Echinocandin treatment of *Candida albicans* biofilms enhances neutrophil extracellular trap (NET) formation.

Echinocandins enhance NET formation to biofilm

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ABSTRACT
The nosocomial pathogen Candida albicans forms biofilms on medical devices that persist in the face of antifungals and host defenses. Echinocandins, the most effective anti-biofilm drugs, have recently been shown to augment the activity of neutrophils against biofilms through an unknown mechanism. Here we show that treatment of C. albicans biofilms with sub-inhibitory concentrations of echinocandins promotes the formation of neutrophil extracellular traps (NETs), structures of DNA, histones, and antimicrobial proteins with antifungal activity.
Candida albicans forms resilient biofilms on vascular catheters, urinary catheters, and dentures (1, 2). In these protective communities, C. albicans resists immune attack and withstands high concentrations of antifungals (3-8). Biofilm formation frequently complicates the treatment of candidiasis, and retained infected devices can lead to prolonged infection and poor outcome (9, 10). Given the profound multi-drug resistance observed for C. albicans biofilms, novel approaches to treatment are of great interest.

One anti-biofilm tactic is to devise strategies that augment the host immune response to these infections. Neutrophils, which are critical for control of numerous fungal infections, exhibit very little activity against C. albicans biofilms (6-8). However, Katragkou et al. found that anidulafungin, an antifungal of the echinocandin drug class, could enhance the activity of neutrophils against C. albicans biofilms (11). In the current report, we analyze neutrophil-biofilm interactions to identify the mechanism of the synergy observed between echinocandins and neutrophils against biofilms.

We first identified sub-inhibitory concentrations of echinocandins against mature C. albicans biofilms to use in biofilm-neutrophil studies. We utilized a XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay to examine the impact of caspofungin (Apexbio Technology LLC), anidulafungin (Apexbio Technology LLC), and micafungin (Astellas) on 24 h C. albicans SC5314 biofilms (12, 13). Antifungals diluted in RPMI 1640 (without phenol red) were applied to biofilms for 24 h. The results of XTT metabolic assays revealed a dose-dependent inhibition of mature C. albicans biofilm following exposure to each echinocandin (Figure 1A-C). The sub-
inhibitory concentrations selected for further study were not statistically different than
the no treatment controls and were 4-fold lower than the lowest inhibitory
concentrations. These included 0.05 μg/ml, 0.025 μg/ml, and 0.0015 μg/ml, for
micafungin, caspofungin, and anidulafungin, respectively.

As echinocandin treatment can influence fungal morphology, we examined the cellular
architecture of biofilms that had been exposed to these sub-inhibitory concentrations by
scanning electron microscopy using a coverslip model of biofilm formation (6). Briefly C.
albicans biofilms were grown in RPMI-MOPS on coverslips for 24 h at 37°C prior to a 24
h treatment with media containing sub-inhibitory concentrations of echinocandins,
followed by processing for imaging (6). By scanning electron microscopy, biofilms
treated with these concentrations were similar in appearance, consisting primarily of
hyphae (Figure 1D). Abnormalities in cellular morphology were not observed.

To gain insight into the synergism observed between neutrophils and echinocandins
against C. albicans biofilms, we co-cultured neutrophils with untreated biofilms and
those treated with sub-inhibitory concentrations of echinocandins. Human peripheral
blood was obtained from donors with written informed consent through a protocol
approved by the University of Wisconsin Internal Review Board (IRB), and neutrophils
were purified using the MACSxpress Neutrophil Isolation and MACSxpress Erythrocyte
Depletion kits (Miltenyi Biotec Inc., Auburn, CA). Neutrophils (5 x 10⁵) in RPMI 1640
supplemented with 2% heat-inactivated fetal bovine serum (FBS) and glutamine (0.3
mg/ml) were added to coverslip biofilms for 4 h and processed for imaging (6). By
scanning electron microscopy, neutrophils applied to untreated biofilms for 4 h
appeared rounded, as previously described (6). In contrast, neutrophils exposed to
echinocandin-treated biofilms produced web-like lattices consistent with the formation of
NETs (Figure 2A) (14).

