, Srinivasan A, Uppuluri P, Ramasubramanian AK, Lopez-Ribot JL. 2013. Antifungal therapy with an emphasis on biofilms. Curr Opin Pharmacol 13:726-30. https://doi.org/10.1016/j.coph.2013.08.008.

29. Boon C, Deng Y, Wang LH, He Y, Xu JL, Fan Y, Pan SQ, Zhang LH. 2008. A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. ISME J 2:27-36. https://doi.org/10.1038/ISMEJ.2007.76.

30. Zhao S, Huang JJ, Sun X, Huang X, Fu S, Yang L, Liu XW, He F, Deng Y. 2018. (1-aryloxy-2-hydroxypropyl)-phenylpiperazine derivatives suppress *Candida albicans* virulence by interfering with morphological transition. Microb Biotechnol 11: 1080-1089. https://doi.org/10.1111/1751-7915.13307.

31. Dongari-Bagtzoglou A, Kashleva H, Dwivedi P, Diaz P, Vasilakos, J. 2009. Characterization of mucosal *Candida albicans* biofilms. PLoS One 4: e7967.

https://doi.org/10.1371/journal.pone.0007967.

32. Zarif L, Graybill JR, Perlin D, Najvar L, Bocanegra R, Mannino RJ. 2002. Antifungal activity of amphotericin B cochleates against *Candida albicans* infection in a mouse model. Antimicrob Agents Chemother 44: 1463-1469. https://doi.org/10.1128/AAC.44.6.1463-1469.2000.

33. Clement M, Fournier H, de Repentigny L, Belhumeur, P. 2000. Characterization of CaGSP1, the *Candida albicans* RAN/GSP1 homologue. Gene 250: 159-169. https://doi.org/10.1016/S0378-1119(00)00173-6.

FIGURE LEGENDS

FIG. 1 Effects of 2-alkylaminoquinoline derivatives on *C. albicans* SC5314 hyphal formation. Each experiment was performed at least three times in triplicate, and each time, at least 400 cells were counted for each treatment. Compounds were dissolved in DMSO, and the amount of DMSO used as the solvent for the compounds was used as a control. Data represent the means ± standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired *t* test).

FIG. 2 Effects of 2-alkylaminoquinoline derivatives on *C. albicans* SC5314 virulence using a cell line. (A) Analysis of the toxicity of compounds to A549 cells. The compounds were dissolved in DMSO, and the amount of DMSO used as the solvent for the compounds was used as a control. (B) Analysis of the effects of the compounds on the cytotoxicity of *C. albicans* to A549 cells. Cytotoxicity was detected and measured as LDH release. The LDH released by A549 cells after inoculation with *C. albicans* in the absence of compounds was defined as 100% to normalize the LDH release ratios of the other treatments. Data represent the means ± standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired *t* test).

FIG. 3 Influence of 2-alkylaminoquinoline derivatives on *C. albicans* SC5314 morphology. (A) Structures of compounds 1 and 12. (B) Effects of compounds 1 and

12 (100 μM, equivalent to 21.2 μg/mL and 22.6 μg/mL, respectively) on the growth rate of *C. albicans* cells. (C) Effects of compounds 1 and 12 (100 μM, equivalent to 21.2 μg/mL and 22.6 μg/mL, respectively) on hyphal formation in *C. albicans*. *C. albicans* cells were grown under noninduction conditions (30°C) or under induction conditions (37°C). The photos were taken 6 h after induction. (D) Effects of compounds 1 and 12 (100 μM, equivalent to 21.2 μg/mL and 22.6 μg/mL, respectively) on the colony morphology of *C. albicans*.

FIG. 4 Effects of different concentrations of 2-alkylaminoquinoline compounds 1 and 12 on *C. albicans* SC5314 hyphal formation (A) and virulence (B). The LDH released by A549 cells in (B) after inoculation with *C. albicans* in the absence of compounds was defined as 100% to normalize the LDH release ratios of the other treatments. Data represent the means ± standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired *t* test).

FIG. 5 Efficacy of 2-alkylaminoquinoline compound 12 (100 μM, equivalent to 22.6 μg/mL) against *C. albicans* SC5314 in the mouse oral mucosal infection model. Pathological sections were evaluated to determine the effect on *C. albicans* infection.

FIG. 6 Effect of 2-alkylaminoquinoline compound 12 on the signaling pathways involved in the hyphal development process of *C. albicans* SC5314. (A) Comparison of relative transcript levels of regulator-encoding genes between *C. albicans* cells with and without the addition of the compound. qRT-PCR results were normalized using the CT values obtained for GSP1 amplifications run in the same plate. The relative levels of the gene transcripts were determined from standard curves. Data represent the means ± standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired *t* test). (B) Schematic diagram of the signaling pathways that govern hyphal morphogenesis in *C. albicans* affected by compound 12.

FIG. 7 Analysis of the hyphal formation (A, B) and virulence (C) of different *Candida* spp. isolates (*C. albicans* ATCC 90028, *C. albicans* ATCC 10231, *C. albicans* ATCC

14053, *C. tropicalis* ATCC 750 and *C. glabrata* ATCC 2001) in the absence or presence of compound 12 (100 µM, equivalent to 22.6 µg/mL). Data represent the means ± standard deviation of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (unpaired *t* test).