Inhibition of Nucleic Acid Biosynthesis Makes Little Difference to Formation of Amphotericin B-Tolerant Persisters in \textit{Candida albicans} Biofilm

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\textit{Candida albicans} persisters constitute a small subpopulation of biofilm cells 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, nongrowing cells. Persister cells are difficult to isolate and study not only due to their low levels in \textit{C. albicans} biofilms but also due to their transient, reversible phenotype. In this study, we tried to induce persister formation by inducing \textit{C. albicans} cells into a dormant state. \textit{C. albicans} cells were pretreated with 5-fluorocytosine (planktonic cells, 0.8 \(\mu\)g ml\(^{-1}\); biofilm cells, 1 \(\mu\)g ml\(^{-1}\)) for 6 h at 37°C, which inhibits nucleic acid and protein synthesis. Biofilms and planktonic cultures of eight \textit{C. albicans} strains were surveyed for persisters after amphotericin B treatment (100 \(\mu\)g ml\(^{-1}\) for 24 h) and CFU assay. None of the planktonic cultures, with or without 5-fluorocytosine pretreatment, contained persisters. Persister cells were found in biofilms of all tested \textit{C. albicans} strains, representing approximately 0.01 to 1.93% of the total population. However, the persister levels were not significantly increased in \textit{C. albicans} biofilms pretreated 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 \textit{C. albicans} biofilms.

\textit{Candida albicans} is the most prevalent opportunistic pathogen in humans, causing endogenous infections ranging from superficial to seriously deep-seated mycoses (1, 2). \textit{Candida} species are also the most common fungal pathogens causing hospital-acquired infections (3–5). While increasing rates of candidemia caused by non-\textit{Candida albicans} species have been reported worldwide, \textit{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 \textit{Candida} infections involve biofilm formation and often pose a poor prognosis due to drug tolerance; these infections are associated with a high rate of mortality, approaching 40 to 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 upregulation 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 \textit{C. albicans} biofilm, a small population of persistor cells that can withstand lethal antifungal treatment is believed to be responsible for the poor prognosis (26, 27). However, the mechanism of \textit{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).

\textit{C. albicans} persisters have so far been detected only in biofilms, comprising only 0.01% to 2% of mature biofilms (26, 29, 32–34). Persisters exhibit a nonhereditary, multidrug tolerance to antifungals and are described as dormant, nondividing 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 pretreatment of \textit{Escherichia coli} with antibiotics that can halt transcription, translation, or ATP synthesis dramatically increased persistence (37). The main mechanisms underlying persister formation seem 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 this study, we attempted to induce \textit{C. albicans} cells into dormancy with 5-FC to investigate whether inhibition of nucleic acid synthesis could increase \textit{C. albicans} persister formation.

MATERIALS AND METHODS

Strains and growth conditions. Eight \textit{C. albicans} strains were tested in this study (Table 1). \textit{C. albicans} 3153A (29) and YEM30 (38, 39) are wild-type strains. \textit{C. albicans} DSY653 and DSY654 (39, 40) are CDR1 and CDR2 mutants. DSY364, DSY366, and DSY368 are CDR1, CDR2, and CDR3 mutants, respectively. 

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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, 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.** The LIVE/DEAD Bacterial Viability Kit (Invitrogen) is a kit containing SYTO9 and propidium iodide, used to distinguish between live and dead yeast cells. The former enters both living and dead cells, while the latter only enters cells with damaged membranes. The SYTO9 green fluorescent nucleic acid stain generally labels all of the yeast cells in a population, while propidium iodide penetrates only yeast cells with damaged membranes, causing a reduction in the SYTO9 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 the 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% SYTO9 and 1% propidium iodide and incubated for 15 to 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 fungal cells in all phases of growth, making it the optimum choice to isolate persisters, regardless of whether 5-FC pretreatment 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 the 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 and C), while biofilms contained a small number of survivors (Fig. 2E and G). The CLSM analysis provided further evi-
Evidence that *C. albicans* persister cells were detected only in biofilms. For all tested *C. albicans* strains, the persister percentage was approximately 0.01 to 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 pretreatment 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 inhibition of 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 pretreatment of *Escherichia coli* with some antibiotics that can halt transcription, translation, or ATP synthesis can increase persistence dramatically (37). Hence, we speculated that maybe 5-FC could be used to

**FIG 1** Persister levels in biofilms with or without 5-FC pretreatment. Biofilms were pretreated with 5-FC (1 μg ml⁻¹) for 6 h prior to amphotericin B treatment (100 μg ml⁻¹). Shown are the growth and persister levels in biofilms of *C. albicans* strains 3153A (A), YEM30 (B), DSY653 (C), DSY659 (D), CJN308 (E), CJN459 (F), 11E (G), and 23A (H). Neither growth nor persister levels were changed significantly in biofilms and planktonic cultures after 5-FC pretreatment (*P* > 0.05). Error bars indicate standard deviations; results are from 16 independent experiments.
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 pretreatment 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 amphotericin B treatment (100 μg ml⁻¹). CLSM micrographs were taken at a magnification of 200×; the bar represents 20 μm. Shown are images of nonpretreated planktonic cells (A), pretreated planktonic cells (B), nonpretreated planktonic cells after amphotericin B treatment (C), pretreated planktonic cells after amphotericin B treatment (D), nonpretreated biofilm cells (E), pretreated biofilm cells (F), nonpretreated biofilm cells after amphotericin B treatment (G), and pretreated biofilm cells after amphotericin B treatment (H). No significant difference was observed between nonpretreated and pretreated cultures.

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 pretreated and nonpretreated 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 a 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,
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 pretreated 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. 5). Although only three C. albicans strains were 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 fungal resistance (44, 49, 50). It should be noted that combination therapy using amphotericin B with 5-FC 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 bac-

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 an increase in number, in comparison with that of the initial inocula (Fig. 5A). In contrast, cells in the nonpretreated biofilms presented as yeast

**FIG 4** Prolonged amphotericin B treatment of C. albicans biofilms. Biofilms of C. albicans 3153A were pretreated with 5-FC (1 μg ml⁻¹) for 6 h prior to amphotericin B treatment (100 μg ml⁻¹). Survivor cell 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 pretreated 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 a magnification of 200×; the size bar represents 20 μm. (A) Images of initial inocula, pretreated biofilm, and pretreated biofilm after amphotericin B treatment. (B) Growth and persister levels in the pretreated 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 pretreated biofilms ($P > 0.05$). Error bars indicate standard deviations; results are from 16 independent experiments.
aterial 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 fungal persisters. Additional studies are needed to investigate the mechanism of fungal persister formation.

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