Inhibition of nucleic acids biosynthesis makes little difference to amphotericin B-tolerant persister formation in *Candida albicans* biofilm

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Running title: Effect of arrested DNA/RNA synthesis on persistence
Abstract

*Candida albicans* persisters constitute a small sub-population of biofilm and play a major role in recalcitrant chronic candidiasis; however, the mechanism underlying persister formation remains unclear. Persisters are often described as dormant, multidrug-tolerant, non-growing cells. Persister cells are difficult to isolate and study not only owing to their low levels in *C. albicans* biofilms, but also owing to their transient, reversible phenotype. In this study, we tried to induce persister formation by inducing *C. albicans* cells into a dormant state. *C. albicans* cells were pre-treated with 5-Fluorocytosine (planktonic cells: 0.8 μg ml\(^{-1}\); biofilm cells: 1 μg ml\(^{-1}\)) for 6 h at 37°C, which inhibits nucleic acid and protein synthesis. Biofilms and planktonic cultures of eight *C. albicans* strains were surveyed for persisters after amphotericin B treatment (100 μg ml\(^{-1}\), 24 h) and colony forming unit (CFU) assay. None of the planktonic cultures, with and without 5-Fluorocytosine pre-treatment, contained persisters. Persister cells were found in biofilms of all tested *C. albicans* strains, representing approximately 0.01%–1.93% of the total population. However, the persister levels were not significantly increased in *C. albicans* biofilms pre-treated with 5-Fluorocytosine. These results suggest that inhibition of nucleic acid synthesis did not seem to increase the formation of amphotericin B-tolerant persisters in *C. albicans* biofilms.

**Key words:** *Candida albicans*; persisters; biofilm
Introduction

*C. albicans* is the most prevalent opportunistic pathogen in humans, causing endogenous infection from superficial to seriously deep-seated mycoses (1, 2). *Candida* species are also the most common fungal pathogens causing hospital-acquired infections (3-5). While increasing rates of candidemia caused by non-*albicans* Candida species have been reported worldwide, *C. albicans* is still the most common pathogen responsible for candidemia (3, 6-8). Although candidemia rates differ between countries, recent epidemiological data from the United States, Europe, and Latin America show an overall increase in the incidence of candidemia in the last decade (5, 9-12). Most *Candida* infections involve biofilm formation and often pose poor prognosis owing to drug tolerance; these infections are associated with a high rate of mortality, approaching 40%-70% (1, 2, 13-19). Biofilm-related drug tolerance may be attributed to high cell density and low growth rates within the biofilm, the presence of exopolymer matrix, and up-regulation of drug efflux pumps and stress responses (2, 20-25). Indeed, increasing evidence indicates that the mechanism of biofilm-related drug tolerance is multifactorial (23-25). Within the *C. albicans* biofilm, a small population of persister cells that can withstand lethal antifungal treatment is believed to be responsible for the poor prognosis (26, 27). However, the mechanism of *C. albicans* persister formation remains unknown and the low levels of persisters and their transience make them difficult to isolate. The inability of an antifungal agent to eradicate persisters is thought to result from the quiescent physiological state because antifungal agents need a
physiologically/metabolically active target to function (2, 27, 28). Persisters may be the main culprit in the recurring symptoms of fungal infections because the persisters can produce a new biofilm with a similar persister level and an equivalent antifungal susceptibility (25, 29-31).

*C. albicans* persisters have so far been detected only in biofilms, comprising only 0.01%–2% of mature biofilms (26, 29, 32-34). Persisters exhibit a non-hereditary, multi-drug tolerance to antifungals and are described as dormant, non-dividing phenotypic variants (27-30, 32, 35). The dormant cells are characterized by quiescent metabolisms such as nucleic acid synthesis and protein synthesis (26, 27, 36). Kwan reported recently that the pre-treatment of *Escherichia coli* with some antibiotics that can halt transcription, translation, or ATP synthesis, dramatically increased persistence (37). The main mechanisms underlying persister formation seems to be inhibition of anabolism and dissipation of the proton motive force (36). Therefore, we speculated that induced dormancy could increase the formation of antifungal-tolerant persisters. 5-Fluorocytosine (5-FC) is a nucleoside analog with antifungal activities that inhibits nucleic acid and protein synthesis. In the study, we attempted to induce *C. albicans* cells into dormancy with 5-FC to investigate whether inhibition of nucleic acid synthesis could increase *C. albicans* persister formation.

