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Enhanced pathogenicity of *Candida albicans* pre-treated with subinhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis

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***Objectives*: To investigate the relative pathogenicity of *Candida albicans* treated with subinhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis. Previous studies indicate that these cells secrete 10 times more farnesol than do untreated cells. In our usage, subinhibitory means a concentration which causes a prominent decrease in turbidity but still allows some cell growth.**

***Methods*: *C. albicans* A72 cells were grown overnight in 0–5.0 μM fluconazole, washed, and inoculated in mice by tail vein injection. Groups of 15 or 16 mice were injected with 1.3×10^6 cells and mortality was recorded for 7 days post-inoculation. The levels of farnesol in control and treated *C. albicans* were determined by GC/MS.**

***Results*: The MIC_{50} for strain A72 was 0.125 mg/L (0.4 μM). Mice administered *C. albicans* pre-treated with 0.5 to 1.0 μM fluconazole died 2.5 to 4 days earlier and had 2 to 4 times higher mortality rates than mice given untreated *C. albicans*. Fluconazole (0.5 to 1.0 μM) pre-treated cells were 4.2 to 8.5 times more lethal ($P < 0.001$) than untreated cells. The extracellular, membrane bound, and intracellular farnesol concentrations of cells pre-treated with 1.0 μM fluconazole were 12-, 2- and 6-times those of untreated cells.**

***Conclusions*: The effects of fluconazole on *C. albicans* are very concentration-dependent. The enhanced pathogenicity of fluconazole pre-treated *C. albicans* in mice should be relevant to the therapeutic and prophylactic use of fluconazole. Further research is needed to explore whether farnesol production by *C. albicans* is a virulence factor.**

Keywords: farnesol, virulence, candidaemia

Introduction

The dimorphic fungus *Candida albicans* is one of the most common fungal pathogens of humans. Candidiasis associated with *C. albicans* is primarily an opportunistic infection of immunocompromised patients.[1] Additionally, infections with *C. albicans* account for 78% of fungal nosocomial infections, representing over 10% of all nosocomial infections in the USA.[1] The annual cost of treating candidiasis in the United States has been estimated at one billion dollars.[2] Fluconazole is the most common antifungal drug prescribed by practitioners and it has been the drug of choice for mucosal and disseminated candidiasis.[3] Fluconazole is also a recommended prophylactic drug to prevent fungal invasion in leucopenic patients[3,4] and to avoid opportunistic and nosocomial candidiasis in AIDS patients.[4] The mode of action of fluconazole involves inhibition of lanosterol 14α demethylase in ergosterol biosynthesis.[5] Previous work in our laboratory has shown that *C. albicans* excretes farnesol, a C_{15}-isoprenoid, and that exogenous farnesol prevents mycelial development in both a growth morphology assay and a differentiation assay.[6] Thus, in the presence of farnesol, *C. albicans* appears as actively budding yeasts without influencing cellular growth rates.[6] *C. albicans* synthesizes farnesol from farnesyl pyrophosphate (FPP).[7] Because FPP is the biosynthetic precursor of both farnesol[7] and ergosterol,[5] we hypothesized that drugs blocking the sterol biosynthetic pathway after FPP might lead to the accumulation of FPP, which, in turn, could

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lead to enhanced farnesol production. This hypothesis proved to be correct; treatment of *C. albicans* with subinhibitory concentrations of either zaragozic acid B or fluconazole resulted in 8-fold[7] and 10-fold[8] increases, respectively, in the amount of farnesol produced by *C. albicans*. A similar response was observed when *C. albicans* was treated with three other azole antifungals.[8]

Since all of our previous work had been done *in vitro*, we were interested in extending the effect of fluconazole treatment and farnesol production to a mouse model of disseminated infection. We hypothesized that by virtue of its effect on farnesol biosynthesis, treatment of *C. albicans* with subinhibitory concentrations of fluconazole before parenteral administration might affect the outcome of the infection. The concept that growth conditions influence *C. albicans* virulence in the host has precedent. Antley and Hazen[9] showed that cells grown at room temperature are significantly more virulent than cells grown at 37°C. Here we show that mice intravenously administered *C. albicans* pre-treated with subinhibitory concentrations of fluconazole died significantly earlier and had higher mortality rates than mice in control groups administered untreated *C. albicans*.

