Synergistic Effect of Calcineurin Inhibitors and Fluconazole against *Candida albicans* Biofilms[▽]

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Calcineurin is a Ca^{2+} -calmodulin-activated serine/threonine-specific protein phosphatase that governs multiple aspects of fungal physiology, including cation homeostasis, morphogenesis, antifungal drug susceptibility, and virulence. Growth of *Candida albicans* planktonic cells is sensitive to the calcineurin inhibitors FK506 and cyclosporine A (CsA) in combination with the azole antifungal fluconazole. This drug synergism is attributable to two effects: first, calcineurin inhibitors render fluconazole fungicidal rather than simply fungistatic, and second, membrane perturbation by azole inhibition of ergosterol biosynthesis increases intracellular calcineurin inhibitor concentrations. *C. albicans* cells in biofilms are up to 1,000-fold more resistant to fluconazole than planktonic cells. In both in vitro experiments and in an in vivo rat catheter model, *C. albicans* cells in biofilms were resistant to individually delivered fluconazole or calcineurin inhibitors but exquisitely sensitive to the combination of FK506-fluconazole or CsA-fluconazole. *C. albicans* strains lacking FKBP12 or expressing a dominant FK506-resistant calcineurin mutant subunit (Cnb1-1) formed biofilms that were resistant to FK506-fluconazole but susceptible to CsA-fluconazole, demonstrating that drug synergism is mediated via direct calcineurin inhibition. These findings reveal that calcineurin contributes to fluconazole resistance of biofilms and provide evidence that synergistic drug combinations may prove efficacious as novel therapeutic interventions to treat or prevent biofilms.

The dimorphic fungus *Candida albicans* is a commensal of the human oral, gastrointestinal, vaginal, cutaneous, and mucosal surfaces. In immunocompetent as well as immunocompromised individuals, *C. albicans* causes cutaneous or subcutaneous infections such as vaginitis or oral thrush or infections of the nails and skin. In patients receiving broad-spectrum antibiotics and undergoing cancer chemotherapy, *C. albicans* can enter the bloodstream to cause serious systemic invasive disease (6, 38). Due to the difficulty in identifying antifungal targets unique to fungi that are not shared with the human host, only a restricted number of antifungal agents have been widely used for treating *C. albicans* systemic infections (12, 32). One such target unique to fungi is the sterol cell membrane component ergosterol.

Fluconazole is a member of the azole class of drugs that target an essential enzyme (Erg11; lanosterol 14 α -demethylase) in the ergosterol biosynthetic pathway (36, 37). Fluconazole is the most commonly used antifungal agent for prevention and treatment of candidiasis. However, prolonged use of fluconazole in recent years has contributed to the development of drug resistance in *C. albicans* and other species. One selective pressure contributing to the emergence of drug resistance is the fungistatic rather than fungicidal nature of fluconazole action.

Difficulty in the treatment of *C. albicans* infections is com-

pounded by the fact that *C. albicans* biofilm cells are resistant to many major classes of antifungal drugs, including azoles. Biofilm resistance to fluconazole has been attributed to a compromise in *C. albicans* cell membrane integrity caused by reduced sterols (16, 20). A recent report also demonstrated that cell wall β -1,3 glucan levels contribute to biofilm resistance to fluconazole (22). Emerging resistance in both planktonic as well as biofilm-associated *C. albicans* isolates poses challenges to the successful use of fluconazole as a single-drug treatment option (16, 24). However, a caveat for two-drug combination strategies (where one drug is fluconazole) is that some drug combinations can have disparate effects on *C. albicans* planktonic and biofilm cells. Fluconazole in combination with amphotericin B (AmB) has a synergistic effect on *C. albicans* planktonic cells but does not alter AmB activity against biofilms (1, 2, 24). Also, fluconazole and caspofungin have an antagonistic effect against biofilms but not with planktonic cells (2). Given these concerns, identifying antifungal drugs that are synergistic with fluconazole for the treatment of both drug-resistant *C. albicans* planktonic cells and biofilms is of importance.

Recent studies have documented that the calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506) are dramatically synergistic with azoles, resulting in potent fungicidal activity (8, 11, 18, 23, 30, 35). Calcineurin is a Ca^{2+} -calmodulin-activated phosphatase that governs fungal physiology, including regulation of cell cycle progression, morphogenesis, mating and cytokinesis, recovery from pheromone arrest, cation homeostasis, cell wall biosynthesis, antifungal drug resistance, and virulence (13, 19, 27, 29, 34, 35, 39). Mutant strains lacking calcineurin are markedly hypersensitive to azoles (11, 32). When combined with fluconazole, calcineurin inhibitors

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render azole drugs fungicidal rather than fungistatic. Also, fluconazole-mediated membrane perturbation (due to inhibition of ergosterol biosynthesis) increases calcineurin inhibitor intracellular concentrations (11). These results stimulated an interest in determining whether synergism of fluconazole-calcineurin inhibitor drug combinations could be extended to *C. albicans* biofilms.

