Ergosterol Biosynthesis Inhibitors Become Fungicidal when Combined with Calcineurin Inhibitors against *Candida albicans*, *Candida glabrata*, and *Candida krusei*

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Azoles target the ergosterol biosynthetic enzyme lanosterol 14α-demethylase and are a widely applied class of antifungal agents because of their broad therapeutic window, wide spectrum of activity, and low toxicity. Unfortunately, azoles are generally fungistatic and resistance to fluconazole is emerging in several fungal pathogens. We recently established that the protein phosphatase calcineurin allows survival of *Candida albicans* during the membrane stress exerted by azoles. The calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506) are dramatically synergistic with azoles, resulting in potent fungicidal activity, and mutant strains lacking calcineurin are markedly hypersensitive to azoles. Here we establish that drugs targeting other enzymes in the ergosterol biosynthetic pathway (terbinafine and fenpropimorph) also exhibit dramatic synergistic antifungal activity against wild-type *C. albicans* when used in conjunction with CsA and FK506. Similarly, *C. albicans* mutant strains lacking calcineurin B are markedly hypersensitive to terbinafine and fenpropimorph. The FK506 binding protein FKBP12 is required for FK506 synergism with ergosterol biosynthesis inhibitors, and a calcineurin mutation that confers FK506 resistance abolishes drug synergism. Additionally, we provide evidence of drug synergy between the nonimmunosuppressive FK506 analog L-685,818 and fenpropimorph or terbinafine against wild-type *C. albicans*. These drug combinations also exert synergistic effects against two other *Candida* species, *C. glabrata* and *C. krusei*, which are known for intrinsic or rapidly acquired resistance to azoles. These studies demonstrate that the activity of non-azole antifungal agents that target ergosterol biosynthesis can be enhanced by inhibition of the calcineurin signaling pathway, extending their spectrum of action and providing an alternative approach by which to overcome antifungal drug resistance.

Infections caused by *Candida* species are the most prevalent fungal infections of humans and are a serious concern for patients with compromised immune systems. *Candida albicans* is the causative agent of most candidiasis, but other *Candida* species, including *C. glabrata* and *C. krusei*, are emerging as serious nosocomial threats to patient populations (28, 32). *Candida* subspecies are a normal component of human flora and reside on mucosal surfaces. In immunocompetent and immunocompromised hosts, *Candida* spp. can cause superficial mucosal infections such as vaginitis, thrush, and esophagitis. However, immunocompromised patients are also susceptible to severe systemic infections. Risk factors include human immunodeficiency virus (HIV) infection, solid-organ transplants, abdominal surgery, indwelling catheters, late-onset diabetes, and broad-spectrum antibiotic use (4, 29).

Compared to bacterial infections, few drugs are available with which to treat fungal infections. This is largely attributable to the eukaryotic nature of fungal cells and the difficulty in identifying unique targets not shared with human hosts. Most therapies designed to treat fungal infections target the ergo-sterol biosynthetic pathway or its final product, ergosterol, a sterol cell membrane component that is unique to fungi (Fig. 1). The most commonly used drug in both the treatment and prevention of candidiasis is fluconazole, a member of the azole family of drugs that targets the essential enzyme Erg11, lanosterol 14α-demethylase, in the ergosterol biosynthetic pathway (Fig. 1) (38, 39). The standard therapy for the treatment of fluconazole-resistant fungal infections is amphotericin B, which binds ergosterol and permeabilizes the plasma membrane. These treatments are extremely effective against *C. albicans*, but resistant *Candida* strains and species are emerging and new treatments for systemic infections need to be developed (37; reviewed in reference 42). A particular difficulty with azole treatment is the inherent resistance, or rapid development of resistance, found in several non-*C. albicans Candida* species, such as *C. glabrata* and *C. krusei* (2, 10, 28, 31, 32, 34, 40, 41, 43). Therefore, there exists a clear demand for more effective treatment of infections caused by these emerging fungal pathogens.

Several classes of drugs have been developed that target enzymes other than Erg11 in the ergosterol biosynthetic pathway. Morpholines and allylamines inhibit specific ergosterol biosynthetic enzymes. For example, terbinafine is in the allylamine class of drugs and targets the enzyme squalene epoxidase (Erg1) (Fig. 1) (14). Terbinafine, available in both oral

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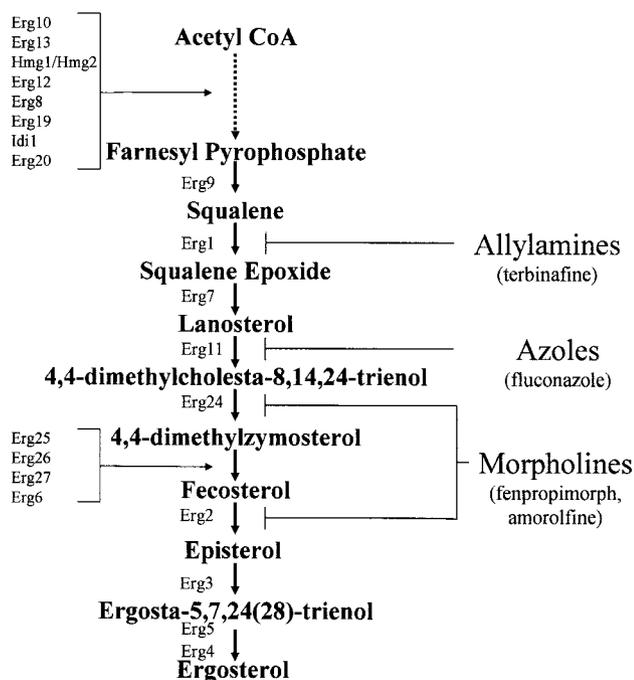
FIG. 1. Linear model of the ergosterol biosynthetic pathway adapted from *Saccharomyces cerevisiae*. Significant intermediates are in boldface, and inhibitors are shown on the right. Commonly used drugs in the allylamine, azole, and morpholine classes are listed beneath the class names. CoA, coenzyme A.