Materials and methods

C. albicans strain, growth conditions and fluconazole treatment

C. albicans strain A72 was kindly provided by Patrick A. Sullivan, University of Otago, New Zealand. It is available as ATCC MYA-2430, Rockville, MD, USA. It is a well characterized farnesol-producing and farnesol-responsive strain that has been used in previous *in vitro* studies.[6–8] In this regard, it is typical of other laboratory and clinical isolates of *C. albicans*.[8] The fluconazole MIC values for strain A72 were determined by standard NCCLS M27 protocol[10] by the Fungus Testing Laboratory (San Antonio, TX, USA). For challenge, *C. albicans* cells were grown overnight in 50 mL of modified glucose/salts/biotin (mGSB) medium at 30°C with aeration as previously described.[6] For fluconazole pre-treatment, *C. albicans* cells were grown overnight (16 h) in 50 mL of mGSB medium supplemented with fluconazole (gift from Pfizer Pharmaceuticals, Sandwich, UK) at 0, 0.25, 0.5, 1.0 or 5.0 μM. All of the cells had a typical yeast morphology. Control and treated cells were harvested by centrifugation at 4750 **g** for 10 min, washed once with 50 mL of sterile, non-pyrogenic normal saline (Abbott Laboratories, North Chicago, IL, USA) and resuspended in 10 mL of saline before measuring cell numbers with a Petroff-Hausser counting chamber. These cell suspensions were adjusted to the final concentration in non-pyrogenic sterile saline for parenteral administration. Viability was assessed by determining the number of cfu per mL of dilutions of 0.1 mL samples of treated and untreated cell suspensions plated in triplicate on BiGGY agar (BBL, Becton Dickinson, Sparks, MD, USA),[11] and counting after 48 h of incubation at 30°C.

Determination of farnesol in C. albicans fractions

C. albicans A72 was grown for 24 h at 25°C or 30°C in 100 mL of mGSB medium[6] with and without 1.0 μM fluconazole. The cells were harvested by centrifugation at 4100 **g** for 5 min at 25°C. The pellets were washed once and resuspended in distilled water. Dry weights were determined using pre-weighed watch glasses and baking the samples in an oven at 180°C for 1 h. Extracellular farnesol was analysed by gas chromatography/mass spectroscopy (GC/MS) as previously described.[6,8] To determine intracellular farnesol, the cell pellets were mixed with 5 g of 0.5 mm glass beads and the cells were disrupted by vortexing for 3 min. Cell breakage was greater than 95% as determined by microscopic examination. Cell debris was collected by centrifugation at 4100 **g** for 5 min at 25°C and the supernatants with the intracellular farnesol were treated and analysed as referenced for the extracellular fraction. To determine the amount of farnesol in the cellular membranes, the cell debris from above was extracted directly into 100% ethyl acetate and vortexed for 3 min. The samples were centrifuged at 11 400 **g** for 20 min at 25°C and the resulting supernatants (ethyl acetate) were analysed as described previously.[6,8] Saponification of the remaining, ethyl acetate extracted membrane fractions gave no further release of farnesol.

Mice and mouse inoculation

Outbred, 4- to 6-week-old (20–25 g), CF-1® female mice (Charles River Laboratories, Wilmington, MA, USA) were randomly allocated to groups of five or six mice in polycarbonate cages with stainless steel wire tops. After a 7- to 10-day acclimatization period, groups of mice were inoculated with 0.1 mL containing the appropriate numbers of *C. albicans* by intravenous (iv) injection in the lateral caudal tail vein using a 27 gauge needle. Clinical signs of illness were monitored four times daily. The experimental protocol, housing, and care of the mice were in accordance with approved guidelines of the University of Nebraska–Lincoln Institutional Animal Care and Use Committee.