We found that both in vitro and in an in vivo rat catheter model, *C. albicans* calcineurin mutants formed biofilms comparable to those of the wild-type strain. Fluconazole or calcineurin inhibitors delivered individually had no effects on wild-type biofilms. On the other hand, biofilms were exquisitely sensitive to either FK506-fluconazole or CsA-fluconazole combinations. *C. albicans* strains lacking the FK506 binding protein (FKBP12) or expressing a dominant FK506-resistant calcineurin mutant subunit (Cnb1-1) formed biofilms that were resistant to FK506-fluconazole but susceptible to CsA-fluconazole. These results document that drug synergism is mediated via direct inhibition of *C. albicans* calcineurin. In summary, these studies reveal that calcineurin contributes to fluconazole resistance of biofilms, and they identify a synergistic drug combination for treating or preventing biofilms.

MATERIALS AND METHODS

***C. albicans* strains.** *C. albicans* strains used in this study were SC5314, K1, and DAY185, *C. albicans* calcineurin B homozygous mutant *cnb1/cnb1* (DAY364) and its complemented strain *cnb1/cnb1+CNB1* (MCC85), *C. albicans* zinc finger transcription factor mutant *crz1/crz1* (OCC1.1) and its complemented strain *crz1/crz1+CRZ1* (OCC7), the FK506-resistant calcineurin mutant *CNB1-1/CNB1* (JRB61), and the *rbp1/rbp1* *C. albicans* strain lacking FKBP12 (YAG171). All strains except K1 are congeneric with the SC5314 strain background. The strains were routinely maintained and grown in YPD liquid medium (20 g of peptone, 10 g of yeast extract, 20 g of glucose per liter) at 30°C. All strains had normal and comparable growth rates.

Biofilm growth conditions. Two in vitro models were used to assess biofilm growth and drug susceptibility. The first model utilized a 96-well polystyrene plate as a substrate, as previously described (22, 28). Briefly, cells were grown in YPD overnight at 37°C and resuspended in RPMI buffered with HEPES at a concentration of 10^6 cells/ml based on hemocytometer calculations. An inoculum (100 μ l) was added to each well of a 96-well flat-bottom plate. After a 24-h incubation at 37°C, the wells were washed with phosphate-buffered saline (PBS) three times to remove any nonadherent cells.

In the second model, biofilms were also grown on silicon elastomer (SE) surfaces as described previously (31). Briefly, strains were grown overnight in YPD medium at 30°C and diluted to an optical density at 600 nm of 0.5 in Spider medium. The suspension was added to a sterile 12-well or a 48-well plate containing bovine serum (B-9433; Sigma)-treated SE squares (cardiovascular instrument silicone sheets; PR72034-06N). The inoculated plates were incubated at 37°C for 90 min at 150 rpm agitation for the initial adhesion of cells. The squares were washed with PBS, transferred to fresh plates containing fresh Spider medium, and incubated at 37°C for 48 h at 150 rpm agitation to allow biofilm formation.

Quantitative measurement of biofilms. Biofilms were quantified by both dry weight measurements and colorimetrically by a 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT; Sigma) reduction assay. For dry weight measurements, the SE squares were weighed prior to biofilm development. After 48 h of biofilm formation, the biofilms were washed gently with sterile PBS and air dried at 37°C for 24 h. The total biomass of each biofilm was calculated by subtracting the weight of the silicone prior to biofilm growth from the weight of the silicone after biofilm growth.

XTT solution (1 mg/ml) was prepared in PBS; menadione solution (0.4 mM; Sigma) was prepared in acetone. The two solutions were added to the wells containing PBS-washed biofilms at a ratio of 5:1 (vol/vol), and the plates were incubated for 3 h at 37°C in the dark. After incubation, the liquid was removed from each well and clarified by centrifugation, and XTT formazan production was measured by determining the absorbance at 492 nm.

