Calcineurin Promotes Infection of the Cornea by *Candida albicans* and can be Targeted to Enhance Fluconazole Therapy

Running Title: Calcineurin Targeted Treatment of *C. albicans* Keratomycosis

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Abstract

In an established *Candida albicans* murine keratitis model, combination therapy with ophthalmic preparations of fluconazole and cyclosporine A (CsA) demonstrated *in vivo* drug synergy and effectively resolved wild-type *C. albicans* infection more rapidly than monotherapy with either drug. Calcineurin, the target of CsA, was also found to contribute to pathogenicity.
Fungal infections of the cornea (fungal keratitis or keratomycosis) cause significant morbidity and can progress to endophthalmitis, with subsequent risk for visual loss (6). In temperate climates, *Candida albicans* is the most frequent etiology of keratitis caused by yeast-like fungi (6, 13, 15). *C. albicans* keratomycosis is associated with pre-existing ocular or systemic conditions, such as epithelial defects, contact lens use, poor eyelid closure, neurotrophic cornea, diabetes, immunosuppression, and/or following corneal transplantation (6, 15). Clinical management of these infections is largely dependent upon antifungal drug efficacy and penetration into corneal tissue (7, 9, 14, 15). Fungistatic azole drugs that target ergosterol biosynthesis and perturb cell membrane integrity are relatively successful in managing a variety of *Candida* disease manifestations (11). However, *Candida* has evolved sophisticated azole drug-resistance mechanisms, which complicate disease management (16-18). Consequently, novel approaches need to be employed to expand antifungal treatment options.

In *C. albicans*, calcineurin, a serine/threonine phosphatase is required for survival in the presence of azoles and for virulence in a murine disseminated candidiasis model (1, 4, 10, 12). We previously demonstrated that azoles act synergistically with the calcineurin inhibitors FK506 or CsA to inhibit *C. albicans in vitro* (4). Here, we explore the potential of applying this drug synergy to a murine model of *C. albicans* keratomycosis (19). We discovered that the efficacy of topical fluconazole therapy was enhanced by genetic or pharmacological inhibition of calcineurin.

BALB/c mice were immunosuppressed with methylprednisolone (100 mg/kg) five days before, one day before, and one day after inoculation to rapidly establish and maintain infections (19). An intramuscular injection of a ketamine (10 mg/ml)/xylazine (1 mg/ml) mixture was given, and the right cornea of each animal was scarred with a 28.5 gauge needle. A five-
microliter suspension containing $10^6$ *C. albicans* wild-type (SC5314) (5) cells was evenly
distributed over the scarred cornea. A disease grading scale from zero (no disease) to four
(severe disease) was established by an ophthalmologist who was blinded to the infecting *C.
*albicans* strain and drug treatment (Figure 1). Animals were randomly assigned to treatment
groups, and treatment was begun when at least one animal in each group achieved a grade 3 or
higher infection (see Figure 1). Treated animals received six doses of 2% CsA (10 µg/dose),
0.2% fluconazole (1 µg/dose), or 0.2% fluconazole (1 µg/dose) + 2% CsA (10 µg/dose) over a
four-day period. For combination therapy, drugs were administered in succession with at least
two minutes between doses. Corneas were observed at 1.6X magnification with a Zeiss bio-
microscope slit-lamp and scored daily. Results from two independent experiments were
combined and analyzed.

All treatment groups exhibited comparable median disease scores prior to treatment (data
not shown). The median disease scores of animals treated with combination therapy declined
more rapidly than all other treatment groups (p<0.0001) (Figure 2). Compared to untreated
animals, combination therapy significantly reduced median disease scores in two days
(p<0.0001), while fluconazole monotherapy required three days (p = 0.0085) (Figure 2). Thus,
combination therapy improved corneal infections more rapidly than fluconazole monotherapy.
The daily change in median disease scores for untreated and CsA-treated animals did not differ
(p> 0.2) (Figure 2). When only grade 4 infections were considered, the disease resolution pattern
of animals receiving combination therapy resembled the collective group (data not shown).
Therefore, combination treatment was effective regardless of disease severity.

Animals were also infected with the *C. albicans cnb1/cnb1* calcineurin mutant strain
(JRB64) (3) or the *cnb1/cnb1+CNB1* complemented calcineurin mutant strain (MCC85) (4).
The calcineurin mutant is avirulent in murine disseminated candidiasis models (3, 10), and none of the cnb1/cnb1 mutant infections reached grade 3 (see Figure 1). By day 2, the median disease score of untreated cnb1/cnb1-infected animals was significantly lower than the wild-type (p = 0.002) and the CNB1-complemented mutant strains (p = 0.006) (Figure 3A). Thus, the absence of functional calcineurin diminished C. albicans pathogenicity and accelerated disease resolution in this infection model.

