

**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

Chiatogu Onyewu³, Natalie A. Afshari^{4*}, and Joseph Heitman^{1-3*}

Departments of Molecular Genetics and Microbiology,¹ Medicine,² and
Pharmacology and Cancer Biology,³ and Duke University Eye Center,⁴

Duke University Medical Center, Durham, North Carolina 27710

Abstract: 50 words

Text: 996 words

*Correspondence to either:

Dr. Afshari at Department of Ophthalmology

Wadsworth Bldg, Box 3802, Erwin Road

Duke University Eye Center, Durham, NC 27710

Telephone: (919) 684-3799 Fax: (919) 681-7661

Email: afsha003@mc.duke.edu

OR

Dr. Heitman at Department of Molecular Genetic and Microbiology

322 Carl Building, Box 3546, Research Drive

Duke University Medical Center, Durham, NC 27710

Telephone: (919) 684-2824 Fax: (919) 684-5458

Email: heitm001@duke.edu

Abstract

2 In an established *Candida albicans* murine keratitis model, combination therapy with
ophthalmic preparations of fluconazole and cyclosporine A (CsA) demonstrated *in vivo* drug
4 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.

6

ACCEPTED

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-

1 microliter suspension containing 10^6 *C. albicans* wild-type (SC5314) (5) cells was evenly
2 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.*
4 *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
6 higher infection (see Figure 1). Treated animals received six doses of 2% CsA (10 μ g/dose),
0.2% fluconazole (1 μ g/dose), or 0.2% fluconazole (1 μ g/dose) + 2% CsA (10 μ g/dose) over a
8 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-
10 microscope slit-lamp and scored daily. Results from two independent experiments were
combined and analyzed.

12 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
14 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
16 ($p < 0.0001$), while fluconazole monotherapy required three days ($p = 0.0085$) (Figure 2). Thus,
combination therapy improved corneal infections more rapidly than fluconazole monotherapy.
18 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
20 of animals receiving combination therapy resembled the collective group (data not shown).
Therefore, combination treatment was effective regardless of disease severity.

22 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).

1 The calcineurin mutant is avirulent in murine disseminated candidiasis models (3, 10), and none
2 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 ($p =$
4 0.002) and the *CNBI*-complemented mutant strains ($p = 0.006$) (Figure 3A). Thus, the absence of
functional calcineurin diminished *C. albicans* pathogenicity and accelerated disease resolution in
6 this infection model.

Fluconazole therapy caused the median disease scores of all infections to decline more
8 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
10 ($p < 0.004$) (Figure 3B). The disease profile of fluconazole-treated *cnb1/cnb1* and
cnb1/cnb1+*CNBI* mutant infections were comparable ($p \geq 0.07$) (Figure 3B). Because the
12 complemented mutant strain only carries one copy of the wild-type *CNBI* gene, it may exhibit
partial phenotypic complementation. Therefore, reduction in calcineurin activity substantially
14 increased the fluconazole susceptibility of the *cnb1/cnb1*+*CNBI* mutant strain, while complete
loss of calcineurin reduced the infectivity of the *cnb1/cnb1* mutants in this corneal infection
16 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
18 hypersensitivity previously demonstrated by calcineurin mutants (1, 3, 4, 12). Thus, the absence
of functional calcineurin diminished *C. albicans* pathogenicity and accelerated disease
20 resolution. This observation provided additional support that a calcineurin-dependent
mechanism was responsible for the enhanced clearing observed when wild-type infections were
22 treated with the fluconazole + CsA combination (Figure 2).

2 This murine fungal keratitis model provided a unique setting to explore *in vivo* drug
3 efficacy against *C. albicans* infection. Recent studies have demonstrated that specific host
4 conditions can dictate strain infectivity and antifungal drug efficacy. Although *C. albicans*
5 calcineurin mutants are avirulent in a murine model of disseminated candidiasis and demonstrate
6 reduced pathogenicity in the cornea, as shown here, these mutants are not attenuated in murine
7 vaginal or pulmonary infection models (1-3, 12). Thus, the role calcineurin plays in *C. albicans*
8 pathogenicity is dependent on host niche. Despite being a fungistatic drug, fluconazole exhibited
9 fungicidal activity against *Candida* species under *in vitro* conditions that simulate the vaginal
10 microenvironment (8). These findings demonstrate *in vitro* studies can be applied to specific *in*
11 *vivo* conditions to predict *Candida* susceptibility to certain antifungal therapies. Our findings
12 may have broad implications given that fluconazole and CsA are already used clinically and may
13 be applicable to a wide range of fungi.

14
15 We are grateful to Sandra Stinnett for statistical data analysis and to the Duke University
16 Medical Center Pharmacy for compounding drug formulations. This study was supported by
17 RO1 grants AI42159 and AI050438 from the NIH/NIAID to Joseph Heitman and in part by NIH
18 Supplement AI050438-04S1 from the NIAID to Chiatogu Onyewu and the Research to Prevent
19 Blindness Award to Natalie Afshari. Chiatogu Onyewu is a UNCF/Merck Graduate Student
20 Fellow.