Experimental design

The LD_{50} value for *C. albicans* A72 at day three post-inoculation (PI) was 1.0×10^7 cells. Therefore to increase the likelihood of observing a difference in lethality between control and treated *C. albicans*, mice were inoculated with a sub-lethal challenge consisting of 1.3×10^6 cells, in all subsequent experiments. Initial experiments (Trial 1) examined the effect of pre-treatment of *C. albicans* with 1.0 μM fluconazole on mouse survival post-challenge. Groups of 15 mice each were inoculated with either untreated *C. albicans* or *C. albicans* pre-treated with fluconazole and clinical signs were monitored for 7 days PI. Additional experiments (Trial 2) examined the effect of pre-treatment of *C. albicans* with increasing concentrations of fluconazole, 0, 0.25, 0.5, 1.0 and 5.0 μM, on mouse survival post-challenge. In this experiment, groups of 16 mice each were inoculated with sub-lethal numbers of either control untreated *C. albicans* or *C. albicans* pre-treated with fluconazole and monitored for 7 days PI. In each experiment, a group of five mice inoculated with saline without *C. albicans* served as a negative control.

Statistical analysis

The main parameter measured was the time (h) from iv inoculation to death or euthanasia because of severe clinical signs. The data were plotted as Kaplan–Meier survival curves and analysed using LIFETEST and proportional hazards regression (PHREG) procedures.[12] The LIFETEST assesses model assumptions using three non-parametric tests, Log-rank, Wilcoxon and Likelihood ratio tests, to determine whether two or more treatment groups are different. PHREG performs Cox regression of survival data using proportional hazards model. The resulting regression coefficients are an estimate of the exponent of the hazard ratios or relative risk.

Results and discussion

Cellular farnesol in C. albicans treated with fluconazole

The fluconazole pre-treated cells used in our mouse inoculations had been washed in sterile saline prior to injection to remove all extracellular fluconazole and farnesol. However, the washed cells

Table 1. Increased farnesol production by fluconazole-treated cells

Farnesol location	Farnesol (mg/g dry weight)[a] no fluconazole	1.0 μM fluconazole
Extracellular	0.115 ± 0.023	1.40 ± 0.595
Intracellular	0.009 ± 0.007	0.054
Membrane-associated	0.106 ± 0.032	0.208

[a]Values for untreated cells and extracellular fluconazole-treated cells are the average of seven experiments with standard deviations shown. The intracellular and membrane-associated samples for cells treated with 1 μM fluconazole are the average of two experiments agreeing within ±10%. The 12-fold increase in extracellular farnesol by fluconazole-treated cells agrees with previously reported values.[8] In the units for extracellular farnesol (mg/g dry weight), the 'g dry weight' refers to the cells which produced the extracellular farnesol.

still contained high cellular concentrations of farnesol (Table 1). Following pre-treatment with 1.0 μM fluconazole, the concentrations of intracellular and membrane-associated farnesol increased 6-fold and 2-fold, respectively (Table 1). Therefore, the fluconazole pre-treated cells appeared pre-programmed to release higher levels of farnesol than untreated cells.

Mouse lethality of C. albicans pre-treated with fluconazole

C. albicans pre-treated with 0 to 5 μM fluconazole were compared for their pathogenicity in the mouse model. The 0.5 and 1.0 μM pre-treated cells displayed enhanced lethality when compared with untreated cells. The onset of mortality was 60–90 h sooner and by day 5 PI they showed 6- to 8-fold higher mortality than mice inoculated with untreated cells (Figure 1). As expected, control mice inoculated with saline alone had no mortality on day 7 PI. The data were analysed according to the Log-rank, Wilcoxon and Likelihood ratio tests. All three tests revealed that the survival curves for the 0.5 and 1.0 μM fluconazole pre-treated cells were different from the survival curves for the control cells ($P < 0.001$). The 0.5 and 1.0 μM pre-treated survival curves were not different from each other, but they were both significantly different ($P < 0.01$) from the 0.25 and 5.0 μM pre-treated groups. Furthermore, from the PHREG, hazard ratio estimates of 8.454 and 95% confidence interval (2.308–30.965), indicated that the 0.5 and 1.0 μM fluconazole pre-treated cells had 4.7 and 4.2 times higher lethality than the untreated cells, respectively. A separate trial which only used 1.0 μM fluconazole showed that the fluconazole pre-treated cells had 8.5 times higher lethality than untreated cells (Figure 1). Interestingly, the concentrations of fluconazole that caused 8.5-fold greater mouse lethality (0.5–1.0 μM) coincided precisely with those that caused maximal enhancement of farnesol production *in vitro*.[8]