In vitro biofilm drug susceptibility. Biofilms were formed in 96-well plates as described above. After 24 h, fresh medium (RPMI/HEPES) and drugs were added to wells. Dilutions of fluconazole (0.25 to 1,000 μ g/ml), FK506 (0.005 to 300 μ g/ml), and cyclosporine (1.2 to 300 μ g/ml) were examined alone and in combination in a checkerboard format. The ranges of concentrations of the drugs used included the standard doses given to humans. After 24 h of incubation at 37°C, biofilms were washed twice with PBS. Measurement of biofilm cell metabolic activity using the XTT reduction assay was performed as previously described (22, 28). Briefly, 90 μ l of XTT (1 mg/ml) and 10 μ l phenazine methosulfate (320 μ g/ml) were added to each well, and the plate was incubated at 37°C for 2 h. Absorbance at 492 nm was measured using an automated plate reader. Assays were performed in triplicate. We determined the drug concentration associated with a 50% reduction in optical density compared to the no-drug control wells (EC_{50}). The fractional inhibitory concentration (FIC) was then calculated as follows: [(EC_{50} of drug A in combination)/(EC_{50} of drug A alone)] + [(EC_{50} of drug B in combination)/(EC_{50} of drug B alone)]. Values of ≤ 0.5 revealed synergy, those of >0.5 but <2 indicated no interaction, and those of >2 were antagonistic (22).

Under many conditions, drug susceptibility testing was also performed for cells in biofilms grown on SE. Mature biofilms were washed gently with sterile PBS and transferred to fresh 12-well plates containing Spider medium supplemented with the appropriate concentration(s) of a single drug or a combination of two drugs. The biofilms were further incubated for 48 h, and metabolic activities of biofilms were measured by the XTT reduction assay. The MIC was defined as the antifungal concentration that caused a 50% reduction in metabolic activity of a *C. albicans* biofilm compared with the control (incubated in the absence of drug). The drugs used were fluconazole (Sigma, St. Louis, MO), FK506 (Prograf; Astellas Pharma US, Inc., Deerfield, IL), and CsA (Alexis Corporation, San Diego, CA).

In vivo biofilm drug susceptibility. A rat central venous catheter infection model was selected for in vivo biofilm studies (1, 21). Specific-pathogen-free Sprague-Dawley rats weighing ~ 400 g were used (Harlan Sprague-Dawley, Indianapolis, IN). A heparinized (100 U/ml) polyethylene catheter was surgically inserted into the external jugular vein and advanced to a site above the right atrium (2-cm length). The catheter was secured to the vein, and the proximal end was tunneled subcutaneously to the midscapular space and externalized through the skin. The catheters were implanted 24 h prior to inoculation. Infection was achieved by intraluminal instillation of 500 μ l *C. albicans* strain K1 (10^6 cells/ml). After 4 h, the catheters were flushed and maintained with heparinized 0.15 M NaCl for 24 h to allow for biofilm formation. Catheters were then treated with fluconazole (125 μ g/ml), FK506 (20 μ g/ml), or a combination for 16 h. These concentrations were picked based on their efficacies in the in vitro drug combination experiments. For controls, animals were treated with saline. Two animals were used per time and treatment point. At the end of the observation period, the catheters were removed and processed for electron microscopy, as described below.

Confocal microscopy. Biofilms were stained with 25 μ g/ml concanavalin A-Alexa Fluor 594 conjugate (C-11253; Molecular Probes, Eugene, OR) for 1 h in the dark at 37°C. Confocal scanning laser microscopy was performed with a Zeiss LSM 510 upright confocal microscope using a Zeiss Achroplan 40 $\times$, 0.8-W objective. Concanavalin A conjugate staining was observed using a HeNe1 laser with an excitation wavelength of 543 nm. Images were assembled into side and depth views using the Zeiss LSM Image Browser v4.2 software. Artificially colored depth view images indicate cell depth using a color gradient, where cells closest to the SE are represented in blue and the cells farthest away are represented in red.

Scanning electron microscopy. Silicon elastomer squares containing biofilms were introduced without fixation into the environmental scanning electron microscope (Philips XL30 ESEM TMP; FEI Company, Hillsboro, OR; NSF award DBI-0098534). The ESEM allows examination of any specimen, wet or dry, in situ and close to its natural state. Samples were examined at 10 kV, 5 torr pressure, and at a temperature of 3°C. The samples remained hydrated throughout the process. Catheter segments were processed for scanning electron microscopy as previously described (1). After overnight fixation (4% formaldehyde, 1% glutaraldehyde in PBS), catheter segments were washed for 5 min in PBS and placed in 1% osmium tetroxide for 30 min. Drying was accomplished using a series of alcohol washes followed by critical point drying. Catheter segments were mounted and gold coated. Images were obtained with a scanning electron microscope (JEOL JSM-6100) in the high-vacuum mode at 10 kV. The images were assembled using Adobe Photoshop 7.0.1.

FIG. 1. Calcineurin is not required for *C. albicans* biofilm formation. Biofilms were grown as described in Materials and Methods, metabolic activity was measured by XTT assay (A), and biomass was measured based on dry weight (B). Assays were performed in triplicate. Biofilms were not significantly different from each other ($P > 0.3$, analysis of variance).

