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EXPERIMENTAL THERAPEUTICS

Inhibiting Fungal Echinocandin Resistance by Small-Molecule Disruption of Geranylgeranyltransferase Type I Activity

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ABSTRACT Echinocandin resistance in *Candida* is a great concern, as the echinocandin drugs are recommended as first-line therapy for patients with invasive candidiasis. However, therapeutic efforts to thwart echinocandin resistance have been hampered by a lack of fungal specific drug targets. Here, we show that deleting *CDC43*, the β subunit of geranylgeranyltransferase type I (GGTase I), confers hypersensitivity to echinocandins, which renders GGTase I a tractable target in combatting echinocandin resistance. The membrane localization of Rho1, which is critical for (1,3)-β-D-glucan synthase Fks1 activation, is disrupted in the *cdc43* mutant, resulting in decreased amounts of glucans in the cell wall, thereby exacerbating the cell wall stress upon caspofungin addition. Guided by this insight, we found that selective chemical inhibition of GGTase I by L-269289 potentiates echinocandin activity and renders echinocandin-resistant *Candida albicans* responsive to treatment *in vitro* and in animal models for disseminated infection. Furthermore, L-269289 and echinocandins also act in a synergistic manner for the treatment of *Candida tropicalis* and *Candida parapsilosis*. Importantly, deletion of *CDC43* is lethal in *Candida glabrata*. L-269289 is active on its own to kill *C. glabrata*, and its fungicidal activity is enhanced when combined with caspofungin. Thus, targeting GGTase I has therapeutic potential to address the clinical challenge of echinocandin-resistant candidiasis.

KEYWORDS *Candida*, GGTase I, antifungal resistance, antifungal therapy, drug development

Fungi infect more than 300 million people worldwide and kill nearly 1.5 million people annually (1). *Candida* species account for most of the hospital-acquired fungal infections, and the mortality rates from systemic infections remain high (2). Only three classes of antifungal drugs are available to treat systemic fungal infections (3). Among them, the echinocandins, including caspofungin, micafungin, and anidulafungin (4), are recommended as the first-line drugs for the treatment of systemic candidiasis (5). Echinocandins target the (1,3)-β-D-glucan synthase, Fks1, resulting in loss of cell wall integrity to kill the fungus. Echinocandin resistance is increasingly reported, which poses grave concern. Amino acid substitutions at Ser645 and Phe641 in the hot spot regions of Fks1 account for most of the resistance in *Candida albicans* (6). Although a fair number of stress response inhibitors have been used to enhance antifungal efficacy and reverse drug resistance (7–10), their therapeutic exploitation is hampered by a lack of fungal selectivity and with host toxicity. This highlights the urgent need for novel treatments that can tackle echinocandin-resistant candidiasis.

Geranylgeranyltransferase type I (GGTase I) is a heterodimeric zinc metalloenzyme comprising the α and β subunits encoded by *RAM2* and *CDC43*, respectively, in *Saccharomyces cerevisiae*, which transfers the geranylgeranyl pyrophosphate (GGPP) to the cysteine present in a C-terminal CaaX consensus motif, in which "a" is any aliphatic

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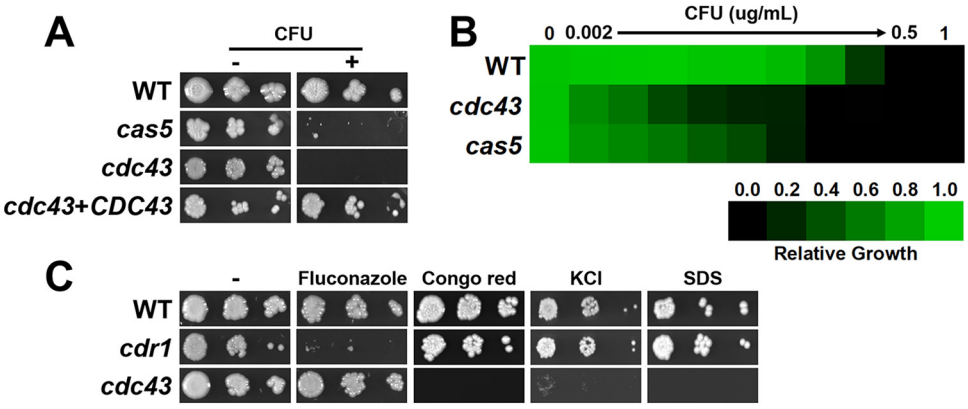

FIG 1 The *cdc43* mutant is susceptible to caspofungin and cell wall pressures. (A) Cells of wild-type (SC5314), *cdc43* (YLC12), *cdc43*+*CDC43* (YLC13), and *cas5* mutant strains were serially diluted 10-fold and spotted onto YPD solid medium with or without 500 ng/ml caspofungin. Photographs were taken after 2 days of growth at 30°C. (B) Caspofungin susceptibility assays were conducted in YPD medium. Growth was measured by absorbance at 600 nm after 48 h at 30°C. Optical densities were averaged from duplicate measurements. Data are quantitatively displayed in heat map format (see color bar). (C) Cdc43 is required for cell wall stress tolerance. Wild-type (SC5314), *cdr1*, and *cdc43* strains were treated with cell wall stress agents, including 1 μg/ml fluconazole, 200 μg/ml Congo red, 1.5 M KCl, and 0.025% SDS. Photographs were taken after 2 days of growth at 30°C.

amino acid and X is Leu (11, 12). Either *RAM2* or *CDC43* is an essential gene in *S. cerevisiae* (13). *RAM2* also encodes the α subunit of farnesyltransferase (FTase) and its β subunit is encoded by *RAM1* (13). Therefore, deletion of *RAM2* will disrupt the activity of both GGTase I and FTase. *C. albicans* GGTase I shares low amino acid identity (30%) with its human counterparts, suggesting the possibility of identifying fungus-specific GGTase I inhibitors for the treatment of fungal infections (14). However, unlike its essential role in nonpathogenic yeasts, GGTase I activity is not necessary for viability of the pathogen *C. albicans* (15). Here, we report that deletion of *CDC43*, the β subunit of GGTase I, confers hypersensitivity to echinocandins. Disruption of GGTase I blocks the membrane localization of Rho1, the regulatory subunit of Fks1, leading to decreased amounts of glucan in the cell wall, thereby exacerbating the cell wall stress induced by echinocandins. Guided by this insight, we found that a fungal selective GGTase I inhibitor (L-269289) enhances echinocandin efficacy and resensitizes echinocandin-resistant *Candida* strains to echinocandins *in vitro* and in animal models for disseminated infection. Thus, targeting GGTase I represents an efficient therapeutic strategy in fungal infectious disease.