References

- 2 1. **Bader, T., B. Bodendorfer, K. Schroppel, and J. Morschhauser.** 2003. Calcineurin is
4 essential for virulence in *Candida albicans*. *Infect. Immun.* **71**:5344-5354.
- 6 2. **Bader, T., K. Schroppel, S. Bentink, N. Agabian, G. Kohler, and J. Morschhauser.**
8 2006. Role of calcineurin in stress resistance, morphogenesis, and virulence of a *Candida*
10 *albicans* wild-type strain. *Infect. Immun.* **74**:4366-4369.
- 12 3. **Blankenship, J. R., F. L. Wormley, M. K. Boyce, W. A. Schell, S. G. Filler, J. R.**
14 **Perfect, and J. Heitman.** 2003. Calcineurin is essential for *Candida albicans* survival in
16 serum and virulence. *Eukaryot. Cell* **2**:422-430.
- 18 4. **Cruz, M. C., A. L. Goldstein, J. R. Blankenship, M. Del Poeta, D. Davis, M. E.**
20 **Cardenas, J. R. Perfect, J. H. McCusker, and J. Heitman.** 2002. Calcineurin is
22 essential for survival during membrane stress in *Candida albicans*. *EMBO J.* **21**:546-559.
- 24 5. **Gillum, A. M., E. Y. Tsay, and D. R. Kirsch.** 1984. Isolation of the *Candida albicans*
26 gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3*
28 and *E. coli* *pyrF* mutations. *Mol. Gen. Genet.* **198**:179-182.
- 30 6. **Klotz, S. A., C. C. Penn, G. J. Negvesky, and S. I. Butrus.** 2000. Fungal and parasitic
32 infections of the eye. *Clin. Microbiol. Rev.* **13**:662-685.
- 34 7. **Manzouri, B., G. C. Vafidis, and R. K. Wyse.** 2001. Pharmacotherapy of fungal eye
36 infections. *Expert. Opin. Pharmacother.* **2**:1849-1857.
- 38 8. **Moosa, M. Y., J. D. Sobel, H. Elhalis, W. Du, and R. A. Akins.** 2004. Fungicidal
40 activity of fluconazole against *Candida albicans* in a synthetic vagina-simulative
42 medium. *Antimicrob. Agents. Chemother.* **48**:161-167.
- 44 9. **O'Day, D. M.** 1996. Fungal keratitis. In J. S. Pepose, G. N. Holland, K. R. Wilhemus,
(ed.), *Ocular infections and immunity*. Mosby, St. Louis, Missouri.
10. **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.
11. **Rex, J. H., T. J. Walsh, J. D. Sobel, S. G. Filler, P. G. Pappas, W. E. Dismukes, and**
J. E. Edwards. 2000. Practice guidelines for the treatment of candidiasis. Infectious
Diseases Society of America. *Clin. Infect. Dis.* **30**:662-678.
12. **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.

- 2 13. **Tanure, M. A., E. J. Cohen, S. Sudesh, C. J. Rapuano, and P. R. Laibson.** 2000.
4 Spectrum of fungal keratitis at Wills Eye Hospital, Philadelphia, Pennsylvania. *Cornea*
6 **19:307-312.**
- 8 14. **Thomas, P. A.** 2003. Current perspectives on ophthalmic mycoses. *Clin. Microbiol. Rev.*
10 **16:730-797.**
- 12 15. **Thomas, P. A.** 2003. Fungal infections of the cornea. *Eye* **17:852-856.**
- 14 16. **White, T. C.** 1998. Antifungal drug resistance in *Candida albicans*. *ASM News* **63:427-**
16 **433.**
- 18 17. **White, T. C., S. Holleman, F. Dy, L. F. Mirels, and D. A. Stevens.** 2002. Resistance
20 mechanisms in clinical isolates of *Candida albicans*. *Antimicrob. Agents. Chemother.*
22 **46:1704-1713.**
- 18 18. **White, T. C., K. A. Marr, and R. A. Bowden.** 1998. Clinical, cellular, and molecular
20 factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11:382-402.**
- 22 19. **Wu, T. G., K. R. Wilhelmus, and B. M. Mitchell.** 2003. Experimental keratomycosis in
a mouse model. *Invest. Ophthalmol. Vis. Sci.* **44:210-216.**

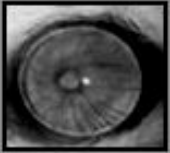


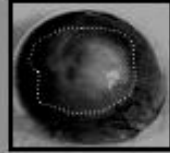
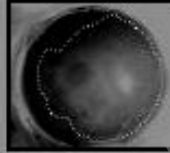
Figure legends

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
4 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
6 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
8 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
12 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
14 ($p = 0.0085$). 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
16 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
18 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
20 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.

Clinical Presentation					
Disease score	0	1	2	3	4
Clinical Description	Clear cornea	Slightly cloudy cornea	Cloudy cornea; iris & pupil visible	Cloudy cornea; Opacity not yet uniform	Nearly uniform opacity of cornea
% Opacity	0%	1%-25%	26%-50%	51%-75%	75%-95%
Degree of Disease	No disease	Mild	Moderate	Moderate to Severe	Severe