RESULTS

Calcineurin is not required for *C. albicans* biofilm formation in vitro or in vivo. Calcineurin governs several important physiological properties in *C. albicans*, including *C. albicans* survival in serum, virulence in a murine model of systemic candidiasis, and resistance to azole antifungals (7, 9, 11, 23, 32). Azole tolerance is mediated in part via the calcineurin target Crz1 (15, 25, 33). Although calcineurin does not control *C. albicans* properties of adhesion or filamentation (3, 4, 9), two properties important for biofilm formation, given its broad physiological roles we hypothesized that calcineurin might be involved in *C. albicans* biofilm formation. To test this, *C. albicans* *cnb1/cnb1* (lacking calcineurin B) and *crz1/crz1* (lacking a transcription factor controlled by calcineurin) mutants were first used to develop biofilms in vitro on silicone elastomer squares. After 48 h of development at 37°C, biofilms were quantitated by two independent methods, dry weight measurements and XTT assays. No significant difference ($P > 0.3$) was observed between the biofilms formed by the *C. albicans* wild-type strain and the *C. albicans* calcineurin or *crz1* mutant strains (Fig. 1). Also, as expected, the complemented strains of the *C. albicans* calcineurin and the *crz1* mutants developed biofilms as robustly as the *C. albicans* wild-type strain (Fig. 1).

The ability of the *C. albicans* *cnb1/cnb1* mutant to form a biofilm in vivo was examined using the venous catheter animal model (Fig. 2). Similar to the wild-type strain, *cnb1/cnb1* formed a mature biofilm 24 h after inoculation. Scanning electron microscopy imaging demonstrated similar biofilm architecture between the wild-type and mutant strains. Both biofilms were of comparable thickness and consisted of a combination of adherent yeast and hyphae surrounded by matrix and host components.

FIG. 2. The *C. albicans* calcineurin mutant forms a biofilm in vivo. *C. albicans* wild type and *cnb1/cnb1* were instilled in rat venous catheters. The ability to form a biofilm and biofilm architecture were evaluated by scanning electron microscopy imaging at 24 h. The top row represents a 50× magnification (bar, 600 μm), and the bottom row represents 1,000× magnification (bar, 30 μm). Experiments were performed in duplicate. Biofilm thickness and architecture were similar between the two strains.

Calcineurin is involved in *C. albicans* biofilm resistance to fluconazole. *C. albicans* *cnb1/cnb1* and *crz1/crz1* mutant strains are known to be hypersensitive to fluconazole compared to wild-type cells under planktonic growth conditions (23, 25, 30, 33). The fact that these mutants formed morphologically wild-type biofilms allowed us to test if these strains were also azole hypersensitive under biofilm conditions. Mature biofilms were treated for 48 h with a range of fluconazole concentrations (0.25 μg/ml to 1,024 μg/ml). The MIC₅₀ of fluconazole for biofilm cells was calculated based on results from the XTT assay. *C. albicans* *cnb1/cnb1* biofilm cells were at least 15-fold more sensitive to fluconazole than wild-type biofilms, and those formed by the *C. albicans* *crz1/crz1* mutant were at least 8-fold more sensitive (Table 1). Complementation of the *cnb1* and *crz1* mutations with the wild-type gene restored wild-type levels of azole resistance in biofilms (Table 1). These results are complementary to earlier reports that, under planktonic conditions, *crz1/crz1* mutants are less fluconazole hypersensi-

TABLE 1. Calcineurin is involved in *C. albicans* biofilm resistance to fluconazole

Strain	Fluconazole EC ₅₀ ^a (μg/ml)
SC5314.....	>1,024
<i>cnb1/cnb1</i>	62.5
<i>cnb1/cnb1</i> + CNB1	>512
<i>crz1/crz1</i>	128
<i>crz1/crz1</i> + CRZ1	>1,024
<i>rbp1/rbp1</i>	512
CNB1-1/CNB1	512

^a EC₅₀, the fluconazole concentration at which XTT absorbance was reduced by 50%.