RESULTS

Disruption of GGTase I confers hypersensitivity to caspofungin. GGTase I plays a critical role in controlling cell morphology in *S. cerevisiae* (16). A putative protein substrate of GGTase I is Rho1, which has been identified as the regulatory subunit of (1,3)-β-D-glucan synthase (17). Given that echinocandins target Fks1 to disrupt the synthesis of (1,3)-β-D-glucan, resulting in loss of cell wall integrity and imparting severe cell wall stress, we predicted that inhibiting GGTase I in *C. albicans* would potentiate echinocandin antifungal efficacy. To test this hypothesis, we constructed a *cdc43* null mutant in *C. albicans* by sequential gene disruption. Cdc43 is the β subunit of GGTase I, and mutation of this subunit is expected to cripple protein geranylgeranylation, whereas farnesyltransferase activity remains intact. Compared to that of wild-type cells, the *cdc43* mutant displayed no obvious growth defect on yeast extract-peptone-dextrose (YPD) agar plates at 30°C (Fig. 1A) as well as in liquid YPD medium at both 30°C and 37°C (see Fig. S1 in the supplemental material), in agreement with the notion that GGTase I activity is not essential for viability in *C. albicans* (15). However, we found that the *cdc43* mutant is hypersensitive to caspofungin, and the growth defect of the *cdc43* mutant in caspofungin was rescued by integration a wild-type copy of *CDC43* (Fig. 1A). We further showed that deletion of *CDC43* resulted in an ~64-fold decrease

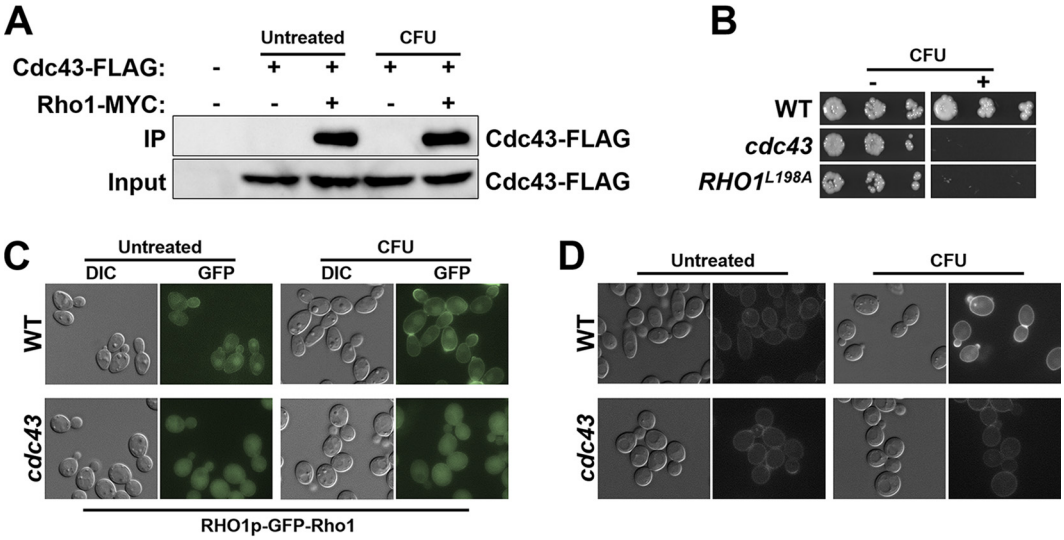

FIG 2 Deletion of *CDC43* mislocalizes Rho1 and reduces glucan synthesis in response to caspofungin. (A) Rho1 interacts with Cdc43 *in vivo*. Protein lysates from strains YLC17 (Cdc43-FLAG, Rho1-Myc) and YLC14 (Cdc43-FLAG) were subjected to immunoprecipitation with anti-Myc antibody, and the precipitated proteins were probed with anti-FLAG antibody. As an input control, cell lysates were analyzed by Western blotting with the anti-FLAG antibody. (B) Mutations in L198 in the CaaL motif of Rho1 confer hypersensitivity to caspofungin. Cells of wild-type, *RHO1*^{L198A} (YLC18), or *cdc43* strains were serially diluted 10-fold and spotted onto YPD solid medium at 30°C with or without 500 ng/ml caspofungin. (C) Wild-type strain expressing GFP-Rho1 under its own promoter (YLC19) was grown to log phase in liquid YPD medium at 30°C and then treated with or without 25 ng/ml caspofungin for 15 min. Cell morphology and Rho1 localization were observed by using DIC and fluorescence microscopy. (D) Wild-type (SC5314) and *cdc43* mutant strains were grown in YPD medium for 15 min with or without 25 ng/ml caspofungin. Cells were stained with 1% aniline blue for 5 min and imaged by DIC and fluorescence microscopy.

in the MIC for caspofungin in YPD broth (Fig. 1B). The hypersensitivity of the *cdc43* mutant to all three echinocandins (caspofungin, micafungin, and anidulafungin) was confirmed by using the CLSI M27-A3 method (see Fig. S2). Our results indicated that disrupting GGTase I activity in *C. albicans* confers hypersensitivity to echinocandins. Cdc43 levels were similar in the presence or absence of caspofungin regardless of temperature (see Fig. S3), suggesting that caspofungin tolerance in *C. albicans* is not mediated by changing Cdc43 levels. Next, we compared the impact of GGTase I with that of Cas5, an important transcription factor that regulates cell wall stability during periods of cell wall stress (18, 19), on caspofungin sensitivity. As shown in Fig. 1A and B, deletion of *CDC43* had a more profound impact on caspofungin tolerance than deletion of *cas5*. In addition to caspofungin, the *cdc43* mutant also displayed increased sensitivity to the cell wall stress agents, including Congo red, KCl, and SDS (Fig. 1C). However, unlike the *cdr1* mutant which was susceptible to the azole antifungal fluconazole (Fig. 1C) (20), deletion of *CDC43* had little effect on its sensitivity to fluconazole, suggesting that the GGTase I activity is specifically required for echinocandin tolerance. These results indicate that disruption of GGTase I activity enhances caspofungin antifungal efficacy.