**Materials and Methods**

**Strains and growth conditions**

Eight *C. albicans* strains were tested in this study (Table 1). *C. albicans* strains
3153A (29) and YEM30 (38, 39) are wild types. *C. albicans* strains DSY653 and DSY654 (39, 40) are CDR1 and CDR2 knockout strains donated by Prof. K. Lewis from Northeastern University, Boston, MA, USA. *C. albicans* strains CJN308 and CJN459 (41) are biofilm-deficient strains. *C. albicans* strain 11E and 23A are clinical isolates, whose origins have been described previously (26). *C. albicans* strain 11E is a high-persister clinical isolate, while 23A is a low-persister clinical isolate (26). Stock cultures of *C. albicans* strains were routinely grown in yeast extract peptone dextrose (YPD; 1% yeast extract, 2% peptone, 2% glucose) solid medium containing 1.5% agar and incubated at 37°C for 24 to 48 h. Yeast inocula were prepared by transfer of a single colony into YPD medium and overnight incubation at 37°C in an incubator shaker at about 100 rpm. Cells were harvested by centrifugation at 6000 × g for 3 min and washed twice in sterile phosphate-buffered saline (PBS; pH 7.2–7.4). Then, the cells were re-suspended in RPMI 1640 medium (Gibco, USA) with L-glutamine, buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, USA), and adjusted to the desired density after counting with a hemocytometer.

**Minimum inhibitory concentration (MIC) assay**

CLSI M27-A3 microdilution methodology was used to test in vitro susceptibility to amphotericin B (Amresco, USA) and 5-FC (Sigma-Aldrich, USA) (42). The inoculated cultures were incubated for 24 h at 37°C, and the MIC₉₀ was determined by observing growth inhibition, as measured by turbidity. Experiments were performed in quadruplicate and repeated with at least four independent cultures.
Growth and 5-FC pre-treatment of planktonic cells

Planktonic cultures of *C. albicans* were prepared according to the methods described by Al-Dhaheri and Douglas (32). Briefly, *C. albicans* cells were harvested by centrifugation, washed twice in sterile PBS and re-suspended in RPMI 1640 medium plus MOPS at a density of 1.0 × 10⁷ cells ml⁻¹. The planktonic cells were either determined the persister levels or pre-treated with 5-FC and then determined the persister levels. Pre-treated planktonic cells were exposed to 5-FC (0.8 µg ml⁻¹) for 6 h at 37°C. The pre-treatment concentration and duration was optimized for maximum survivability (approximately 95% to 100%; p > 0.05).

Growth and 5-FC pre-treatment of biofilms

*C. albicans* biofilm formation was induced as described by Ramage et al. (29, 43). Briefly, *C. albicans* cells were harvested from an overnight culture by centrifugation, washed twice in sterile PBS, and re-suspended in RPMI 1640 medium plus MOPS at a density of 1.0 × 10⁶ cells ml⁻¹. One hundred microliters of the suspension was dispensed into the wells of 96-well microtiter plates, and the plates were incubated for 24 h at 37°C at approximately 100 rpm. The biofilms were either determined the persister levels or pre-treated with 5-FC and then determined the persister levels. The pre-treated biofilm cells were exposed to 5-FC (1 µg ml⁻¹) for 6 h at 37°C. The pre-treatment concentration and duration was optimized for maximum survivability (approximately 95% to 100%; p > 0.05).

*C. albicans* cells were pre-treated by exposure to 5-FC throughout biofilm formation.
Briefly, *C. albicans* cells were harvested, washed and re-suspended in RPMI 1640 medium containing 5-FC (0.55 µg ml\(^{-1}\)) at a density of 1.0 × 10\(^6\) cells ml\(^{-1}\). One hundred microliters of the suspension was dispensed into the wells of 96-well microtiter plates, and the plates were incubated for 24 h at 37°C. The pre-treatment concentration of 5-FC was optimized for maximum survivability (approximately 95% to 100%; p > 0.05).