TABLE 2. Calcineurin and FKBP12 are targets of calcineurin inhibitor-fluconazole synergistic activity under *C. albicans* biofilm growth conditions

Strain and fluconazole concn range (μg/ml)	Calcineurin inhibitor, concn range (μg/ml)	FIC index ^a
Wild type (SC5314)		
62.5–1,000	FK506, 1.2–300	0.08
62.5–1,000	FK506, 0.005–1.17	0.1
0.25–62.5	FK506, 0.58–9.38	0.13
0.25–62.5	CsA, 18.75–300	0.53
62.5–1,000	CsA, 1.2–300	0.13
<i>rbp1/rbp1</i>		
8.0–1,000	FK506, 0.5–1.17	2.0
8.0–62.5	FK506, 0.58–9.38	2.0
8.0–62.5	CsA, 1.2–50	0.31
<i>CNB1-1/CNB1</i>		
8.0–1,000	FK506, 0.5–1.17	1.51
8.0–62.5	FK506, 0.58–9.38	2.0
8.0–62.5	CsA, 1.2–50	0.37

^a FIC index = [(EC₅₀ of fluconazole-calcineurin inhibitor combination)/(EC₅₀ of fluconazole alone)] + [(EC₅₀ of fluconazole-calcineurin inhibitor combination)/(EC₅₀ of calcineurin inhibitor alone)]. A FIC of <0.5 is indicative of synergism.

tive than *cnb1/cnb1* mutants (but both strains are significantly more sensitive than wild-type *C. albicans* cells) (15, 25).

***C. albicans* biofilms are hypersensitive to calcineurin inhibitor-fluconazole combinations.** The increased fluconazole susceptibility of the *cnb1/cnb1* mutant biofilms indicated that calcineurin may be responsible for the resistance of *C. albicans* biofilms to fluconazole. This hypothesis was further tested by exposing wild-type *C. albicans* biofilms to a combination of calcineurin inhibitors and fluconazole. Previous studies demonstrated that *C. albicans* planktonic cells are hypersensitive to

combinations of calcineurin inhibitor (either FK506 or cyclosporine A) and fluconazole (11, 17, 18). If calcineurin were indeed responsible for mediating fluconazole resistance, then biofilms cells might also exhibit sensitivity to the drug combination.

Measurement of metabolic activity of biofilm cells in an XTT assay revealed that biofilm cells were resistant to CsA (1 to 300 μg/ml), FK506 (0.005 to 300 μg/ml), or fluconazole (0.25 to 1,024 μg/ml) delivered individually (Table 2). However, biofilm cells were found to be exquisitely sensitive to either an FK506-fluconazole or CsA-fluconazole combination, with FIC indexes as low as 0.08 and 0.13 for the respective drug combinations, indicating a synergistic interaction (Table 2).

In addition to the XTT assays, we performed confocal scanning laser microscopy (CSLM) to examine the architecture of biofilms treated with the FK506-fluconazole combination compared to individual drug treatment. Side and depth biofilm analyses revealed no significant difference between the biofilms formed by the wild-type untreated, fluconazole-treated, or FK506-treated cells (~300 μm) (Fig. 3). However, the biofilms treated with the FK506-fluconazole combination were significantly reduced in thickness by ~5-fold to 20% the depth produced by cells under the other conditions (Fig. 3).

To examine the impact of the FK506-fluconazole combination in vivo, we used a rat venous catheter biofilm model and imaged treated and control biofilms by scanning electron microscopy (Fig. 4). Addition of fluconazole alone (125 μg/ml) or FK506 alone (20 μg/ml) had minimal impact on mature wild-type *C. albicans* biofilms, compared to a saline-treated control. However, imaging of the two-drug combination demonstrated the complete inhibition of the biofilm.

To test if calcineurin is involved in the toxic effect of the FK506-fluconazole combination, two FK506-resistant strains

FIG. 3. *C. albicans* biofilms are hypersensitive to the tacrolimus (FK506)-fluconazole combination. *C. albicans* wild-type biofilms (untreated) (A) or treated with fluconazole (B), FK506 (C), or a combination of fluconazole and FK506 (D) were stained with concanavalin A conjugate for CSLM visualization, and image reconstructions were created to provide side views (A to D). CSLM depth views for the wild-type biofilm (E) and biofilm treated with the two-drug combination (G) were artificially colored: blue represents cells closest to the silicone, and red represents cells farthest from the silicone. SEM images of the latter two conditions were also obtained (F and H). Biofilms treated with only fluconazole or only FK506 showed depth view and SEM patterns similar to the wild type and are not included in the figure. Bars in SEM images, 100 μm.