Geranylgeranylation of Rho1 by GGTase I is critical for echinocandin tolerance. Rho1 is a small GTPase that plays dual roles in the activation of cell wall stress responses and in β-glucan biosynthesis (21). Since *C. albicans* Rho1 contains a CaaL motif that is recognized by GGTase I, we proposed that GGTase I regulates echinocandin tolerance through modulation of Rho1 activity. We first determined if Cdc43 interacts with Rho1. Cdc43 was fused at its C terminus with a 3FLAG tag. Using strains carrying the Rho1-13Myc, we found that immunoprecipitation of Rho1 with a Myc antibody was able to pull down Cdc43-3FLAG in a caspofungin-independent manner (Fig. 2A). We then tested whether geranylgeranylation of Rho1 is required for GGTase I-mediated echinocandin tolerance. Leu198 that is located in the CaaL motif of Rho1 was mutated to Ala using CRISPR-Cas9 (22). *RHO1*^{L198A} could not be geranylgeranylated by GGTase I, because Leu198 is the determinant responsible for the recognition of Rho1 by GGTase

I. As shown in Fig. 2B, *RHO1*^{L198A} was as highly sensitive to caspofungin as the *cdc43* mutant, suggesting that the major function of GGTase I in echinocandin tolerance is the geranylgeranylation of Rho1.

Rho1 is required for activation of Fks1, which depends on its interaction with Fks1 at the cell membrane (23). It is known that protein prenylation enhances membrane affinity (24), implying that GGTase I regulates cell wall integrity by modulating Rho1 localization. We therefore determined Rho1 localization in wild-type and *cdc43* mutant cells. Green fluorescent protein (GFP)-Rho1 was expressed from its own promoter. We found that a significant portion of Rho1 accumulated in cell membranes of wild-type cells (Fig. 2C). Furthermore, the membrane accumulation of Rho1 was more obvious after the addition of caspofungin (Fig. 2C). In contrast, Rho1 was diffuse in the cytosol in the *cdc43* mutant regardless of caspofungin (Fig. 2C), suggesting that geranylgeranylation of Rho1 by GGTase I is required for its membrane localization. Given that mislocalization of Rho1 would impair Fks1 activity, we monitored glucan levels with aniline blue in wild-type and *cdc43* mutant cells. In wild-type cells, an increase in fluorescence intensity upon staining with aniline blue was detected after short exposure to caspofungin (Fig. 2D), whereas deletion of *CDC43* did not alter aniline blue staining by addition of caspofungin (Fig. 2D), indicating that disruption of GGTase I activity abolished the caspofungin-responsive increase in glucan levels. In addition to regulating Fks1 activity, Rho1 is required for activation of cell wall stress responses, including the protein kinase C (PKC) signaling cascade (21), which is also critical for caspofungin tolerance. However, the caspofungin-responsive induction of Pkc1 pathway genes was independent of GGTase I activity, as the expression of genes involved in this pathway increased significantly in the *cdc43* mutant by addition of caspofungin (see Fig. S4). Therefore, we suggest that the major function of GGTase I regarding caspofungin tolerance is modulating Rho1-dependent Fks1 activity. Altogether, these results indicate that disrupting GGTase I activity impairs Rho1 membrane localization, resulting in the reduction of glucans levels, which in turn exacerbates caspofungin-induced cell wall stresses.

GGTase I inhibitor L-269289 enhances echinocandin efficacy. The hypersensitivity to caspofungin of the *cdc43* mutant suggested that GGTase I in *C. albicans* might represent a therapeutic target to enhance echinocandin efficacy. A previous study identified a set of structurally diverse compounds which exhibited selective inhibition for *C. albicans* GGTase I versus human GGTase I (14). Among them, only L-269289 exhibited sufficient cell permeability (14). The structure of L-269289 is shown in Fig. 3A. Using a checkerboard assay, we corroborated the synergy between caspofungin and L-269289 against *C. albicans*. L-269289 had marginal antifungal activity alone but enhanced caspofungin efficacy against *C. albicans*, as the MIC for caspofungin decreased dramatically when L-269289 was added (Fig. 3B). We next assessed the impact of L-269289 on cell wall physiology in response to caspofungin by monitoring levels of glucan with aniline blue. Similar to that in the *cdc43* mutant, we did not observe an increase in glucan levels in response to caspofungin when L-269289 was added (Fig. 3C). Therefore, we suggest that L-269289 potentiates echinocandin efficacy by inhibiting GGTase I activity, leading to the decrease in glucan levels in the cell wall. To provide further genetic support for GGTase I as the target responsible for L-269289 action, we measured the growth of wild-type and *cdc43* mutant cells in caspofungin-containing medium in the presence or absence of L-269289. Addition of L-269289 decreased the growth of wild-type cells in caspofungin-containing medium, although the reduction in growth was not as much as the reduction seen when *CDC43* was deleted (Fig. 3D). Furthermore, L-269289-treated *cdc43* mutant cells grew the same as *cdc43* mutant cells treated with vehicle (Fig. 3D), suggesting that GGTase I as the major target responsible for the enhanced caspofungin efficacy induced by L-269289.

L-269289 counteracts echinocandin resistance. Owing to its ability to potentiate caspofungin activity, we predicted that L-269289 would restore echinocandin sensitivity to resistant strains. First, we confirmed the resistance phenotype of the *FKS1*^{F641S} or