**Persister cells determination**

Amphotericin B was dissolved in RPMI 1640 medium plus MOPS at 100 µg ml\(^{-1}\), which exceeded 10 × MIC to minimize survival of potential spontaneous resistant mutants (26, 29, 32, 33). The planktonic cells were harvested, washed twice in PBS, aliquoted into wells of microtiter plates at 100 µl per well and treated for 24 h with amphotericin B (100 µg ml\(^{-1}\)) in RPMI 1640 medium plus MOPS. The biofilms were washed twice in PBS and treated with amphotericin B (100 µg ml\(^{-1}\)) for 24 h. The number of persister cells was determined based on an assessment of cell viability after amphotericin B treatment, by harvesting and serially diluting *C. albicans* cells in sterile PBS and plating 5 µL drops on YPD solid medium and counting colonies (26, 29, 32-34). The percentage of persisters was determined by comparing the number of persisters with the total number of viable cells in an untreated culture.

**Live/dead cell staining and confocal laser scanning microscopy**

LIVE/DEAD® FungaLight™ yeast viability kit (Invitrogen, USA), which contains SYTO®9 and propidium iodide, was used to distinguish between live and dead yeast cells.
When used alone, the SYTO®9 green-fluorescent nucleic acid stain generally labels all the yeast cells in a population, while propidium iodide penetrates only yeast cells with damaged membranes, causing a reduction in the SYTO®9 stain fluorescence when both dyes are present. As a result, live cells stain fluorescent green, whereas dead cells stain fluorescent red. Biofilms and planktonic cells of C. albicans 3153A strain were washed twice with sterile Tris-HCl buffer (pH 6.8) before and after amphotericin B treatment. Then the samples were treated with 100 μl Tris-HCl buffer containing 1‰ SYTO®9 and 1‰ propidium iodide and incubated for 15–30 min in the dark at 37°C. After incubation with the dyes, stained samples were viewed with a Zeiss LSM780 confocal laser scanning microscope (CLSM).

Results and Discussion

C. albicans persisters were first reported to occur following treatment with amphotericin B or chlorhexidine at a high concentration (29). Dose-dependent killing has been the only effective and straightforward method for identification and isolation of C. albicans persisters (26, 29, 32-34, 37). In the present study, we used the same protocol with amphotericin B to identify persister levels. Amphotericin B is the most effective antifungal agent and can kill fungi cells in all phases of growth, making it the optimum choice to isolate persisters regardless of whether 5-FC pre-treatment inhibits replication. (26, 29, 32, 33).

Presence of persisters in C. albicans biofilms and planktonic cultures
Biofilms and planktonic cells of eight *C. albicans* strains were surveyed for the presence of persisters after amphotericin B treatment. *C. albicans* persisters were detected in biofilms of all tested *C. albicans* strains (Fig. 1), while none of planktonic cultures contained persisters. CLSM micrographs of live/dead cell staining (*C. albicans* strain 3153A) also showed that after exposure to amphotericin B, the planktonic cells were effectively killed, without detectable survivors stained fluorescent green (Fig. 2A, C), while biofilms contained a small number of survivors (Fig. 2E, G). The CLSM analysis provided further evidence that *C. albicans* persister cells were detected only in biofilms. For all tested *C. albicans* strains, the persister percentage was approximately 0.01%–1.93% of the total population of mature biofilms. In agreement with previous data (26, 29, 32-34), our results further confirm that *C. albicans* persister cells seem to be present only in biofilms and are absent in planktonic cultures.

**5-FC pre-treatment makes little difference to *C. albicans* persister formation**

5-FC is a nucleoside analog with antifungal activities that inhibits nucleic acid and protein synthesis. The antifungal activity of 5-FC is a consequence of two metabolites, 5-fluorodeoxyuridine monophosphate and 5-fluorouridine triphosphate, which result in an inhibition in the synthesis of nucleic acid and protein (44-48). Currently, 5-FC is the only antifungal agent in use that can inhibit replication, transcription and translation. Recently researchers found that pre-treatment of *Escherichia coli* with some antibiotics which can halt transcription, translation, or ATP synthesis can increase persistence dramatically (37). Hence, we speculated that maybe 5-FC could be used to induce *C. albicans* cells into a
dormant state to increase persister formation by inhibiting nucleic acid and protein synthesis.