Viability of fluconazole-treated cells

The cells which had been pre-treated with 0.25 or 5.0 μM fluconazole showed no enhanced lethality (Figure 1). Low doses of fluconazole (0.25 μM) might not have inhibited lanosterol 14α demethylase sufficiently to result in detectable farnesol accumulation,[8] whereas the cells pre-treated with the highest concentration of fluconazole (5.0 μM) might have been unable to replicate in

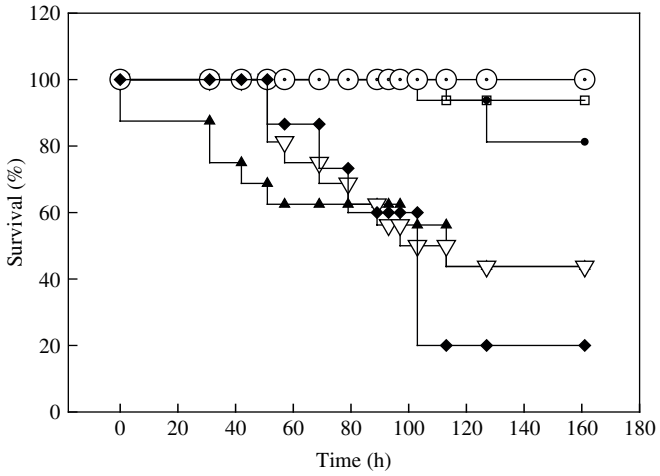

Figure 1. Effect of pre-treatment of *C. albicans* with fluconazole on the survival of mice inoculated intravenously at time 0 with a sub-lethal dose of *C. albicans* cells (1.3×10^6). All data shown are for Trial 2 except the filled diamonds for 1.0 μM fluconazole. Symbols: open squares, 0.25 μM fluconazole; filled triangles, 0.5 μM fluconazole; filled diamonds, 1.0 μM fluconazole (Trial 1); open inverted triangles, 1.0 μM fluconazole; open circles, 5.0 μM fluconazole; filled circles, untreated *C. albicans* (both trials). A control group consisting of five mice inoculated with saline intravenously had no mortality on day 7 post-inoculation (data not shown). For the last two time points, the filled triangle and open inverted triangle data coincide. The two controls with untreated *C. albicans* (both $n = 15$) conducted 2 months apart gave identical results.

mice and therefore were eliminated rapidly. In keeping with the fact that fluconazole is fungistatic rather than fungicidal,[5,8] cells pre-treated with fluconazole (0.25–5.0 μM) were still viable by plate count assays. *C. albicans* cells pre-treated with 0, 0.25, 0.5, 1.0 or 5.0 μM fluconazole showed 7.3×10^6, 6.1×10^6, 4.4×10^6, 2.0×10^6 and 9.0×10^5 cfu/mL, respectively. For reference, the MIC_{50} value at both 24 and 48 h for *C. albicans* A72 towards fluconazole was 0.125 mg/L (0.41 μM). This MIC represents the lowest concentration which prominently decreased turbidity[10] and a value of 0.125 mg/L indicates that strain A72 is highly susceptible to fluconazole.[4] We previously showed that 4–8 mg/L (13–26 μM) fluconazole prevented growth entirely.[13]

The observation that subinhibitory concentrations of fluconazole enhance virulence might be relevant to the therapeutic use of fluconazole as well as prolonged, prophylactic administration of fluconazole and to otherwise unexplained failures of fluconazole treatment in certain patients.[3,5] The potential side effects of exposure of *C. albicans* to subinhibitory concentrations of fluconazole have not been investigated previously. Exposure to subinhibitory concentrations of fluconazole correlated with an increased concentration of intracellular farnesol in *C. albicans* treated cells. Therefore, we propose that the fungal quorum sensing molecule farnesol might indirectly play a role in the pathogenesis of disseminated candidiasis. Farnesol is not an innocuous molecule. It has been reported to influence mammalian cells in several ways,[14] including blocking calcium channels, triggering apoptosis, targeting HMG CoA reductase for degradation, and stimulating cell differentiation. Thus, future research aimed at determining the role of farnesol and quorum sensing in disseminated candidiasis is needed.