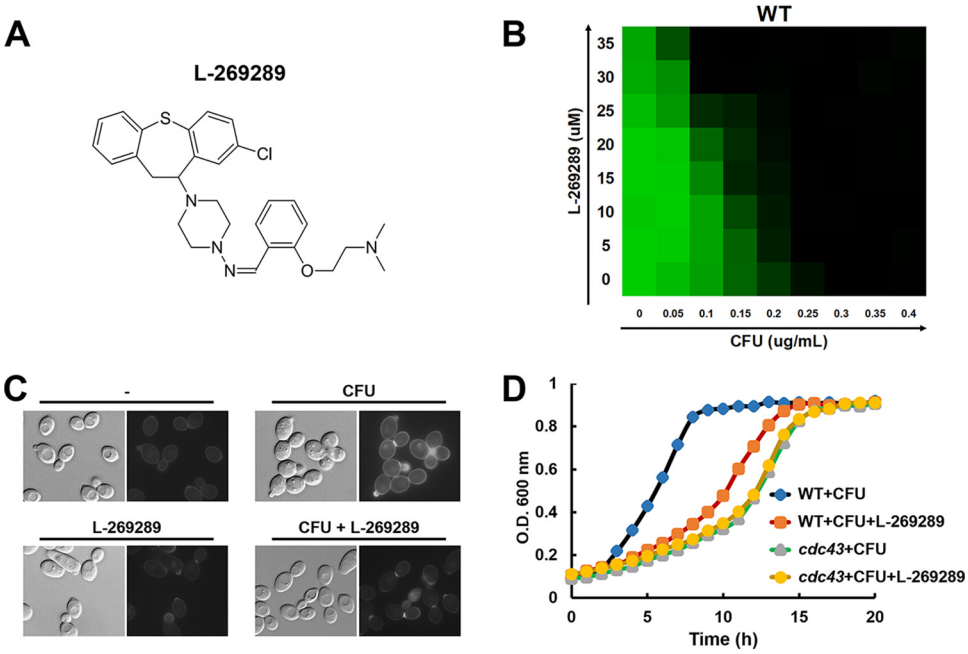

FIG 3 GGTase I inhibitor L-269289 potentiates caspofungin antifungal efficacy. (A) Structure of L-269289. (B) Wild-type (SC5314) cells were cultured in YPD containing different concentrations of caspofungin and L-269289. Growth was measured by absorbance at 600 nm after 48 h at 30°C. Data represent the means from two independent experiments. (C) L-269289 abolishes the caspofungin-responsive increase in glucan levels. Wild-type (SC5314) cells were grown in YPD with or without 25 ng/ml caspofungin and/or 50 μM L-269289 for 15 min. Cells were stained and imaged as described for Fig. 2D. (D) L-269289 treatment phenocopies the deletion of *CDC43*. Change in optical density over time as a measure of growth was monitored in liquid cultures of wild-type (SC5314) and *cdc43* mutant strains. Assays were performed in YPD with 20 ng/ml caspofungin and L-269289 (100 μM) added as indicated. Data are derived from one representative experiment. Three independent experiments yielding similar results were performed.

FKS1^{S645F} strain to caspofungin. The wild-type strain was sensitive to caspofungin, whereas the *FKS1*^{F641S} or *FKS1*^{S645F} strain grew robustly in the presence of caspofungin (Fig. 4A). We next deleted *CDC43* in both *FKS1*^{F641S} and *FKS1*^{S645F} strains and found that disruption of GGTase I resensitized *FKS1*^{F641S} and *FKS1*^{S645F} strains to caspofungin (Fig. 4A). Finally, echinocandin-resistant strains *FKS1*^{F641S} and *FKS1*^{S645F} were tested for their sensitivity to caspofungin by checkerboard assay with increasing concentrations of L-269289. As expected, L-269289 restored caspofungin sensitivity to *FKS1*^{F641S} and *FKS1*^{S645F} resistant strains in a concentration-dependent manner (Fig. 4B). In addition to caspofungin, micafungin and anidulafungin also displayed synergy with L-269289 against either wild-type or echinocandin-resistant *C. albicans* (see Fig. S5). Using the CLSI M27-A3 method, we detected a dramatic decrease in MIC for all three echinocandins in both *FKS1*^{F641S} and *FKS1*^{S645F} resistant strains when L-269289 was added (see Fig. S6), indicating that the GGTase I inhibitor reversed echinocandin resistance. Biofilms also offer a refuge from antifungal drugs, which facilitate the development of persister cells able to tolerate high concentrations of drug (25). Because the extracellular biofilm matrix is composed predominantly of glucan, we suppose that inhibiting GGTase I would impair biofilm formation. As expected, the defect in biofilm formation was exhibited by either deletion of *CDC43* or addition of L-269289 (see Fig. S7A and B), indicating that, like echinocandins (26), L-269289 could also disrupt biofilm to thwart drug resistance. Our data demonstrate that combination of L-269289 and echinocandins is a new and efficient strategy to counteract echinocandin resistance.

L-269289 potentiates echinocandin therapeutic efficacy. The potent synergy of L-269289 with echinocandins suggests that it may have potential for use as a therapeutic in combination therapy to combat drug resistance. To test this, we used a well-established murine model of *C. albicans* disseminated infection. Prior to perform-

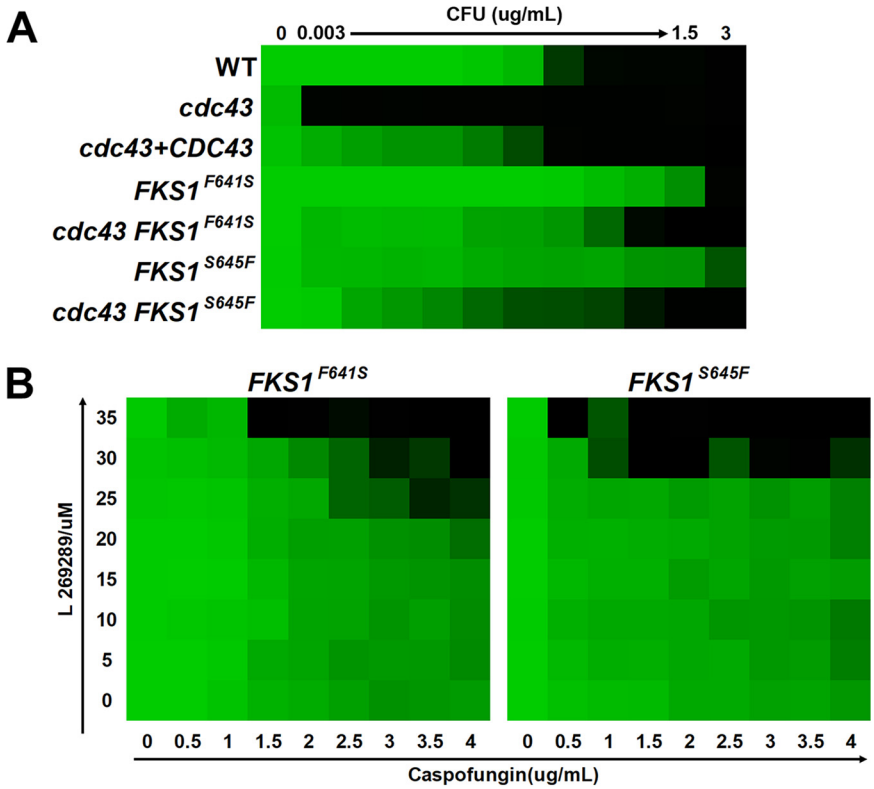

FIG 4 L-269289 resensitizes echinocandin-resistant strains to caspofungin. (A) Deletion of *CDC43* increased the susceptibility of echinocandin-resistant *C. albicans* to caspofungin. Assays were performed as described for Fig. 1B. (B) L-269289 renders echinocandin-resistant *C. albicans* responsive to treatment. Assays were performed on indicated echinocandin-resistant strains as described for Fig. 3B.