To quantify the production of NETs, we utilized Sytox Green staining of free DNA
(Figure 2B) (6). Neutrophils were added to echinocandin-treated and untreated biofilms
in a 96-well plate to a final concentration of 2x10^5 cell/well. After a 4 h incubation, Sytox
Green was added at a final concentration of 1 μM and fluorescence (excitation 500
nm/emission 528 nm) was measured. Background fluorescence for each condition was
subtracted from total fluorescence values. In response to untreated biofilms very little
free DNA was observed, suggesting minimal NET formation, as previously described for
biofilm-neutrophil interactions (6). However, treatment of biofilms with sub-inhibitory
concentrations of echinocandins resulted in elevated free DNA, approximately 6-8-fold
above the levels measured for untreated biofilms, and reaching up to a third of the
levels observed in response to 100 nM phorbol 12-myristate 13-acetate (PMA), a potent
stimulus for NET formation. These findings are consistent with the release of NETs, an
observation confirmed by scanning electron microscopy (Figure 2A).

Neutrophils have been shown to form NETs in response to non-biofilm C. albicans, and
the importance of this process for control of candidiasis and other invasive fungal
infections has been demonstrated (15-17). However, biofilm formation by C. albicans
inhibits this process, which contributes to the resistance of biofilms to neutrophil attack
Interestingly, biofilms exhibit susceptibility to NETs if induced through other stimuli, suggesting that they would be an effective mechanism of killing if initiated on biofilm. Here, we show that neutrophils release NETs in response to echinocandin-treated biofilms. This altered response likely contributes to the synergy observed for neutrophils uniquely with this drug class (11).

During planktonic growth, treatment of C. albicans with sub-inhibitory concentrations of echinocandins disrupts cell wall assembly and leads to a greater exposure of β-glucan, a proinflammatory polysaccharide that is normally masked (20). This unmasking of glucan occurs preferentially in hyphal cells and is unique to treatment with the echinocandin drug class (21). As β-glucan has been shown to induce neutrophils to form NETs, unmasked glucan following echinocandin treatment of biofilms may serve as a trigger for NET release (22). Considering that echinocandin treatment of Candida parapsilosis biofilms also augments the activity of neutrophils, a similar mechanism of neutrophil activation may be triggered in response to this pathogen as well (23).

Understanding how antifungal therapy modulates the innate immune response may open new avenues to augment the host response to invasive fungal infections.

Acknowledgements
This work was supported by the National Institutes of Health (K08 AI108727), the Burroughs Wellcome Fund (1012299) and the Doris Duke Charitable Foundation (112580130).
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factor on antifungal activity of human polymorphonuclear neutrophils against


**FIGURE LEGENDS**

Figure 1. Identification of sub-inhibitory concentrations of echinocandins. (A-C) The impact of 24 h of echinocandin treatment on 24 h *C. albicans* was assessed by XTT assay, *n=3, replicate with SD is shown, *P<0.05 by Student's t-test compared to no drug control.* (D) By scanning electron microscopy, treatment of *C. albicans* biofilms with micafungin (0.05 μg/ml), caspofungin (0.025 μg/ml), and anidulafungin (0.0015 μg/ml) did not impact biofilm architecture. The measurement bars represent 10 μm and 1 μm for the 2,000x and 10,000x images, respectively.

Figure 2. Echinocandin treatment of *C. albicans* biofilms triggers neutrophils to form NETs. *C. albicans* biofilms were incubated in the presence of sub-inhibitory concentrations of echinocandins for 24 h and then co-cultured with human neutrophils for 4 h. (A) By scanning electron microscopy, NETs were triggered in response to echinocandin-treated biofilms. The measurement bars represent 10 μm and 1 μm for the 2,000x and 10,000x images, respectively. (B) NET release was estimated by Sytox Green detection of free DNA. NET formation in response to a potent inducer of
NETs, PMA, is shown for comparison. Experiments were performed in triplicate on six occasions, SEM shown, *P<0.05 by paired t-test