FIG. 4. Impact of fluconazole and FK506 alone and in combination as lock therapy against *C. albicans* biofilm cells in an in vivo catheter model. The biofilms were grown for 24 h followed by intraluminal drug treatment for 16 h. Following compound exposure, the catheters were removed for SEM processing. For each of the four panels, the top row represents a 50 $\times$ magnification (bar, 600 μ m) and the bottom row represents 1,000 $\times$ magnification (bar, 30 μ m). Panel columns: no treatment, images from a control biofilm treated with saline; fluconazole, images after a 125- μ g/ml fluconazole exposure; FK506, images from catheters exposed to FK506 at 20 μ g/ml; fluconazole + FK506, images from a combination of fluconazole and FK506 at 125 μ g/ml and 20 μ g/ml, respectively.

were analyzed: first, a strain lacking the *RBPI* gene, whose product is required to bind FK506 and inhibit calcineurin (10, 11), and second, *CNB1-I*, a mutant strain that expresses a dominant drug-resistant form of calcineurin B that has reduced affinity for FKBP12-FK506 but wild-type sensitivity to cyclophilin A-CsA (11, 14). If calcineurin is required for the observed synergistic interaction between FK506 and fluconazole in *C. albicans*, then both the *rbp1/rbp1* and *CNB1-I* mutations should confer resistance to the drug combination while continuing to be sensitive to the CsA-fluconazole combination. Indeed, both the *rbp1/rbp1* and *CNB1-I* strains were completely resistant to the FK506-fluconazole combination (FIC, 2.0) but remained sensitive to the CsA-fluconazole combination (FIC, 0.31 to 0.37), confirming that calcineurin is directly involved in biofilm resistance to fluconazole (Tables 1 and 2).

DISCUSSION

Calcineurin is a Ca^{2+} -calmodulin-dependent protein phosphatase that controls myriad processes in fungi, including cation homeostasis, morphogenesis, virulence traits, and antifungal drug resistance (13, 27, 29, 34). In *C. albicans*, calcineurin is required for survival in serum, virulence, and resistance to azole antifungals, in part via its downstream target, Crz1 (7, 9, 11, 15). Given these broad physiological roles, we tested if calcineurin or Crz1 had any role in *C. albicans* biofilm formation in vitro and in an in vivo rat catheter model. Dry weight measurements and XTT assays of in vitro biofilms and SEM images of in vivo biofilms revealed that both calcineurin as well as Crz1 were dispensable for biofilm formation in *C. albicans*. Also, when wild-type biofilms were grown in the presence of the calcineurin inhibitors FK506 (Fig. 2 and 4) or CsA (data not shown), there was no negative effect on biofilm development. These results were not surprising in light of the fact that

calcineurin does not control *C. albicans* adhesion or filamentation—properties critical for biofilm development (11, 31).

Although calcineurin mutants and Crz1 were not found to be involved in *C. albicans* biofilm growth, the biofilms they formed were more susceptible to the azole drug fluconazole (Table 1). The sensitivity of calcineurin mutants to fluconazole has been reported previously under planktonic conditions (11, 28, 35). We have shown here that this phenomenon also applies to the biofilm setting.

To confirm that calcineurin is indeed involved in biofilm resistance to fluconazole, we treated the wild-type biofilm cells with a combination of calcineurin inhibitors and fluconazole. The calcineurin inhibitors CsA and FK506 are immunosuppressive drugs that exhibit potentials as antifungal agents, either singly or in combination with other antifungal agents, such as azoles (8, 18, 21). Both CsA-fluconazole and FK506-fluconazole combinations were highly synergistic in action against *C. albicans* wild-type biofilms both in vitro and in vivo (Table 2; Fig. 3 and 4), and calcineurin and FKBP12 were the exclusive targets of the FK506-fluconazole combination (Table 2).

In *C. albicans* planktonic cells, fluconazole treatment significantly reduces levels of ergosterol within the cell, leading to cell membrane perturbation (11). The resulting alteration in the permeability of the plasma membrane allows increased concentrations of calcineurin inhibitors to penetrate the cell, resulting in cell death. We speculate that fluconazole similarly decreases the sterol concentration in biofilms and, thereby, its combination with calcineurin inhibitors renders the combination fungicidal.

Inhibition of *C. albicans* biofilms by this combination is significant in light of the studies that have reported on the failure of various antifungal drug combinations against biofilms. These drug-resistant biofilms, especially those that form on indwelling medical devices, are essentially untreatable, thereby making removal of the colonized device the only treatment

option available. Calcineurin inhibitor-fluconazole combinations could prove an effective antifungal regimen to prevent or eradicate these biofilm-associated cells and may thus allow for longer retention of implanted devices.

The mechanism of *C. albicans* biofilm resistance to fluconazole is considered multifactorial, encompassing contributions of cell density, impermeability of drugs through the extracellular matrix, activation of multidrug efflux pumps, increases in cellular β -1,3 glucan levels, and decreases in sterol levels (5, 20, 22, 26). Our study reveals that calcineurin is an important component in mediating fluconazole resistance in biofilms of *C. albicans*. We also suggest that calcineurin inhibitor-fluconazole drug combinations may prove to be a novel and effective therapeutic option.