ing mammalian studies, we sought to evaluate the potential toxicity of L-269289 in mammalian cells. Human 293 cells treated with L-269289 revealed toxicity only at high concentrations of L-269289 (50% inhibitory concentration [IC_{50}] $>$ 50 μM) (Fig. 5A). Mice were inoculated with a *C. albicans FKS1*^{F641S} resistant strain by tail vein injection and were dosed peritoneally with caspofungin (0.05 mg kg^{-1}), L-269289 (0.5 mg kg^{-1}), a combination of the two, or vehicle; survival was monitored every 24 h. In the absence of treatment, most of the mice succumbed to the infection within 5 days, demanding rapid therapeutic effects. The single agent L-269289 did not significantly increase survival compared to that with vehicle, while caspofungin had a minor benefit for survival (Fig. 5B). Cotreatment with L-269289 and caspofungin significantly increased survival, as 80% of the mice were still alive after the 20-day trial (Fig. 5B). Our results indicate that L-269289 has profound therapeutic benefits in combination with echinocandins to tackle echinocandin-resistance candidiasis in mammalian infection models.

L-269289 enhances echinocandin efficacy against diverse *Candida* species. Given the significant sequence similarity shared by GGTase I in diverse fungi, we supposed that inhibiting GGTase I would also enhance echinocandin efficacy against non-*albicans Candida* species. To test this, *CDC43* was first deleted in diploid yeasts *C. tropicalis* and *C. parapsilosis*. As previously reported, *C. parapsilosis* displayed high MIC values for caspofungin relative to other *Candida* species (Fig. 6A), because a polymorphism at Pro649 occurs in this fungus (27). Similar to that in *C. albicans*, the *cdc43* null mutant displayed a dramatic increase in sensitivity to caspofungin compared to that of wild-type cells, as MIC values for caspofungin were reduced ~32-fold when *CDC43* was deleted in either *C. tropicalis* or *C. parapsilosis* (Fig. 6A). Correspondingly, L-269289 enhanced caspofungin efficacy against *C. tropicalis* and *C. parapsilosis* (Fig. 6B). We also tried to delete *CDC43* in *C. glabrata* but failed to get the deletion mutant, suggesting that GGTase I activity might be essential for *C. glabrata* viability. To confirm this notion,

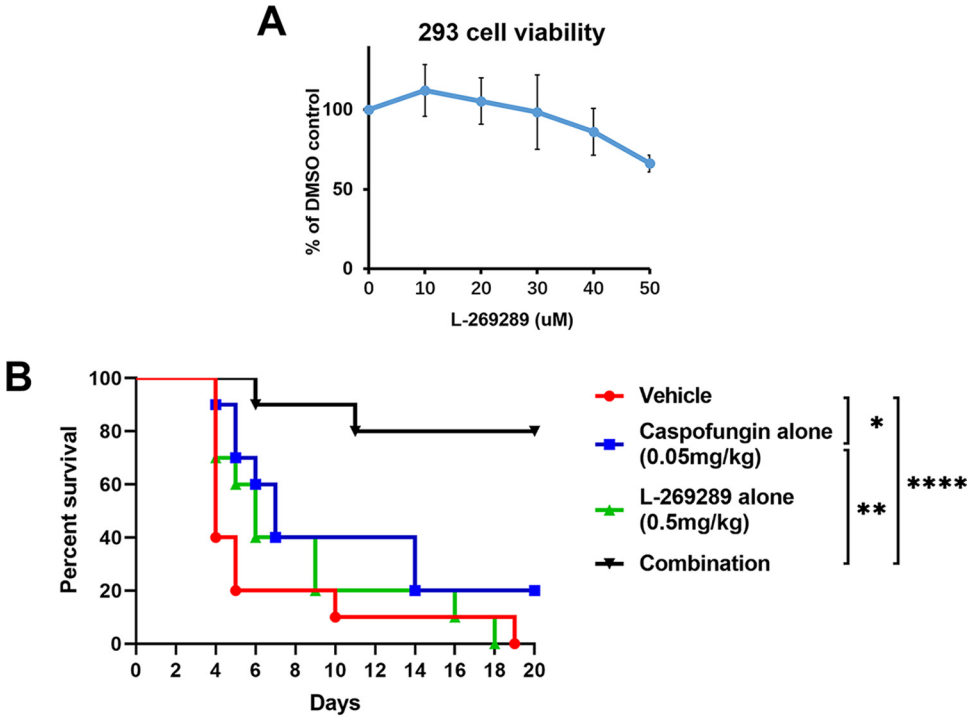

FIG 5 L-269289 as a cotherapeutic in models of disseminated echinocandin-resistant candidiasis. (A) L-269289 inhibits viability of 293 cells at high concentrations ($IC_{50} > 50\ \mu M$). The means from 3 replicates are shown; error bars represent means ± standard deviations. (B) Female BALB/c mice were infected with 7.5×10^5 cells of the echinocandin-resistant *C. albicans* (*FKS1*^{F641S}) via tail vein injections. Caspofungin, L-269289, or a combination was administered intraperitoneally, as indicated, starting 4 h after infection and then daily for a total of five doses. ****, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

we replaced the endogenous promoter of *CDC43* by a *MET3* promoter. As shown in Fig. 6C, the isogenic *C. glabrata* strain grew normally, similarly to wild-type cells, in the absence of methionine and cysteine when *CDC43* was expressed. In contrast, when *CDC43* was repressed in methionine-containing medium, the isogenic strain displayed a severe growth defect, as only a few colonies grew on the plate. This indicates that, like in *S. cerevisiae*, deletion of *CDC43* is lethal in *C. glabrata*. Consistent with this result, we found that L-269289 alone was able to kill *C. glabrata* (Fig. 6D). While active on its own, L-269289 was more effective when combined with caspofungin against *C. glabrata* (Fig. 6E). Therefore, GGTase I represents a new drug target for treating *C. glabrata* infection. Overall, our discovery of L-269289 plus an echinocandin provides an efficient broad-spectrum drug combination for treating *Candida* infections.