Fluconazole therapy caused the median disease scores of all infections to decline more rapidly than their untreated counterparts (Figures 3). By day 2, the median disease score of fluconazole-treated cnb1/cnb1 infections was significantly lower than wild-type infections (p<0.004) (Figure 3B). The disease profile of fluconazole-treated cnb1/cnb1 and cnb1/cnb1+CNB1 mutant infections were comparable (p ≥ 0.07) (Figure 3B). Because the complemented mutant strain only carries one copy of the wild-type CNB1 gene, it may exhibit partial phenotypic complementation. Therefore, reduction in calcineurin activity substantially increased the fluconazole susceptibility of the cnb1/cnb1+CNB1 mutant strain, while complete loss of calcineurin reduced the infectivity of the cnb1/cnb1 mutants in this corneal infection model (Figure 3). In addition, the median disease score for cnb1/cnb1 mutant infections reached zero one day sooner with fluconazole treatment (Figure 3), likely owing to the fluconazole hypersensitivity previously demonstrated by calcineurin mutants (1, 3, 4, 12). Thus, the absence of functional calcineurin diminished C. albicans pathogenicity and accelerated disease resolution. This observation provided additional support that a calcineurin-dependent mechanism was responsible for the enhanced clearing observed when wild-type infections were treated with the fluconazole + CsA combination (Figure 2).
This murine fungal keratitis model provided a unique setting to explore *in vivo* drug efficacy against *C. albicans* infection. Recent studies have demonstrated that specific host conditions can dictate strain infectivity and antifungal drug efficacy. Although *C. albicans* calcineurin mutants are avirulent in a murine model of disseminated candidiasis and demonstrate reduced pathogenicity in the cornea, as shown here, these mutants are not attenuated in murine vaginal or pulmonary infection models (1-3, 12). Thus, the role calcineurin plays in *C. albicans* pathogenicity is dependent on host niche. Despite being a fungistatic drug, fluconazole exhibited fungicidal activity against *Candida* species under *in vitro* conditions that simulate the vaginal microenvironment (8). These findings demonstrate *in vitro* studies can be applied to specific *in vivo* conditions to predict *Candida* susceptibility to certain antifungal therapies. Our findings may have broad implications given that fluconazole and CsA are already used clinically and may be applicable to a wide range of fungi.

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References


Figure legends

2 Figure 1. Ocular grading scale used to determine disease score. The right eye of immunosuppressed animals was scarred and inoculated with *C. albicans* cells. Infections were photographed using a 35 mm camera mounted to a Zeiss bio-microscope slit-lamp. White dotted lines designate the area of focal lesions. Photographs and corresponding clinical criteria describe the degree of infection. Scores that deviated from the whole-number grading scale were assigned fractional values in 0.25 increments in reference to the nearest whole number score. Photographs represent criteria for grading scale, and do not reflect the disease progression in a single animal.

10 Figure 2. The fluconazole-CsA combination rapidly clears wild-type infections. By day 2, the change from baseline median disease score for animals treated with combination therapy was significantly different from untreated animals (p<0.0001), while fluconazole-treated animals required three days of treatment to exhibit a significant difference from untreated animals (p=0.0085). Descriptive statistics of the median (plus 1st and 3rd quartiles) disease scores were obtained for each day based on drug treatment using two-sided tests. The significance of the median changes from baseline was assessed within groups using the Wilcoxon signed rank test. The differences among groups with respect to response at each time point were assessed using the Kruskal-Wallis test for medians. Pair-wise comparisons between groups were assessed using the Wilcoxon Rank sum test for medians. Boniferonni’s adjusted alpha levels were applied to sets of pair-wise tests. Analyses were carried out using the Statistical Analysis System (SAS) and graphs were created with PRISM 4.0 software (GraphPad Software, San Diego, California).

22 Animals were untreated (n=28) or treated with CsA (n=13), fluconazole (n=17), or both drugs (n=22). Values in parentheses represent the 1st and 3rd quartiles, respectively. Intersecting data
points with only one label indicate identical values. “*” designates p-value ≤ 0.008 when compared to untreated animals.

**Figure 3. Calcineurin promotes C. albicans pathogenicity in the cornea.** (A) The disease severity of cnb1/cnb1 mutant infections differed from the wild-type strain. By day 2, the median disease score of calcineurin mutant infections was significantly less than the wild-type and the complemented calcineurin mutant (cnb1/cnb1+CNB1) strains (p = 0.002 and p = 0.006, respectively). (B) Fluconazole treatment enhanced disease resolution for all strains. By day 2, cnb1/cnb1 mutant infections exhibited a median disease score of zero, and differed significantly from wild-type infections (p = 0.004). Fluconazole treatment lowered the median disease scores of cnb1/cnb1+CNB1 mutant infections to zero by day 3. The wild-type and complemented mutant infections, which persisted with mock treatment (see Figure 3A), declined to zero with fluconazole therapy by day 3 or 4. Descriptive statistics of the median disease scores were obtained as in Figure 2. Animals were infected with wild-type (n=23), cnb1/cnb1 (n=15), or cnb1/cnb1+CNB1 (n=15) strains. Values in parentheses represent the 1st and 3rd quartiles, respectively. Intersecting data points with only one label indicate identical values. “**” designates p-value ≤ 0.017 when compared to wild-type infections. “***” designates p-value ≤ 0.017 when compared to wild-type and cnb1/cnb1+CNB1 infections.
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