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

Dhammika H.M.L.P. Navarathna
University of Nebraska - Lincoln

Jacob M. Hornby
Lewis-Clark State College, Lewiston, ID

Natasha Hoerrmann
Lewis-Clark State College, Lewiston, ID

Anne M. Parkhurst
University of Nebraska - Lincoln, aparkhurst@unl.edu

Gerald E. Duhamel
University of Nebraska - Lincoln, gduhamel1@unl.edu

See next page for additional authors

Follow this and additional works at: http://digitalcommons.unl.edu/vetscipapers

Part of the Veterinary Medicine Commons

http://digitalcommons.unl.edu/vetscipapers/29

This Article is brought to you for free and open access by the Veterinary and Biomedical Sciences, Department of at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Veterinary and Biomedical Science by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.
Authors
Dhammika H.M.L.P. Navarathna, Jacob M. Hornby, Natasha Hoerrmann, Anne M. Parkhurst, Gerald E. Duhamel, and Kenneth Nickerson
Enhanced pathogenicity of Candida albicans pre-treated with subinhibitory concentrations of fluconazole in a mouse model of disseminated candidiasis

Dhammika H. M. L. P. Navarathna1,2, Jacob M. Hornby3, Natasha Hoerrmann3, Anne M. Parkhurst4, Gerald E. Duhamel2 and Kenneth W. Nickerson1*

1School of Biological Sciences, University of Nebraska, Lincoln, NE 68588, USA; 2Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, NE 68583, USA; 3Division of Natural Sciences, Lewis-Clark State College, Lewiston, ID 83501, USA; 4Department of Statistics, University of Nebraska, Lincoln, NE 68583, USA

Received 24 June 2005; returned 5 August 2005; revised 22 September 2005; accepted 23 September 2005

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 MIC50 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 immuno-compromised 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 patients3,4 and to avoid opportunistic and nosocomial candidiasis in AIDS patients.5 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 C15-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 farnesy1 pyrophosphate (FPP).7 Because FPP is the biosynthetic precursor of both farnesol1 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
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 5-fold\(^2\) and 10-fold\(^3\) increases, respectively, in the amount of farnesol produced by *C. albicans*. A similar response was observed when *C. albicans* was treated with three otherazole 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-Haussler 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 spectrometry (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 $1.0 \times 10^7$ 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 $1.3 \times 10^6$ 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

<table>
<thead>
<tr>
<th>Farnesol location</th>
<th>Farnesol (mg/g dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td>0.115 ± 0.023, 1.40 ± 0.595</td>
</tr>
<tr>
<td>Intracellular</td>
<td>0.009 ± 0.007, 0.054</td>
</tr>
<tr>
<td>Membrane-associated</td>
<td>0.106 ± 0.032, 0.208</td>
</tr>
</tbody>
</table>

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. In the units for extracellular farnesol (mg/g dry weight), the ‘g dry weight’ refers to the cells which produced the extracellular farnesol.

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 pretreated 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-treatment 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 farnesol 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.

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, whereas the cells pre-treated with the highest concentration of fluconazole (5.0 µM) might have been unable to replicate in mice and therefore were eliminated rapidly. In keeping with the fact that fluconazole is fungistic rather than fungicidal, 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 × 10⁶, 6.1 × 10⁵, 4.4 × 10⁵, 2.0 × 10⁵ and 9.0 × 10⁴ cfu/mL, respectively. For reference, the MIC₅₀ 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 and a value of 0.125 mg/L indicates that strain A72 is highly susceptible to fluconazole. We previously showed that 4–8 mg/L (13–26 µM) fluconazole prevented growth entirely.

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. 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 associated with inactivation of mammalian cells in several ways, 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.
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. It was suggested that patients infected with fluconazole-resistant isolates of *C. albicans* may have enhanced virulence when exposed to subinhibitory concentrations of fluconazole.

Acknowledgements

We thank Pfizer Pharmaceuticals, Sandwich, UK, for providing the fluconazole used in this study and Annette Fothersgill, Fungus Testing Laboratory, San Antonio, TX, for determining the MIC sensitivities of fluconazole. This work was supported by grants from the National Science Foundation (MCB-0110999) and the University of Nebraska Tobacco Settlement Biomedical Research Enhancement Fund. Work by J. M. H. and N. H. was supported by NIH grant P20 RRO16454 from the INBRE Program of the National Center for Research Resources. Published as Paper No. 14795, Agriculture Research Division, Institute of Agriculture and Natural Resources, University of Nebraska–Lincoln.

Transparency declarations

No declarations were made by the authors of this paper.

References