DISCUSSION

In this study, we established a powerful and broadly effective therapeutic strategy by inhibiting GGTase I to tackle echinocandin-resistant candidiasis. The fungal-selective GGTase I inhibitor L-269289 potentiates echinocandin efficacy and renders echinocandin-resistant pathogens responsive to treatment in mouse infection models. These effects are mediated via impairing Rho1 membrane localization where it interacts with Fks1 and promotes its activity, leading to the decreased production of the major cell wall biopolymer (1,3)-β-D-glucan, thereby aggravating cell wall stress imparted by echinocandins. Our findings have demonstrated the feasibility of targeting GGTase I with a small molecule as a novel therapeutic strategy in fungal infectious disease.

In the fungus *S. cerevisiae*, GGTase I was shown to be essential for growth, but it is not the case for pathogenic yeasts, including *C. albicans*, *C. tropicalis*, and *C. parapsilosis*. Here, we have shown that selective chemical inhibition of GGTase I potentiates

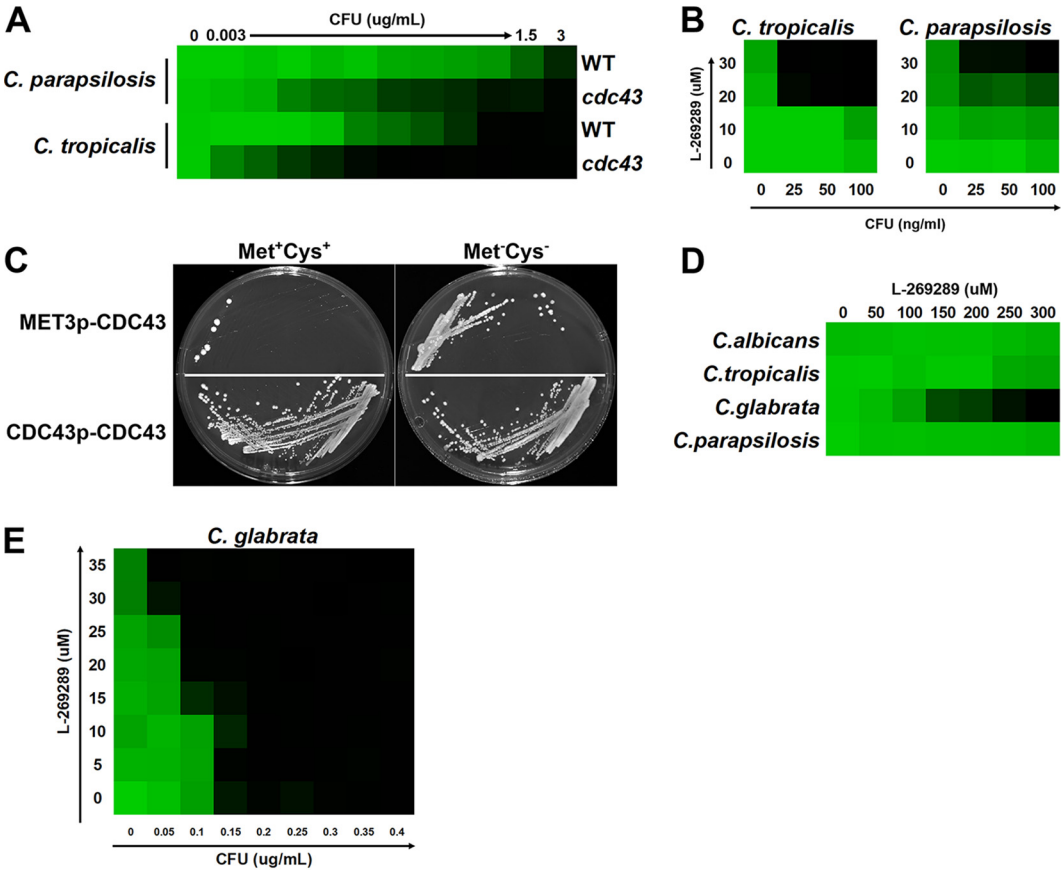

FIG 6 L-269289 potentiates caspofungin efficacy against non-*albicans Candida* species. (A) Deletion of *CDC43* results in increased sensitivity of *C. tropicalis* and *C. parapsilosis* to caspofungin. Assays were performed on indicated strains as described for Fig. 1B. (B) L-269289 enhances caspofungin efficacy against *C. tropicalis* and *C. parapsilosis*. Assays were performed as described for Fig. 3B. (C) Deletion of *CDC43* in *C. glabrata* is lethal. Wild-type and isogenic *C. glabrata* (*CDC43* controlled by a *MET3* promoter) strains were grown on sythetic complete dextrose (SCD) agar plates with or without methionine and cysteine for 2 days. (D) L-269289 is fungicidal to *C. glabrata*. L-269289 susceptibility assays for *C. glabrata* were conducted in YPD medium. Growth was measured by absorbance at 600 nm after 48 h at 30°C. Optical densities were measured and displayed as described for Fig. 1B. (E) *C. glabrata* was cultured in YPD containing different concentrations of caspofungin and L-269289 as described for Fig. 3B. Growth was measured by absorbance at 600 nm after 48 h at 30°C.

echinocandin activity that reverses echinocandin resistance. Prenyltransferase inhibitors have been extensively used to treat cancer, parasitic infections, and other maladies (28). In this study, we identified L-269289 as the first prenyltransferase inhibitor for the treatment of fungal infectious disease. Its ability to potentiate the antifungal activity of echinocandins makes L-269289 plus an echinocandin an ideal broad-spectrum drug combination to attack the fungal cell wall in pathogenic yeasts. A recent report showed that the expression of *CDC43* was required for *C. albicans* pathogenesis (29), suggesting that, in addition to enhancing echinocandin activity, L-269289 alone can reduce *C. albicans* virulence by inhibiting GGTase I. This may explain why a fairly high concentration of L-269289 (>35 μM) was required to significantly reduce the MIC of caspofungin *in vitro* (Fig. 4B), yet a lower dosage (0.5 mg kg^{-1}) of the drug was able to reverse echinocandin resistance in murine model of disseminated infection (Fig. 5B). However, 0.5 mg kg^{-1} L-269289 alone did not significantly increase survival in mice (Fig. 5B), suggesting that a higher concentration of this drug would be required to reduce virulence of *C. albicans* during invasive infection.