and topical forms as Lamisil, is used in the treatment of tinea pedis and onychomycosis but has not been effective as monotherapy for systemic infections. Fenpropimorph is a morpholine that targets two enzymes in the ergosterol biosynthetic pathway, sterol C_8-C_7 isomerase (Erg2) and sterol reductase (Erg24), and is used as a fungicide in agriculture (Fig. 1) (24). Fenpropimorph, like many other morpholines, mainly targets Erg2 but has been shown to inhibit Erg24 as well at higher concentrations (16). Amorolfine is currently the only morpholine in clinical use and is only available in topical forms, primarily for the treatment of onychomycosis. Recent results have indicated that Erg24 is required for virulence of *C. albicans* in a murine model of infection (16). However, to date, no drugs of the morpholine class have been developed for oral therapy in humans.

One drawback of azole drugs is that they are fungistatic rather than fungicidal. This characteristic probably contributes to the development of resistance seen in clinical isolates from immunocompromised patients. Since the cells are allowed to persist and immune function is not sufficient to clear residual fungal cells, a positive selection for drug-resistant mutants is established. A fungicidal drug with low toxicity would be the ideal treatment for these patients, but such therapy does not exist. It has recently been shown, however, that the calcineurin inhibitors cyclosporine A (CsA) and FK506 exhibit a potent fungicidal synergism with the azole class of drugs against *C. albicans* (8, 20, 22, 23). These results stimulated our interest in determining whether additional drugs targeting the ergosterol biosynthetic pathway also exhibit fungicidal synergism with calcineurin inhibitors, thus improving and expanding their an-

tifungal properties. Here we show that both terbinafine and fenpropimorph exhibit a potent fungicidal synergism with calcineurin inhibitors in *C. albicans*, and we present evidence that L-685,818, a nonimmunosuppressive analog of FK506, can also participate in this drug synergy. Additionally, we establish that the molecular basis of synergism with FK506 involves inhibition of calcineurin by the FKBP12-FK506 complex. Importantly, we also illustrate that these synergistic drug combinations are effective against *C. glabrata* and *C. krusei* in vitro, which we previously demonstrated to be largely insensitive to the synergism between azoles and calcineurin inhibitors (8).

MATERIALS AND METHODS

Strains and media. The strains used in this study are listed in Table 1. All of the strains were grown on YPD medium containing 2% (vol/vol) glucose, 2% (wt/vol) Bacto Peptone (Difco Laboratories), and 1% (wt/vol) yeast extract (Difco). YPD agar plates also contained 2% (wt/vol) Bacto Agar (Difco). The top agar used in these assays was 0.7% Bacto Agar (Difco) in water.

Disk diffusion halo assays. Strains were inoculated into liquid YPD medium and grown overnight at 37°C. The cells were then pelleted, washed three times with distilled water, and counted with a hemocytometer. Approximately 2×10^7 cells were inoculated into 8 ml of top agar at 42°C. This cell suspension was then spread onto warmed 150-mm-diameter YPD plates and allowed to dry. Terbinafine (Novartis) dissolved in 100% ethanol, fenpropimorph (Crescent) dissolved in dimethyl sulfoxide, CsA (Novartis) dissolved in 90% ethanol and 10% Tween 20, FK506 (Fujisawa) dissolved in 90% ethanol and 10% Tween 20, L-685,818 (Merck) dissolved in 90% ethanol and 10% Tween 20, or solvent controls were pipetted onto 6-mm-diameter BBL disks (Becton Dickinson & Co.). These disks were then placed on the solidified top agar surface, and the strains were grown at 37°C for 24 to 48 h.

Serial-dilution assays. Strains were inoculated and grown overnight in liquid YPD medium. Cells were pelleted, washed three times with distilled water, and counted with a hemocytometer. Suspensions containing 2.5×10^4 cells per 5 μl were made for each strain. Fivefold serial dilutions were prepared, and 5 μl of each dilution was spotted onto YPD solid medium containing CsA at 100 μg/ml, FK506 at 1 μg/ml, or no drug. The strains were grown at 37°C for 24 h.

Antifungal drug activity testing by NCCLS criteria. MIC assays were performed as previously described (8). Briefly, in vitro testing was performed in RPMI 1640 medium with L-glutamine and without sodium bicarbonate. The final drug concentrations tested were fenpropimorph at 12.5 to 0.01 μg/ml (11 dilutions), terbinafine at 12.5 to 0.01 μg/ml (11 dilutions), CsA at 3.12 to 0.04 μg/ml (7 dilutions), and FK506 at 3.12 to 0.04 μg/ml (7 dilutions). Plates were incubated at 30°C without shaking, and readings were performed following a 48-h incubation. Each plate was shaken for 5 min with an Easy-Shaker EAS 2/4 (SLT Lab Instruments), and the optical density at 600 nm of each well was read by a microtiter plate reader (Thermomax; Molecular Devices, Menlo Park, Calif.). The MIC of each drug in combination or alone was defined as the lowest drug concentration that resulted in a 100% decrease in absorbance (MIC_{100}) compared with that of the control (no drug). The absence of a trailing effect enabled the use of the MIC_{100} as an end point instead of the standard MIC_{80}. Minimum fungicidal concentrations (MFCs) were determined by plating 100 μl from each well with growth inhibition onto YPD plates, which were incubated at 30°C for

TABLE 1. Strains used in this study

Strain	Species	Genotype[a]	Reference or source[b]
SC5314	*C. albicans*	WT	9
DAY364	*C. albicans*	*cnb1/cnb1*	8
MCC85	*C. albicans*	*cnb1/cnb1 CNB1*	8
NJ51-2	*C. albicans*	*erg24/erg24*	16
KPC8	*C. albicans*	*erg6/erg6*	15
YAG171	*C. albicans*	*rbp1/rbp1*	7
YAG237	*C. albicans*	*CNB1-1/CNB1*	8
DUMC111.10/MMRL361	*C. glabrata*	WT	DUMC
MMRL70	*C. krusei*	WT	DUMC