Alternatively, the mechanism of fluconazole-induced enhanced lethality of *C. albicans* might not involve farnesol and thus the changes in intracellular farnesol would be coincidental. Exposure

of *C. albicans* to subinhibitory concentrations of fluconazole can affect the expression of virulence determinants such as the secretory aspartyl proteinases (SAPs). For *C. albicans* isolates obtained from an AIDS patient both before and after the development of fluconazole resistance, it was found that the fluconazole-susceptible isolates had a dose-dependent decrease in extracellular SAP activity whereas the fluconazole-resistant isolates had a dose-dependent increase in SAP activity.[15] It was suggested that patients infected with fluconazole-resistant isolates of *C. albicans* may have enhanced virulence when exposed to subinhibitory concentrations of fluconazole.[15]

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Transparency declarations

No declarations were made by the authors of this paper.

References

1. Kullberg BJ, Filler SG. Candidemia. In: Calderone RA, ed. *Candida and Candidiasis.* Washington, DC, USA: ASM Press, 2002; 327–40.

2. Miller LG, Hajjeh RA, Edwards Jr JE. Estimating the cost of nosocomial candidemia in the United States. *Clin Infect Dis* 2001; **32**: 1110.

3. Rex JH, Rinaldi MG, Pfaller, MA. Resistance of *Candida* species to fluconazole. *Antimicrob Agents Chemother* 1995; **39**: 1–8.

4. Debruyne D, Ryckelynck J-P. Clinical pharmacokinetics of fluconazole. *Clin Pharmacokinet* 1993; **24**: 10–27.

5. Sanlard D, Bille J. Current understanding of the modes of action of and resistance mechanisms to conventional and emergency antifungal agents for treatments of *Candida* infections. In: Calderone RA, ed. *Candida and Candidiasis.* Washington, DC, USA: ASM Press, 2002; 349–83.

6. Hornby JM, Jensen EC, Lisec AD *et al.* Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl Environ Microbiol* 2001; **67**: 2982–92.

7. Hornby JM, Kebaara BW, Nickerson KW. Farnesol biosynthesis in *Candida albicans*: cellular response to sterol inhibition by zaragozic acid B. *Antimicrob Agents Chemother* 2003; **47**: 2366–9.

8. Hornby JM, Nickerson KW. Enhanced production of farnesol by *Candida albicans* treated with four azoles. *Antimicrob Agents Chemother* 2004; **48**: 2305–7.

9. Antley PP, Hazen KC. Role of yeast cell growth temperature on *Candida albicans* virulence in mice. *Infect Immun* 1988; **56**: 2884–90.

10. National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing for Yeasts—Second Edition: Approved Standard M27-A2.* NCCLS, Wayne, PA, USA, 2002.

11. Nickerson WJ. Reduction of inorganic substances by yeasts. *J Infect Dis* 1953; **93**: 43–56.

12. SAS. *SAS/STAT User's Guide. Version 8.* Cary, NC, USA: SAS Institute Inc., 1999.

13. Dumitru R, Hornby JM, Nickerson KW. Defined anaerobic growth medium for studying *Candida albicans*: basic biology and resistance to eight antifungal drugs. *Antimicrob Agents Chemother* 2004; **48**: 2350–4.

14. Edwards PA, Ericsson J. Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. *Annu Rev Biochem* 1999; **68**: 157–85.

15. Wu T, Wright K, Hurst SF *et al.* Enhanced extracellular production of aspartyl proteinase, a virulence factor, by *Candida albicans* isolates following growth in subinhibitory concentrations of fluconazole. *Antimicrob Agents Chemother* 2000; **44**: 1200–8.