Unlike other *Candida* species, deletion of *CDC43* in *C. glabrata* is lethal, which might be attributed to its close phylogenetic relationship with *S. cerevisiae*. L-269289 has a marginal effect on the growth rates of *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, but it is fungicidal to *C. glabrata*. Given that many *C. glabrata* clinical isolates are resistant

to azoles (30), echinocandins are recommended to treat *C. glabrata* infections. However, the emergence of multidrug-resistant *C. glabrata* strains (31) shows the need for novel treatments that can target these resistant populations. Our study shows that small-molecule disruption of GGTase I may be a new therapeutic strategy for addressing the rapidly rising burden of drug-resistant *C. glabrata* infection.

L-269289 inhibits *C. albicans* GGTase I with an IC_{50} of 0.10 μM, displays a >1,000-fold selectivity for *C. albicans* GGTase I over human GGTase I (14), and is toxic to human cell lines only at high concentrations (Fig. 5A). Moreover, L-269289 had little effect on FTase in *C. albicans*, indicating its specificity for the fungal GGTase I. Therefore, inhibition of GGTase I by L-269289 provides an efficient therapeutic strategy with minimal side effects for treating fungal infectious disease. We identified the GTPase Rho1 as a key downstream substrate of GGTase I involved in echinocandin tolerance and resistance, as mutating Leu198 to Ala in Rho1, which blocks its geranylgeranylation by GGTase I, confers hypersensitivity to echinocandins, such as in the *cdc43* mutant (Fig. 2B). Defects in cell wall integrity induced by the echinocandins activate Rho1 that positively regulates multiple effectors, including Pkc1, as well as the echinocandin target, Fks1. Deletion of *CDC43* decreased the amount of glucan in the cell wall (Fig. 2D) but did not affect gene induction in the PKC pathway when exposed to caspofungin (see Fig. S4 in the supplemental material). These data suggest that inhibiting GGTase I by L-269289 mislocalizes Rho1 by preventing it from geranylgeranylation modification, resulting in disruption of (1,3)-β-D-glucan synthase activity, which synergizes with echinocandins against *Candida*. Although the use of prenyltransferase inhibitors has been investigated in a number of diseases, little success was achieved. Our study links the molecular mechanism of L-269289 with its efficacy against fungi and provides an example of how to overcome the major challenge for utilizing prenyltransferase inhibitors in a clinical setting.

There is an urgent need for developing new strategies to enhance the efficacy of existing antifungals to tackle drug-resistant candidiasis. ML316 that inhibits mitochondrial phosphate transport and the natural product beauvericin that inhibits multidrug efflux and TOR signaling have been shown to potentiate azole antifungal activity (10, 32). Romo et al. characterized a small-molecule compound capable of inhibiting *C. albicans* filamentation against candidiasis (33). In this study, we demonstrate that combining L-269289 and echinocandins produced an increased killing effect, which may reduce the pathogen population size and thus the probability of acquiring resistance mutations. Additionally, the echinocandins noncompetitively inhibit the (1,3)-β-D-glucan synthase enzyme encoded by *FKS1*, while L-269289 is competitive for the prenyl acceptor substrate (14) to selectively inhibit GGTase I to potentiate echinocandin activity. Combination of mechanistically distinct antifungals allows for lower individual drug dosage and less probability of evolving resistance to either agent alone. The small molecule iKIX1 was recently found to abrogate azole resistance in *C. glabrata* by disrupting the protein-protein interaction (34). Polvi et al. identified the broad-spectrum chelator diethylenetriaminepentaacetic acid (DTPA), which potentiated echinocandin efficacy against echinocandin-resistant *C. albicans* (35). Given that GGTase I requires Zn^{2+} ions for catalysis (36), we speculate that the enhanced activity of caspofungin when combined with DTPA may result at least partially from chelation of Zn^{2+}, resulting in the inactivation of GGTase I. Our findings illustrate how molecular knowledge of protein posttranslational prenylation modifications can render the process a tractable target in combatting drug resistance.

MATERIALS AND METHODS

Media and growth conditions. *C. albicans* strains were routinely grown at 30°C in YPD (2% Bacto peptone, 2% dextrose, 1% yeast extract). Transformants were selected on synthetic medium (2% dextrose, 0.17% Difco yeast nitrogen base without ammonium sulfate, 0.5% ammonium sulfate, and auxotrophic supplements). RPMI medium (10.4 g/liter RPMI 1640, 3.5% MOPS [morpholinepropanesulfonic acid], and 2% glucose, pH 7) was prepared for some antifungal susceptibility testing. Caspofungin (Selleck), micafungin (Selleck), anidulafungin (Selleck), and fluconazole (Sequoia Research) were added to media as indicated in Results and figure legends.

Plasmid and strain construction. All strains constructed and used in this study are listed in Table S1 in the supplemental material, and all primers used in this study are listed in Table S2. *CDC43* was deleted in *C. albicans* based on the method as described previously (37). The disruption was confirmed by PCR. A PCR product (primers 1 and 2) containing the *CDC43* promoter and coding region was inserted into the BamHI-MluI site of pPR673 (38). The resulting plasmid was digested with KpnI for integration into its own locus to express CDC43-13Myc or digested with StuI for integration into RP10 locus in the *cdc43* mutant to generate its complement strain. A 1.2-kb PCR product (primers 3 and 4) containing the full-length *CDC43* coding region was inserted into the ClaI-KpnI site of pBES116-3FLAG (39). The resulting plasmid was digested with AscI to target integration into the *ADE2* locus to express CDC43-3FLAG. A 0.6-kb PCR product (primers 5 and 6) containing the full-length *RHO1* coding region was inserted into the BamHI-MluI site of pPR671 (40). The resulting plasmid was digested with StuI to target integration into the *RP10* locus to express RHO1-13Myc. Both copies of *RHO1* were replaced by $Rho1^{L198A}$ using CRISPR-Cas9 (22) to construct $Rho1^{L198A}$ mutant strains as follows. The single guide RNA (sgRNA) (primers 7 and 8) was annealed to insert into the pV1093 vector. The resulting plasmid was linearized by digestion with KpnI and SacI and was transformed into the wild type with the repair template (primers 9 and 10). The mutants were verified by sequencing. pHL471-RHO1p-GFP was constructed by inserting the *RHO1* promoter (primers 13 and 14) into the XhoI and HindIII site of plasmid pHL471 (41). The GFP coding sequence was PCR amplified using primers 11 and 12 and then cloned into HindIII and PstI sites to generate pHL471-RHO1p-GFP. Then, the *RHO1* coding sequence (primers 15 and 16) was cloned into the PstI and BamHI sites to generate RHO1p-Rho1-GFP. This plasmid was digested with BglII to target integration into the endogenous locus of *RHO1* to express GFP-Rho1. Both copies of *FKS1* were replaced by $FKS1^{F641S}$ or $FKS1^{S645F}$ using CRISPR-Cas9 to construct *C. albicans* $FKS1^{F641S}$ or $FKS1^{S645F}$ mutant strains. We obtained *CDC43* loss-of-function mutations of *C. tropicalis* and *C. parapsilosis* using the CRISPR system by inserting a stop codon right after the transcription start site.