Cyclosporine is already being broadly marketed for dry eye syndrome and could be readily applied for other indications in ophthalmology. FK506 analogs, such as pimecrolimus, that have less-penetrating properties than FK506 could be readily applied for topical fungal infections in conjunction with existing topical or systemic antifungals (35). Other settings might include troches for oropharyngeal candidiasis or impregnated catheters with these or other inhibitors.

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REFERENCES

- Andes, D., J. Nett, P. Oschel, R. Albrecht, K. Marchillo, and A. Pitula. 2004. Development and characterization of an in vivo central venous catheter *Candida albicans* biofilm model. *Infect. Immun.* **72**:6023–6031.
- Bachmann, S. P., G. Ramage, K. VandeWalle, T. F. Patterson, B. L. Wickes, and J. L. Lopez-Ribot. 2003. Antifungal combinations against *Candida albicans* biofilms in vitro. *Antimicrob. Agents Chemother.* **47**:3657–3659.
- Bader, T., B. Bodendorfer, K. Schroppel, and J. Morschhauser. 2003. Calcineurin is essential for virulence in *Candida albicans*. *Infect. Immun.* **71**:5344–5354.
- Bader, T., K. Schroppel, S. Bentink, N. Agabian, G. Kohler, and J. Morschhauser. 2006. Role of calcineurin in stress resistance, morphogenesis, and virulence of a *Candida albicans* wild-type strain. *Infect. Immun.* **74**:4366–4369.
- Baillie, G. S., and L. J. Douglas. 2000. Matrix polymers of *Candida biofilms* and their possible role in biofilm resistance to antifungal agents. *J. Antimicrob. Chemother.* **46**:397–403.
- Beck-Sague, C., and W. R. Jarvis. 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980–1990. National Nosocomial Infections Surveillance System. *J. Infect. Dis.* **167**:1247–1251.
- Blankenship, J. R., and J. Heitman. 2005. Calcineurin is required for *Candida albicans* to survive calcium stress in serum. *Infect. Immun.* **73**:5767–5774.
- Blankenship, J. R., W. J. Steinbach, J. R. Perfect, and J. Heitman. 2003. Teaching old drugs new tricks: reincarnating immunosuppressants as antifungal drugs. *Curr. Opin. Investig. Drugs* **4**:192–199.
- Blankenship, J. R., F. L. Wormley, M. K. Boyce, W. A. Schell, S. G. Filler, J. R. Perfect, and J. Heitman. 2003. Calcineurin is essential for *Candida albicans* survival in serum and virulence. *Eukaryot. Cell* **2**:422–430.
- Cruz, M. C., A. L. Goldstein, J. Blankenship, M. Del Poeta, J. R. Perfect, J. H. McCusker, Y. L. Bennani, M. E. Cardenas, and J. Heitman. 2001. Rapamycin and less immunosuppressive analogs are toxic to *Candida albicans* and *Cryptococcus neoformans* via FKBP12-dependent inhibition of TOR. *Antimicrob. Agents Chemother.* **45**:3162–3170.
- Cruz, M. C., A. L. Goldstein, J. R. Blankenship, M. Del Poeta, D. Davis, M. E. Cardenas, J. R. Perfect, J. H. McCusker, and J. Heitman. 2002. Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO J.* **21**:546–559.
- Dismukes, W. E. 2000. Introduction to antifungal drugs. *Clin. Infect. Dis.* **30**:653–657.
- Edlind, T., L. Smith, K. Henry, S. Katiyar, and J. Nickels. 2002. Antifungal activity in *Saccharomyces cerevisiae* is modulated by calcium signalling. *Mol. Microbiol.* **46**:257–268.
- Fox, D. S., M. C. Cruz, R. A. Sia, H. Ke, G. M. Cox, M. E. Cardenas, and J. Heitman. 2001. Calcineurin regulatory subunit is essential for virulence and mediates interactions with FKBP12-FK506 in *Cryptococcus neoformans*. *Mol. Microbiol.* **39**:835–849.
- Karababa, M., E. Valentino, G. Pardini, A. T. Coste, J. Bille, and D. Sanglard. 2006. CRZ1, a target of the calcineurin pathway in *Candida albicans*. *Mol. Microbiol.* **59**:1429–1451.
- Kohli, A., Smriti, K. Mukhopadhyay, A. Rattan, and R. Prasad. 2002. In vitro low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. *Antimicrob. Agents Chemother.* **46**:1046–1052.
- Marchetti, O., J. M. Entenza, D. Sanglard, J. Bille, M. P. Glauser, and P. Moreillon. 2000. Fluconazole plus cyclosporine: a fungicidal combination effective against experimental endocarditis due to *Candida albicans*. *Antimicrob. Agents Chemother.* **44**:2932–2938.