[a] WT, wild type.
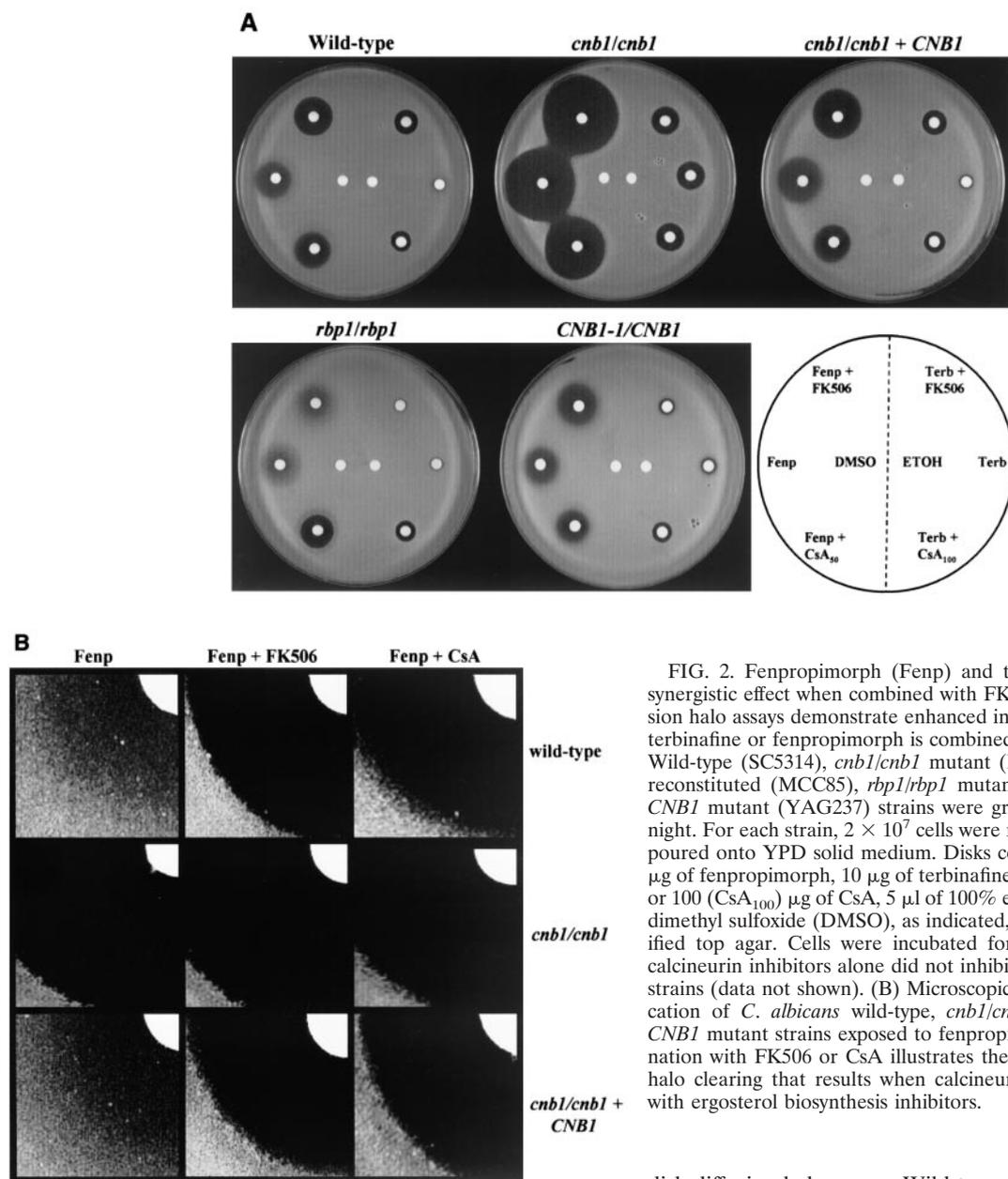[b] DUMC, Duke University Medical Center Stock Center.

FIG. 2. Fenpropimorph (Fenp) and terbinafine (Terb) exhibit a synergistic effect when combined with FK506 or CsA. (A) Disk diffusion halo assays demonstrate enhanced inhibition of *C. albicans* when terbinafine or fenpropimorph is combined with calcineurin inhibitors. Wild-type (SC5314), *cnb1/cnb1* mutant (DAY364), *cnb1/cnb1 CNB1* reconstituted (MCC85), *rbp1/rbp1* mutant (YAG171), and *CNB1-1/CNB1* mutant (YAG237) strains were grown in YPD medium overnight. For each strain, 2×10^7 cells were resuspended in top agar and poured onto YPD solid medium. Disks containing combinations of 1 μg of fenpropimorph, 10 μg of terbinafine, 2 μg of FK506, 50 (CsA_{50}) or 100 (CsA_{100}) μg of CsA, 5 μl of 100% ethanol (ETOH), and 5 μl of dimethyl sulfoxide (DMSO), as indicated, were placed over the solidified top agar. Cells were incubated for 24 to 48 h at 37°C. The calcineurin inhibitors alone did not inhibit the growth of any of these strains (data not shown). (B) Microscopic observation at 4× magnification of *C. albicans* wild-type, *cnb1/cnb1* mutant, and *cnb1/cnb1 CNB1* mutant strains exposed to fenpropimorph alone and in combination with FK506 or CsA illustrates the significant enhancement of halo clearing that results when calcineurin inhibitors are combined with ergosterol biosynthesis inhibitors.