The 1-kb *MET3* promoter (primers 17 and 18) was inserted into the NotI-SacII sites of pSFS2 (42). A 1-kb PCR product (primers 19 and 20) containing the upstream *C. glabrata CDC43* open reading frame (ORF) and the *CDC43* coding sequence (primers 21 and 22) were inserted into the ApaI-XhoI and SacII-SacI sites, respectively. The resulting plasmid was digested with ApaI and SacI to replace the *C. glabrata CDC43* promoter with a *MET3* promoter by homologous recombination.

Cell wall stress assay. Freshly grown cells were serially diluted 10-fold, spotted onto YPD plates with or without 1 μg/ml fluconazole, 200 μg/ml Congo red, 1.5 M KCl, or 0.025% SDS, and incubated for 2 days at 30°C.

Coimmunoprecipitation. Protein extraction was performed as described previously (40). To determine whether Cdc43 interacts with Rho1 *in vivo*, crude extracts of *C. albicans* cells were precleared with protein A agarose (GE) and then incubated with 2.0 μg of anti-c-myc antibody (Sigma). After incubation for 2 h at 4°C, ~40 μl of protein A agarose suspension was added to precipitate the immunocomplex. The proteins were subjected to Western blotting with anti-FLAG antibody (Sigma).

Quantitative reverse transcription-PCR. Total RNA was purified from *C. albicans* cells using the RNeasy Minikit and DNase-treated at room temperature for 15 min using the RNase-free DNase set (Qiagen). cDNA was synthesized using the SuperScript II reverse transcriptase kit (Invitrogen), and quantitative PCR (qPCR) was performed using the iQ SYBR green Supermix (Bio-Rad). Primers 23 and 24 were used to amplify *C. albicans ACT1*. Primers 25 and 26 were used to amplify *C. albicans PGA13*. Primers 27 and 28 were used to amplify *C. albicans ALS1*. Primers 29 and 30 were used to amplify *C. albicans BCK1*. Primers 31 and 32 were used to amplify *C. albicans MKC1*.

Cell wall staining and fluorescence microscopy. An overnight culture was diluted 1:50-fold into YPD at 30°C and grown to log phase. Cells were then treated with or without 25 ng/ml caspofungin for 15 min. For glucan visibility, cells were stained with aniline blue (0.05% [wt/vol]; Sigma). GFP and stained glucan were visualized by fluorescence microscopy and differential inference contrast (DIC) optics. The fluorescence signal in the cells was observed under a Leica DM2500 microscope. Images for a single stain were taken at the same exposure.

Antifungal susceptibility testing. Susceptibility to single antifungal drugs or drug combinations was assayed in 96-well microtiter plates (Thermo) as previously described (9, 43). Assays were performed in a total volume of 0.1 ml/well with various concentrations of each drug in YPD medium. Plates were incubated in the dark at 30°C for 48 h before the optical density at 600 nm (OD_{600}) was determined using a spectrophotometer (BioTek Instruments). For the CLSI M27-A3 method (44), a total volume of 0.1 ml/well with 2-fold dilutions of each drug in RPMI medium was used. Plates were incubated in the dark at 35°C for 48 h. OD_{600} values were determined using a spectrophotometer (BioTek Instruments). Data were displayed as heat maps. L-269289 (TopScience) was dissolved in dimethyl sulfoxide (DMSO). Caspofungin (Selleck) and micafungin (Selleck) were dissolved in double-distilled water (ddH_2O). Anidulafungin (Selleck) was dissolved in ethanol.

293 cell toxicity testing. The human cell line 293 was supplied from the China Center for Type Culture collection (CCTCC) and confirmed negative for mycoplasma contamination by PCR-based testing. 293 cells were seeded at 5.0×10^4 cells per well in 96-well plates in Eagle minimal essential medium (MEM) supplemented with 10% fetal calf serum (EveryGreen). Cells were treated with DMSO (MP Biomedicals) and L-269289 (10 μM, 20 μM, 30 μM, 40 μM, and 50 μM) after culturing for 72 h. The surviving cells were examined with a cell counting kit-8 (APExBIO) after 1 h of incubation, and the survival percentage of cells without L-269289 treatment was set as 100% as a control.

Murine model of systemic infection. Female BALB/c mice (Charles River) were inoculated with 7.5×10^5 cells of *C. albicans* strain $FKS1^{F641S}$, resuspended in 200 μl of sterile phosphate-buffered saline (PBS), by tail vein injection. There were four treatment groups consisting of ten mice each. Test groups

were treated with vehicle, L-269289 alone at 0.5 mg/kg/dose, caspofungin alone at 0.05 mg/kg/dose, and a combination of L-269289 and caspofungin at these doses. Drug doses were administered by intraperitoneal injection starting 4 h postinfection and then every 24 h for a total of five doses. Mice were monitored daily for weight loss and overall health condition and were euthanized upon reaching humane endpoints. The survival curve was calculated using GraphPad Prism. All animal experiments were approved by the IACUC at Wuhan University and as outlined in the guide for the care and use of laboratory animals issued by the Ministry of Science and Technology of the People's Republic of China.

***In vitro* biofilm growth.** The *in vitro* biofilm growth assays were carried out using a previously established protocol with minor modifications (43). In brief, overnight cultures of *C. albicans* strains were grown in YPD at 30°C. Cells were then washed twice with PBS and were diluted to an OD_{600} of 0.5 in 2 ml of Spider medium. The 12-well polystyrene plates were previously treated with fetal bovine serum overnight and washed with 2 ml of PBS. After inoculating with each of the *C. albicans* strains, the plates were incubated at 37°C for 90 min at 120 rpm to allow initial adhesion of cells. Each well was washed once with 2 ml of PBS to remove any nonadhering cells, 2 ml of fresh Spider medium was added to each well, and biofilms were grown for another 48 h as described above. After removing the medium, each well was washed with 2 ml of PBS, dried, and photographed.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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