- Marchetti, O., P. Moreillon, M. P. Glauser, J. Bille, and D. Sanglard. 2000. Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrob. Agents Chemother.* **44**:2373–2381.
- Moser, M. J., J. R. Geiser, and T. N. Davis. 1996. Ca^{2+} -calmodulin promotes survival of pheromone-induced growth arrest by activation of calcineurin and Ca^{2+} -calmodulin-dependent protein kinase. *Mol. Cell. Biol.* **16**:4824–4831.
- Mukherjee, P. K., J. Chandra, D. M. Kuhn, and M. A. Ghannoum. 2003. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect. Immun.* **71**:4333–4340.
- Nett, J., L. Lincoln, K. Marchillo, and D. Andes. 2007. β -1,3 glucan as a test for central venous catheter biofilm infection. *J. Infect. Dis.* **195**:1705–1712.
- Nett, J., L. Lincoln, K. Marchillo, R. Massey, K. Holodyda, B. Hoff, M. VanHandel, and D. Andes. 2007. Putative role of β -1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob. Agents Chemother.* **51**:510–520.
- Onyewu, C., N. A. Afshari, and J. Heitman. 2006. Calcineurin promotes infection of the cornea by *Candida albicans* and can be targeted to enhance fluconazole therapy. *Antimicrob. Agents Chemother.* **50**:3963–3965.
- Onyewu, C., J. R. Blankenship, M. Del Poeta, and J. Heitman. 2003. Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*. *Antimicrob. Agents Chemother.* **47**:956–964.
- Onyewu, C., F. L. Wormley, Jr., J. R. Perfect, and J. Heitman. 2004. The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of *Candida albicans*. *Infect. Immun.* **72**:7330–7333.
- Perumal, P., S. Mekala, and W. L. Chaffin. 2007. Role for cell density in antifungal drug resistance in *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* **51**:2454–2463.
- Prokisch, H., O. Yarden, M. Dieminger, M. Tropschug, and I. B. Barthelmess. 1997. Impairment of calcineurin function in *Neurospora crassa* reveals its essential role in hyphal growth, morphology and maintenance of the apical Ca^{2+} gradient. *Mol. Gen. Genet.* **256**:104–114.
- Ramage, G., K. Vande Walle, B. L. Wickes, and J. L. Lopez-Ribot. 2001. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* **45**:2475–2479.
- Rasmussen, C., C. Garen, S. Brining, R. L. Kincaid, R. L. Means, and A. R. Means. 1994. The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. *EMBO J.* **13**:3917–3924.
- Reedy, J. L., S. Husain, M. Ison, T. L. Pruett, N. Singh, and J. Heitman. 2006. Immunotherapy with tacrolimus (FK506) does not select for resistance to calcineurin inhibitors in *Candida albicans* isolates from liver transplant patients. *Antimicrob. Agents Chemother.* **50**:1573–1577.
- Richard, M. L., C. J. Nobile, V. M. Bruno, and A. P. Mitchell. 2005. *Candida albicans* biofilm-defective mutants. *Eukaryot. Cell* **4**:1493–1502.
- Sanglard, D., F. Ischer, O. Marchetti, J. Entenza, and J. Bille. 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol. Microbiol.* **48**:959–976.
- Santos, M., and I. F. de Larrinoa. 2005. Functional characterization of the *Candida albicans* CRZ1 gene encoding a calcineurin-regulated transcription factor. *Curr. Genet.* **48**:88–100.
- Steinbach, W. J., R. A. Cramer, Jr., B. Z. Perfect, Y. G. Asfaw, T. C. Sauer, L. K. Najvar, W. R. Kirkpatrick, T. F. Patterson, D. K. Benjamin, Jr., J. Heitman, and J. R. Perfect. 2006. Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. *Eukaryot. Cell* **5**:1091–1103.
- Steinbach, W. J., J. L. Reedy, R. A. Cramer, Jr., J. R. Perfect, and J. Heitman. 2007. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nat. Rev. Microbiol.* **5**:418–430.
- Vanden Bossche, H. 1985. Biochemical targets for antifungal azole derivatives: hypothesis on the mode of action. *Curr. Top. Med. Mycol.* **1**:313–351.
- Vanden Bossche, H., G. Willemsens, and P. Marichal. 1987. Anti-*Candida* drugs—the biochemical basis for their activity. *Crit. Rev. Microbiol.* **15**:57–72.
- Wenzel, R. P. 1995. Nosocomial candidemia: risk factors and attributable mortality. *Clin. Infect. Dis.* **20**:1531–1534.
- Yoshida, T., T. Toda, and M. Yanagida. 1994. A calcineurin-like gene *ppb1+* in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. *J. Cell Sci.* **107**:1725–1735.