72 h. The lowest concentration that yielded three or fewer colonies was recorded as the MFC. Fractional inhibitory concentrations (FICs) and FIC indices (Σ FIC) were determined as described previously (8). Drug activity was classified as synergistic (Σ FIC, ≤0.5), additive (Σ FIC, 1), or antagonistic (Σ FIC, >4).

RESULTS

Fenpropimorph and terbinafine are synergistic with calcineurin inhibitors. Fungicidal synergism between azoles and calcineurin inhibitors has previously been demonstrated in *C. albicans* (8, 20, 22, 23). We hypothesized that this synergy might also exist between calcineurin inhibitors and other antifungal drugs that inhibit ergosterol biosynthesis in *C. albicans*. To determine whether synergism between calcineurin inhibitors and fenpropimorph or terbinafine occurs, we employed

disk diffusion halo assays. Wild-type *C. albicans* strain SC5314 was grown in the presence or absence of FK506 or CsA with either fenpropimorph or terbinafine (Fig. 2). Fenpropimorph or terbinafine alone exhibited only modest growth inhibition. As previously demonstrated by Cruz et al. (8), neither FK506 nor CsA alone exhibited any in vitro growth inhibition of the *Candida* strains used in our study (see Fig. 5; data not shown).

Wild-type growth was inhibited by fenpropimorph, but the halo produced was turbid, indicating fungistatic growth inhibition (Fig. 2). However, when fenpropimorph was combined with FK506 or CsA, the halo produced was completely clear, an indication of potential fungicidal activity (Fig. 2). To examine whether calcineurin is the target of the observed synergy with FK506 or CsA, a *cnb1/cnb1* calcineurin B mutant strain (DAY364) and a *cnb1/cnb1 CNB1* reconstituted calcineurin mutant strain (MCC85) were also tested (Table 1). When the calcineurin-deficient (*cnb1/cnb1*) strain was grown in the pres-

TABLE 2. Synergism between ergosterol biosynthesis inhibitors and calcineurin inhibitors is FKBP12 and calcineurin dependent

Genotype (*C. albicans* strain)	MIC_{100} alone (µg/ml)[a]				MIC_{100} combined[a,b]				FIC index[a]			
	CsA	FK506	F	T	CsA/F	CsA/T	FK506/F	FK506/T	CsA/F	CsA/T	FK506/F	FK506/T
Wild type (SC5314)	>3.12	>3.12	3.12	>10.5	1.56/0.04	0.78/3.12	≤0.04/≤0.01	0.09/0.39	0.26	0.24	0.004	0.03
cnb1/cnb1 (DAY364)	>3.12	>3.12	≤0.01	0.39	≤0.04/≤0.01	≤0.04/0.39	≤0.04/≤0.01	≤0.04/0.39	1.003	1.003	1.003	1.003
cnb1/cnb1 CNB1 (MCC85)	>3.12	>3.12	0.78	>12.5	0.78/0.04	0.19/3.12	≤0.04/≤0.01	≤0.04/0.78	0.17	0.15	0.009	0.03
CNB1-1/CNB1 (YAG237)	>3.12	>3.12	3.12	>12.5	1.56/0.19	0.78/3.12	>3.12/3.12	>3.12/>12.5	0.31	0.25	2.0	2.0
rbp1/rbp1 (YAG171)	>3.12	>3.12	3.12	>12.5	0.19/0.78	0.39/1.56	>3.12/3.12	>3.12/>12.5	0.28	0.12	2.0	2.0

[a] F, fenpropimorph; T, terbinafine.
[b] Combined MICs, expressed as [drug 1]/[drug 2] are the minimum concentrations of drugs 1 and 2 required to inhibit cell growth when the two drugs were used in combination.

ence of fenpropimorph alone, we observed a larger halo with clearing that was comparable to that of the wild-type strain exposed to FK506 or CsA in combination with fenpropimorph (Fig. 2). The inhibition profile of the *cnb1/cnb1 CNB1* strain resembled that of the wild type for all drug combinations in the fenpropimorph assay. Thus, inhibition of calcineurin underlies the synergistic drug interaction.

We found terbinafine to be less potent than fenpropimorph, but it also displayed synergism with CsA and FK506. Growth inhibition of the wild-type strain was enhanced, and a clear halo resulted when FK506 or CsA was added to terbinafine. However, the halos produced were significantly smaller than those produced by fenpropimorph, even at a 10-fold higher concentration of terbinafine (Fig. 2). As with fenpropimorph, the *cnb1/cnb1* mutants were hypersensitive to terbinafine, mimicking the effect observed when wild-type cells were exposed to terbinafine in combination with FK506 or CsA. The terbinafine hypersensitivity phenotype of the *cnb1/cnb1* mutant was complemented by reintroduction of the wild-type *CNB1* gene into the *cnb1/cnb1 CNB1* reconstituted strain, restoring the wild-type level of drug sensitivity (Fig. 2).

As shown in Fig. 2B, increased magnification of our disk diffusion assays demonstrated that *C. albicans* cells and microcolonies were present in the turbid halos produced when the wild-type and reconstituted mutant strains were exposed to fenpropimorph alone. However, no cells or microcolonies were observed in the clear halos that resulted from combining fenpropimorph with FK506 or CsA. There was no observable difference in halo clearing in the *cnb1/cnb1* mutant strain exposed to the various drug combinations. For each of the *Candida* strains used in our study, the halo clearing depicted in Fig. 2B is generally applicable to the turbid and clear halos described in all other experiments.

Fenpropimorph and terbinafine are fungicidal in combination with calcineurin inhibitors. We next examined the effects of calcineurin inhibitors in conjunction with fenpropimorph or terbinafine by using standard NCCLS in vitro susceptibility testing methods. The MICs measured supported the results of the halo assays and confirmed the presence of a synergistic interaction between these drug combinations (Table 2). For wild-type *C. albicans*, the MIC of fenpropimorph decreased more than 300-fold when the drug was combined with FK506 and was reduced approximately 100-fold when the drug was combined with CsA. The calculated FIC index of fenpropimorph was 0.004 with FK506 and 0.26 with CsA, denoting a synergistic relationship between fenpropimorph and both calcineurin inhibitors.