In the present study, we tested whether 5-FC pre-treatment of eight *C. albicans* strains would increase persister formation. *C. albicans* cells were exposed to 5-FC (planktonic cells: 0.8 µg ml⁻¹; biofilm cells: 1 µg ml⁻¹) for 6 h prior to colony forming unit (CFU) assay and amphotericin B treatment. Our results showed that the number of *C. albicans* cells neither increased nor decreased significantly after 5-FC pre-treatment (p > 0.05; Fig. 1). CLSM micrographs also showed that there was no significant difference before and after 5-FC pre-treatment, without clear indications of killing cells (Fig. 2A, B, E, and F; Fig. 3A and B). The results indicated that planktonic and biofilm cells of *C. albicans* appeared to be induced successfully into a dormancy-like state by 5-FC pre-treatment.

According to the hypothesis that dormancy results in the development of persister cells, *C. albicans* persisters should be increased significantly in cultures pre-treated with 5-FC. However, contrary to our hypothesis, the formation of persisters was not significantly increased in cultures pre-treated with 5-FC (p > 0.05; Fig. 1, Fig. 2C, D, G and H; Fig. 3C and D).

To provide further evidence that the survivors were “true” persisters, we tested the survivors to see whether they showed the slow loss of viability characteristic of persisters (29). Hence, we exposed the pre-treated and non-pre-treated biofilms to amphotericin B for a prolonged period and quantified the survivors at several time points. Our results showed that the survivors exhibited slow, steady cell death for up to 8 h of prolonged...
amphotericin B treatment (p > 0.05; Fig. 4). Therefore, the persisters detected in the present study are “true” persisters, rather than spontaneous resistant mutants that are able to survive and even grow in the presence of an antifungal agent.

Our results suggest that inhibition of nucleic acid synthesis does not increase C. albicans persister formation. This finding is different from those of earlier studies in bacteria, in which bacterial persisters can be induced by inhibiting transcription or translation (37). Although dormancy appears to be the main mechanism underlying bacterial persister formation (36, 37), our results suggest that dormancy has negligible effect on C. albicans persister formation. Compared with bacterial persisters, the formation of C. albicans persisters is likely to be multifactorial and more complicated.

We also attempted to induce C. albicans cells into dormancy to increase persister formation by exposing C. albicans strains 3153A, 11E and 23A to 5-FC (0.55 µg ml\(^{-1}\)) throughout biofilm formation. Our results showed that after incubation for 24 h, C. albicans cells maintained the yeast form without indications of increase in the number, in comparison with that of the initial inocula (Fig. 5A). In contrast, cells in the non-pre-treated biofilms presented as yeast cells, pseudohyphae, and hyphae and formed a dense network of cells of all morphologies (Fig. 2E). After exposure to amphotericin B (100 µg ml\(^{-1}\)), the proportion of persister cells increased, but the number of persister cells did not increase significantly in the pre-treated biofilms (p > 0.05; Fig. 5B). Given that the number of C. albicans cells did not increase significantly after incubation for 24 h, the increase in the proportion of persisters could be attributed to inhibited growth rather than...
to an increase in persister formation (Fig. 4). Although only three *C. albicans* strain was
tested in this part of the experiment, the results once again clearly indicate that inhibition
of nucleic acid synthesis does not appear to be the mechanism underlying *C. albicans*
persister formation. The formation of *C. albicans* persisters seems more complicated than
that of bacterial persisters; hence, further study is required.

In clinical practice, combination therapy with amphotericin B and 5-FC may be used
to treat candidiasis to achieve antifungal synergy and to prevent the development of
fungus resistance (44, 49, 50). It should be noted that the combination therapy with
amphotericin B with 5-FC remains does not eradicate persisters.

The conclusions of this study are limited because only eight *C. albicans* strains were
tested. Although this is a preliminary study, our results suggest that dormancy seems not
to be the mechanism of *C. albicans* persister formation and that in comparison to
bacterial persisters, the formation of *C. albicans* persisters appears to be multifactorial
and more complicated. However, since dormancy is an important feature of persisters, it
is premature to rule out dormancy as the cause of fungi persisters. Additional studies are
needed to investigate the mechanism of fungal persister formation.