Although less potent than fenpropimorph, terbinafine also exhibited a synergistic relationship when combined with FK506 or CsA. For wild-type *C. albicans* cells, the MIC of terbinafine declined 30-fold when the drug was combined with FK506 and 4-fold when it was combined with CsA. These values resulted in FIC indices of 0.03 with FK506 and 0.24 with CsA, also indicating a synergistic interaction (Table 2). MFC analysis was also performed, and the resulting values were very similar to the MIC data (Table 3). The results of the MFC calculations confirmed that the inhibitory concentrations of these drug combinations were fungicidal (Table 3).

Synergy between calcineurin inhibitors and fenpropimorph or terbinafine is calcineurin dependent. We used FK506-resistant *C. albicans* strains (Table 1) to demonstrate that the loss of calcineurin activity, and not a secondary effect of CsA and FK506, underlies their synergy with ergosterol biosynthesis inhibitors. The *CNB1-1/CNB1* strain contains a two-amino-acid insertion that perturbs the FKBP12-FK506 binding site of the calcineurin B subunit, while the *rbp1/rbp1* mutant lacks the FK506 binding protein FKBP12 (7, 8). FKBP12 is required for FK506 activity against calcineurin. FK506 must first complex with FKBP12 in order to bind and inhibit calcineurin, whereas CsA acts by first binding to cyclophilin A (6, 11, 17–19, 25). The *CNB1-1/CNB1* mutation prevents FKBP12-FK506 binding to calcineurin but has no effect on cyclophilin A-CsA binding (8). The *CNB1-1/CNB1* and *rbp1/rbp1* mutant strains were modestly inhibited by fenpropimorph or terbinafine alone, and addition of FK506 had no further effect on growth inhibition, as determined by halo assay and the MICs and FICs based on NCCLS criteria (Fig. 2A and Table 2). When the *CNB1-1/CNB1* and *rbp1/rbp1* mutants were exposed to CsA in combination with fenpropimorph or terbinafine, enhanced inhibition with halo clearing comparable to that of wild-type cells under the same conditions was observed (Fig. 2A). These findings demonstrate that FK506 synergy with fenpropimorph and terbinafine is mediated via FKBP12-dependent inhibition of calcineurin.

***erg24/erg24* mutants are hypersensitive to calcineurin inhibition.** Although the morpholine drugs target both Erg2 and Erg24, it has previously been demonstrated that Erg2 is the major target of fenpropimorph in *C. albicans* (16). To investigate the synergistic potential of calcineurin inhibitors in combination with morpholine drugs that primarily target Erg24, an Erg24-deficient (*erg24/erg24*) mutant (Table 1) was tested for sensitivity to CsA and FK506. The *erg24/erg24* mutants grew more slowly than the wild type, even in the absence of a drug, and showed hypersensitivity to both CsA and FK506 compared

TABLE 3. Fenpropimorph and terbinafine are potently fungicidal in combination with the calcineurin inhibitors CsA and FK506

Genotype (*C. albicans* strain)	MFC alone (μg/ml)[a]				MFC combined[a,b]				FIC index[a]			
	CsA	FK506	F	T	CsA/F	CsA/T	FK506/F	FK506/F	CsA/F	CsA/T	FK506/F	FK506/F
Wild type (SC5314)	>3.12	>3.12	3.12	>12.5	1.56/0.04	0.78/3.12	≤0.04/≤0.01	0.09/0.39	0.26	0.24	0.004	0.03
cnb1/cnb1 (DAY364)	>3.12	>3.12	≤0.01	0.39	≤0.04/≤0.01	≤0.04/0.39	≤0.04/≤0.01	≤0.04/0.39	1.003	1.003	1.003	1.003
cnb1/cnb1 + *CNB1* (MCC85)	>3.12	>3.12	0.78	>12.5	0.78/0.04	0.19/3.12	≤0.04/≤0.01	≤0.04/0.78	0.17	0.15	0.009	0.03
CNB1-1/CNB1 (YAG237)	>3.12	>3.12	3.12	>12.5	1.56/0.39	1.56/6.52	>3.12/3.12	>3.12/>12.5	0.26	0.49	2.0	2.0
rbp1/rbp1 (YAG171)	>3.12	>3.12	3.12	>12.5	0.39/1.56	0.78/3.12	>3.12/3.12	>3.12/>12.5	0.56	0.25	2.0	2.0

[a] F, fenpropimorph; T, terbinafine.
[b] Combined MFCs, expressed as [drug 1]/[drug 2], are the minimum concentrations of drugs 1 and 2 that resulted in a fungicidal inhibition profile when the two drugs were used in combination.

to the wild type (Fig. 3). As a positive control, the *erg24/erg24* mutant was compared to a *C. albicans* Erg6-deficient (*erg6/erg6*) mutant strain previously found to be hypersensitive to CsA and FK506 (8, 15). The *ERG6* gene encodes an enzyme responsible for methylating zymosterol and converting it to ergosterol (Fig. 1). The *erg24/erg24* mutant was as sensitive to both calcineurin inhibitors as the *erg6/erg6* mutant (Fig. 3). On the basis of these findings, morpholines or other drugs that target Erg24 could potentially exhibit synergistic activity with calcineurin inhibitors.

Fenpropimorph and terbinafine are synergistic with calcineurin inhibitors against *C. krusei* and *C. glabrata*. Two non-*C. albicans Candida* species that are emerging pathogens in the immunosuppressed patient population, *C. glabrata* and *C. krusei*, are inherently resistant or rapidly acquire resistance to azoles. We have recently shown that these species are also largely insensitive to the synergism between azoles and calcineurin inhibitors that is potently fungicidal against the related pathogen *C. albicans* (8). We employed halo assays to determine whether the resistance profile of *C. glabrata* and *C. krusei* could be expanded to include terbinafine and fenpropimorph and found that these species demonstrate a species-specific sensitivity to these antifungal agents that is enhanced by the addition of calcineurin inhibitors (Fig. 4).

Two independently isolated *C. glabrata* strains (DUMC111.10 and MMRL361) were sensitive to the synergism between terbinafine and calcineurin inhibitors but showed limited sensitivity to fenpropimorph alone or in combination with calcineurin inhibitors (Fig. 4 and data not shown). Treatment with terbinafine alone was ineffective against *C. glabrata*, but clear

halos were produced when either FK506 or CsA was added to terbinafine (Fig. 4). *C. krusei*, however, showed sensitivity to both fenpropimorph and terbinafine in combination with FK506. Although fenpropimorph alone showed a striking growth inhibition halo around *C. krusei*, this halo was turbid compared to the clear halo produced by the combination of FK506 and fenpropimorph (Fig. 4). Terbinafine alone had a modest effect on the growth of *C. krusei*. Addition of CsA to either terbinafine or fenpropimorph only produced small, turbid halos indicating minimal interaction with terbinafine and potential antagonism with fenpropimorph in *C. krusei* (Fig. 4).

The nonimmunosuppressive FK506 analog L-685,818 enhances growth inhibition when combined with fenpropimorph or terbinafine. We tested the nonimmunosuppressive FK506 analog L-685,818 for the ability to enhance growth inhibition of *C. albicans* when combined with fenpropimorph or terbinafine. L-685,818 differs from FK506 on the exposed surface of the drug in complex with FKBP12. Substitution of an allyl group with an ethyl group at C_{21} and the presence of a hydroxyl group at C_{18} prevent the resulting human FKBP12–L-685,818 complex from binding mammalian calcineurin. In contrast, the yeast FKBP12–L-685,818 complex can bind and inhibit calcineurin because of additional protein-protein contacts provided by yeast FKBP12 that mitigate the deleterious effects of the C_{18} hydroxyl group. As a consequence, L-685,818 binds to yeast FKBP12 and inhibits fungal growth while sparing calcineurin-dependent immune function in the mammalian host (3, 30, 35). Neither FK506 nor L-685,818 alone inhibited the growth of wild-type *C. albicans* in vitro (Fig. 5). As demonstrated earlier, fenpropimorph or terbinafine alone produced
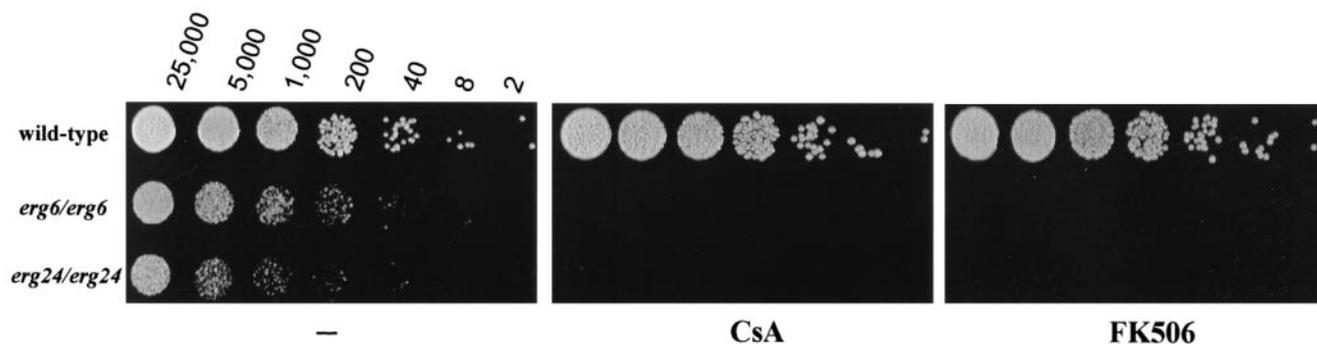

FIG. 3. *erg24/erg24* mutants are hypersensitive to CsA and FK506. *C. albicans* wild-type (SC5314), *erg6/erg6* homozygous mutant (KPC8), and *erg24/erg24* homozygous mutant (NJ51-2) strains were grown in YPD medium overnight. Fivefold serial dilutions of each strain were prepared and spotted onto YPD solid medium containing CsA (100 μg/ml), FK506 (1 μg/ml), or no drug (−). Cells were incubated for 24 h at 37°C.
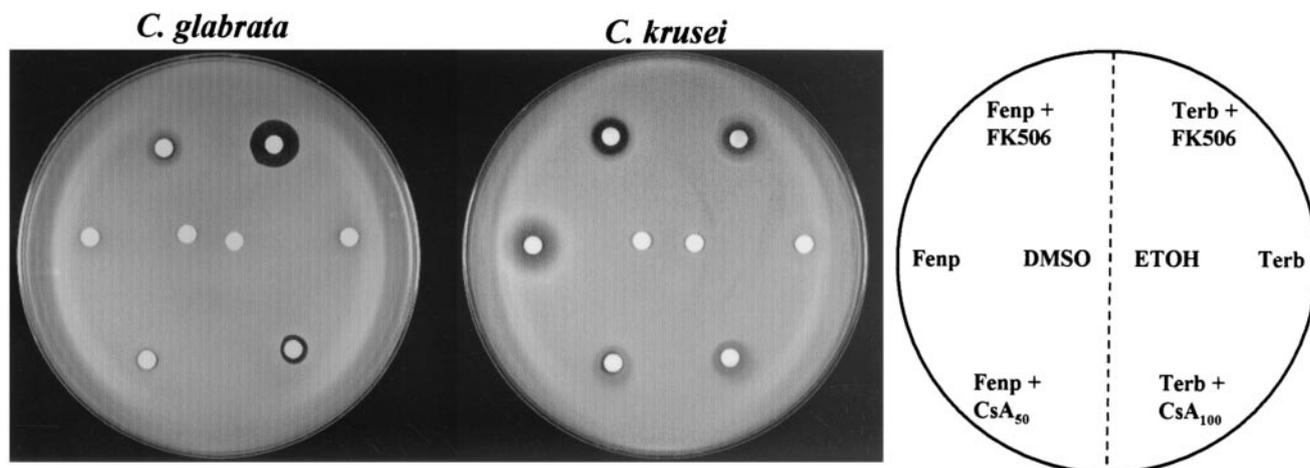
FIG. 4. *C. glabrata* and *C. krusei* isolates are sensitive to the synergism between calcineurin inhibitors and non-azole ergosterol biosynthesis inhibitors. *C. glabrata* shows sensitivity to the synergism between terbinafine (Terb) and CsA or FK506, whereas fenpropimorph (Fenp) only has a slight effect in combination with FK506. *C. krusei* is sensitive to both fenpropimorph and terbinafine in combination with FK506 but demonstrates little synergy with 50 (CsA_{50}) or 100 (CsA_{100}) µg of CsA. *C. glabrata* strain DUMC111.10 and *C. krusei* strain MMRL70 were grown overnight in YPD medium. The treatment of cells and the drug disk concentrations were the same as in the experiment described in Fig. 2. Cells were incubated for 24 to 48 h at 37°C. The calcineurin inhibitors alone did not inhibit the growth of these strains (data not shown). DMSO, dimethyl sulfoxide; ETOH, ethanol.