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References


15. Colombo AL, Nucci M, Park BJ, Nauer SA, Arthington-Skaggs B, da Matta DA,


39. Qi QG, Lafleur MD. 2009. [Roles of drug efflux pump genes in the mechanism of


47. Polak A. 1974. Effects of 5-fluorocytosine on protein synthesis and amino acid pool


Fig. 1 Persister levels in biofilms with or without 5-FC pre-treatment. Biofilms were pre-treated with 5-FC (1 µg ml⁻¹) for 6 h prior to amphotericin B treatment (100 µg ml⁻¹). Growth and persister levels in biofilms of *C. albicans* 3153A (A), *C. albicans* YEM30 (B), *C. albicans* DSY653 (C), *C. albicans* DSY659 (D), *C. albicans* CJN308 (E), *C. albicans* CJN459 (F), *C. albicans* 11E (G) and *C. albicans* 23A (H). Neither growth nor persister levels were changed significantly in biofilms and planktonic cultures after 5-FC pre-treatment (p > 0.05). Error bars indicate standard deviations; results are from 16 independent experiments.

Fig. 2 Live/dead staining of planktonic and biofilm cells with LIVE/DEAD® *FungaLight™* yeast viability kit. Planktonic and biofilm cells were pre-treated with 5-FC (planktonic cells: 0.8 µg ml⁻¹; biofilm cells: 1 µg ml⁻¹) for 6 h prior to amphotericin B treatment (100 µg ml⁻¹). CLSM micrographs were taken at 200 × magnification; bar represents 20µm. Images of non-pre-treated planktonic cells (A), pre-treated planktonic cells (B), non-pre-treated planktonic cells after amphotericin B treatment (C), pre-treated planktonic cells after amphotericin B treatment (D), non-pre-treated biofilm cells (E), pre-treated biofilm cells (F), non-pre-treated biofilm cells after amphotericin B treatment (G), pre-treated biofilm cells after amphotericin B treatment (H). No significant difference was observed between non-pretreated and pre-treated cultures.

Fig. 3 Three-dimensional CLSM images of *C. albicans* 3153A biofilms. Biofilms were pre-treated with 5-FC (1 µg ml⁻¹) for 6 h prior to treatment with amphotericin B.
(100 µg ml\(^{-1}\)). CLSM images were taken at 200× magnification; bar represents 50 µm. Images were processed to obtain a lateral view in order to show biofilm thickness (20 µm; A and C) and a rotated view to present a global perspective (B and D). Images of biofilms before amphotericin B treatment showed that there was no significantly change in the pre-treated biofilm (A and B). Images of biofilms after amphotericin B treatment showed that the survival cells were not increased significantly in the pre-treated biofilm (C and D).

**Fig. 4** Prolonged amphotericin B treatment of *C. albicans* biofilms. Biofilms of *C. albicans* 3153A were pre-treated with 5-FC (1 µg ml\(^{-1}\)) for 6 h prior to amphotericin B treatment (100 µg ml\(^{-1}\)). Survivor levels were unchanged significantly during the prolonged amphotericin B treatment (p > 0.05). Error bars indicate standard deviations; results are from 16 independent experiments.

**Fig. 5** Persister levels in biofilms pre-treated with 5-FC throughout the formation process. Biofilms (24 h) of *C. albicans* 3153A, 11E and 23A were induced in the presence of 5-FC (0.55 µg ml\(^{-1}\)) and then treated with amphotericin B (100 µg ml\(^{-1}\)). CLSM micrographs were taken at 200× magnification; bar represents 20 µm. (A) Images of initial inocula, pre-treated biofilm and pre-treated biofilm after amphotericin B treatment. (B) Growth and persister levels in the pre-treated biofilms of *C. albicans* 3153A, 11E and 23A. The proportion of persisters increased, but the number of persister cells did not change significantly in the
pre-treated biofilms (p > 0.05) Error bars indicate standard deviations; results are
from 16 independent experiments.
TABLE 1 C. albicans strains evaluated in this study

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