turbid halos of inhibition around wild-type *C. albicans* (Fig. 2 and 5). When wild-type cells were exposed to fenpropimorph or terbinafine in combination with L-685,818, enhanced halo clearing was observed (Fig. 5). Although the halo size and

clearing effect produced by the addition of L-685,818 was less dramatic than that of FK506, this analog exhibited in vitro potential as a nonimmunosuppressive alternative to FK506. L-685,818 and similar analogs may prove to be more practical for the clinical application of the drug synergy between calcineurin inhibitors and fenpropimorph or terbinafine.

DISCUSSION

Candida species cause serious systemic disease in the immunocompromised patient population. Current drug treatments are largely effective, but resistant strains and intrinsically resistant species are emerging. Our findings suggest options for expanding the utility of existing antifungal drug classes. In *C. albicans*, it has previously been shown that calcineurin activity is required for survival during membrane perturbation with azoles (8). Therefore, cells exposed to calcineurin inhibitors exhibit increased sensitivity to antifungals that target cell membrane integrity. Here, we explored the synergism between calcineurin inhibitors and fenpropimorph, which is used exclusively in agriculture as a fungicide, and terbinafine, which is used clinically to treat dermatophyte infections.

We demonstrated that fenpropimorph and terbinafine exhibit synergistic fungicidal activity with calcineurin inhibitors against *C. albicans*, *C. glabrata*, and *C. krusei* in vitro. The degree of this drug synergy varied among the different *Candida* species, but we observed significant enhancement of growth inhibition with at least one drug combination for each species. In addition to our laboratory wild-type *C. albicans* strain, six recently obtained *C. albicans* clinical isolates were tested by halo assay for evidence of synergism (data not shown). The six strains varied in the extent to which they were inhibited by the different drug combinations, but all demonstrated synergistic inhibition when exposed to calcineurin inhibitors and either
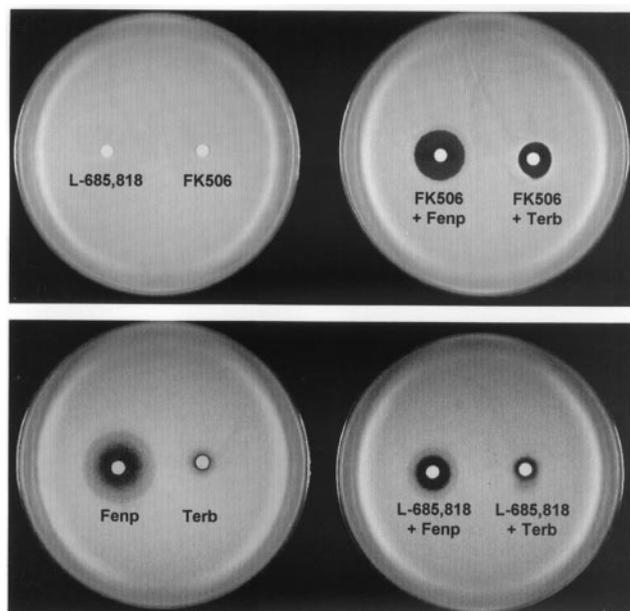

FIG. 5. L-685,818 demonstrates synergy with fenpropimorph (Fenp) and terbinafine (Terb) against wild-type *C. albicans*. Although less potent than FK506, the nonimmunosuppressive FK506 analog L-685,818 demonstrates drug synergy with fenpropimorph and terbinafine against wild-type *C. albicans* in halo assays. Wild-type *C. albicans* (SC5314) cells were grown overnight in YPD medium and treated the same as in the experiment described in Fig. 2. As indicated, disks containing 2 µg of FK506, 4 µg of L-685,818, 2 µg of fenpropimorph, and 10 µg of terbinafine were placed over the solidified top agar. Cells were incubated for 24 to 48 h at 37°C.

terbinafine or fenpropimorph. Although there was an overall difference in the inhibitory profiles of terbinafine and fenpropimorph, both drugs exhibited improved antifungal activity when combined with CsA or FK506.

Our results suggest that antifungal drug classes previously relegated to agricultural purposes or reserved for treatment of superficial infections might be resurrected for treatment of human systemic infections by the prospect of enhanced efficacy through combination therapy. While variability may occur among the different *Candida* strains and species in response to these drug combinations, it is exciting that the fungicidal potential of normally fungistatic drugs can be revealed. In a study by Marchetti and colleagues, the combination of fluconazole and CsA was found to be effective in treating aortic valve endocarditis in a rat model of *C. albicans* infection, thus illustrating the potential therapeutic benefit of drug synergy (22). Such findings serve as evidence of the utility of a combinatorial treatment approach to combating fungal disease. Furthermore, recent studies have revealed the efficacy of azoles in treating cutaneous leishmaniasis (1). If the synergistic relationship of combinatorial treatment with calcineurin inhibitors exhibited in pathogenic yeast is also applicable to parasites, many antifungal agents may find even broader therapeutic applications.

Azoles have been a predominant therapy for *Candida* infections for more than 20 years, but given the emergence of azole-resistant *C. albicans* strains and *Candida* species, there is a need for more antifungal drug options. Terbinafine acts upstream of the azole target Erg11 and fenpropimorph acts downstream of Erg11 in the ergosterol biosynthesis pathway (Fig. 1). Like fluconazole, terbinafine and fenpropimorph have demonstrated synergy with both calcineurin inhibitors. Our in vitro studies have also shown that the combination of terbinafine or fenpropimorph and FK506 can significantly inhibit both *C. glabrata* and *C. krusei*. It is possible that this synergism may provide relief from the nephrotoxicity that results from amphotericin B therapy, the typical alternative to using azoles to treat these resistant species.

Since Erg2 is the primary target of morpholine drugs, our analysis of the inhibition profile of fenpropimorph in combination with FK506 or CsA established Erg2 as a good target for the development of antifungal drugs that participate in this synergy. However, we sought to investigate the practicality of specifically targeting Erg24, the secondary target of fenpropimorph. We independently confirmed the findings of Jia et al. (16) that *erg24/erg24* mutants are hypersensitive to terbinafine and surprisingly found that these mutants were also rather sensitive to fenpropimorph compared to the wild-type strain (data not shown). Erg2 functions downstream of Erg24. Thus, if Erg24 is mutated or inhibited, why does blocking Erg2 activity have any effect on *erg24/erg24* mutants? Although the ergosterol biosynthetic pathway is commonly depicted as a linear set of reactions (Fig. 1), this pathway is an intricate network of enzymes and enzyme product interactions. Either the Erg2 enzyme functions in the absence of Erg24, or fenpropimorph has additional targets other than Erg2 and Erg24. Therefore, inhibiting the *ERG24* gene product may also increase the sensitivity of *C. albicans* to a variety of ergosterol biosynthesis inhibitors. These results serve as evidence that the Erg24 enzyme is a practical target for antifungal drug combinations. With amorolfine only available as topical therapy and

no other morpholines in clinical use, our data demonstrate the therapeutic potential of the development of systemically active morpholines as an alternative to azoles and amphotericin B therapy.

Nonimmunosuppressive analogs of FK506 and CsA also exhibit drug synergy with ergosterol inhibitors and could extend the utility and efficacy of this therapeutic approach (8). The immunocompromised population, including premature neonates, diabetics, HIV-positive patients, and individuals receiving organ transplants or chemotherapy, along with immunocompetent individuals with urinary catheters, indwelling vascular devices, or access sites for hemodialysis are particularly at risk for candidemia and subsequent systemic infection (5, 21, 29, 33, 36). Therefore, administration of calcineurin inhibitors to these already immunosuppressed or hospitalized immunocompetent patients may actually compromise their recovery. The nonimmunosuppressive FK506 analog L-685,818 exploits subtle structural differences between the yeast and mammalian FKBP12 and calcineurin molecules, resulting in growth inhibition of yeast cells and maintenance of host immune function (35). Our experiments demonstrate that L-685,818 enhances growth inhibition of *C. albicans* when combined with fenpropimorph or terbinafine in in vitro halo assays (Fig. 5). Increased amounts of this analog may be required to mimic the potent inhibitory effect of FK506 against wild-type *C. albicans*. However, L-685,818 shows great promise as a nonimmunosuppressive alternative to drug combinations involving FK506.

Immunocompromised patients are substantially more susceptible to opportunistic fungal infections, but our findings also have important implications for the many immunocompetent adult and pediatric patients with nondisseminated *Candida* infections. *C. albicans* is found in the natural flora of the mouth, vaginal tract, and gut. Mucocutaneous candidiasis can result in significant morbidity for burn victims, women with recurring vaginitis, bedridden individuals, postoperative surgical patients, and neonates (5, 12, 21). A pulsed therapy using FK506, CsA, or analogs of either in which a patient is transiently immunosuppressed while receiving an appropriate ergosterol inhibitor might be a practical therapeutic approach for these individuals. Long-term adverse health risks would be less likely given the patients' immunocompetency and the brevity of immunosuppression. Additionally, with the advent of topical FK506 for treatment of atopic dermatitis (27), therapeutic options for superficial fungal infections might be expanded and improved. For example, the topical forms of terbinafine and amorolfine may show improved efficacy against mucocutaneous candidiasis or dermatophytic infections when combined with topical FK506 or CsA.

As drug-resistant microorganisms continue to emerge and the number of patients susceptible to these infections rises, the demand for more effective antimicrobial agents multiplies. In order to combat the growing problem, we must be innovative in our approaches to drug design and vigilant in monitoring current therapies whose properties can be exploited for novel therapeutic purposes. This concept was demonstrated in recent studies in which an antiprotease-based highly active antiretroviral therapy regimen exhibited an anticandidal effect in HIV-positive patients (26). It was also exhibited in a clinical study of immunosuppressed patients infected with *Cryptococcus neofor-*

mans, where the use of FK506 as a primary immunosuppressant appeared to protect against *C. neoformans* infection of the central nervous system (13). These examples illustrate the benefit of evaluating current drugs for the ability to serve in new or alternative clinical roles. Ultimately, improvement in drug design and available therapy options for fungal infections is of key importance now and in the future. Most exciting, however, is the potential to tailor antifungal drug regimens to the specific requirements of individual patient populations.

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