Paradoxical antifungal activity and structural observations in biofilms formed by echinocandin-resistant *Candida albicans* clinical isolates

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Abstract

Echinocandin-resistant clinical isolates of *Candida albicans* have been reported, and key hot spot mutations in the *FKS1* gene, which encodes a major glucan synthase subunit, have been identified in these (caspofungin-resistant [CAS-R]) strains. Although these mutations result in phenotypic resistance to echinocandins in planktonic cells, there is little data on antifungal susceptibilities of CAS-R *C. albicans* strains within biofilms. Thus, we analyzed biofilms formed by 12 *C. albicans* CAS-R clinical strains in which we previously identified *FKS1* hot-spot mutations and compared the sessile antifungal and paradoxical activity of anidulafungin (ANID), caspofungin (CAS), and micafungin (MICA). Biofilms were formed in a 96-well static microplate model and assayed using both tetrazolium-salt reduction and crystal violet assays, as well as examination by scanning electron microscopy. We first sought to assess biofilm formation and structure in these *fks1* mutants and found that the biofilm mass and metabolic activities were reduced in most of the *fks1* mutants as compared with reference strain SC5314. Structural analyses revealed that the *fks1* mutant biofilms were generally less dense and had a clear predominance of yeast and pseudohyphae, with unusual “pit”-like cell surface structures. We also noted that sessile minimum inhibitory concentrations (MICs) to ANID, CAS, and MICA were higher than planktonic MICs of all but one strain. The majority of strains demonstrated a paradoxical effect (PE) to particular echinocandins, in either planktonic or sessile forms. Overall, biofilms formed by echinocandin-resistant clinical isolates demonstrated varied...
PEs to echinocandins and were structurally characterized by a preponderance of yeast, pseudohyphae, and pit-like structures.

**Key words:** antifungal therapy, biofilms, Candida albicans.

## Introduction

The echinocandins anidulafungin (ANID), caspofungin (CAS), and micafungin (MICA) comprise a class of antifungal agents that target the glucan synthase enzyme, which is involved in cell wall synthesis of many medically important fungi. In randomized clinical trials, the echinocandins have demonstrated excellent efficacy against *Candida albicans* and non-*Candida albicans* *Candida* species involved in invasive and disseminated infections [1–4]. Despite excellent overall *in vitro* activity against Candida biofilms, differences in echinocandin efficacy in *in vitro* and animal models have been reported, although the clinical significance of these differences remains unclear [5–8]. Furthermore, a paradoxical effect (PE), defined as an attenuation of activity of these antifungals at higher concentrations despite potency at lower levels, has been observed in *in vitro* and animal models. However, the extent of this effect varies depending on the specific echinocandin, fungal species, and model used in the study [9–13]. It should be noted that there is a paucity of systematic, direct comparative studies that define the differences in echinocandin activity against *Candida* biofilms. In a prior comparative *in vitro* investigation from our lab, we saw a marked PE with CAS, a moderate one with ANID, but none with MICA when used against *C. albicans* reference strain SC5314 [14].

From a resistance standpoint, clinical and laboratory isolates of *C. albicans* and non-*Candida albicans* *Candida* species with high levels of resistance to echinocandins have now been described, and while the number of reported echinocandin-resistant clinical isolates is small, the numbers are increasing [15–20]. Key mutations in the *C. albicans* FKS1 gene encoding a major subunit of the glucan synthase enzyme in these caspofungin-resistant (CAS-R) mutants have been identified [21,22]. In collaboration with the Fungus Testing Laboratory (Department of Pathology, University of Texas Health Science Center at San Antonio), we obtained a collection of *Candida* clinical isolates with *in vitro* CAS-resistance (minimum inhibitory concentration [MIC] > 2 µg/ml), including 12 *C. albicans* isolates, and identified characteristic mutations in FKS1 using both pyrosequencing and Sanger sequencing in these strains [23].

Although certain mutations in *fks1* “hot-spot” regions result in phenotypic resistance to the echinocandins, it is unclear whether the same echinocandin activities and PE occur when the yeast occurs in biofilms. Moreover, little is known regarding the overall characteristics of biofilms produced by these CAS-R *fks1* mutant strains. Therefore, we sought to compare the *in vitro* activity of ANID, CAS, and MICA against *C. albicans* CAS-R clinical isolates and to characterize the occurrence of the PE of each echinocandin within biofilms formed by these strains. In addition, we sought to define structural characteristics of biofilms formed by these echinocandin-resistant *C. albicans* strains.

## Materials and methods

### Strains and antifungal agents used

Starter cultures were routinely grown at 30°C in 1% yeast extract, 2% peptone, 2% glucose broth supplemented with uridine (80 µg/ml). Wild-type *C. albicans* SC5314 was used as a reference strain [24], while *C. albicans* ATCC10231, ATCC14053 (fluconazole-resistant), and ATCC24433 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and used as control strains. Twelve *C. albicans* *fks1* mutant clinical isolates were acquired from the University of Texas Health Sciences Center at San Antonio Fungus Testing Laboratory (San Antonio, TX, USA) and used throughout the study. Characterization of these mutants demonstrated reduced CAS susceptibility (MIC > 2 µg/ml) and were found to be CAS-resistant when MICs were determined in the presence of 50% human serum [23]. FKS1 mutations in these strains were determined by both pyrosequencing and Sanger sequencing [23], as indicated in Tables 1 and 2. The echinocandin powders, that is, ANID (Pfizer Inc., New York, NY), CAS (Merck & Co, Inc., Whitehouse Station, NJ), and MICA (Astellas Pharma, Inc., Deerfield, IL, USA) were purchased from the hospital pharmacy and reconstituted in sterile water.

### Assessment of *in vitro* growth and filamentation

*In vitro* growth rates were assayed in liquid media by first diluting cells from overnight cultures to an optical density of 0.05, read at a wavelength of 600 nm (OD600nm), in complete synthetic media supplemented with uridine (80 µg/ml). The cells were then grown at 30°C using a Synergy H1m (BioTek Instruments, Inc., Winooski, VT, USA) with
Table 1. Comparison of echinocandin activity on planktonic (pMIC<sub>80</sub>) and sessile (sMIC<sub>80</sub>) cells of *Candida albicans* fks1 mutants.

<table>
<thead>
<tr>
<th><em>C. albicans</em> strain (mutation)</th>
<th>Anidulafungin (µg/ml)</th>
<th>Caspofungin (µg/ml)</th>
<th>Micafungin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pMIC&lt;sub&gt;80&lt;/sub&gt;</td>
<td>sMIC&lt;sub&gt;80&lt;/sub&gt;</td>
<td>pMIC&lt;sub&gt;80&lt;/sub&gt;</td>
</tr>
<tr>
<td>2762 (F641S)</td>
<td>≤0.25</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>32746 (F641S)</td>
<td>0.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4254 (F641S)</td>
<td>≤0.25</td>
<td>≥128</td>
<td>2</td>
</tr>
<tr>
<td>4715 (F641S)</td>
<td>≤0.25</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>41509 (F641S)</td>
<td>0.5</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>42996 (F641S)</td>
<td>≤0.25</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>43001 (F641S)</td>
<td>≤0.25</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>41301 (F641S)</td>
<td>≤0.25</td>
<td>≥128</td>
<td>2</td>
</tr>
<tr>
<td>42379 (S645P)</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>53264 (S645P)</td>
<td>0.5</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>42286 (S645P/S)</td>
<td>≤0.25</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5415 (S645P/H)</td>
<td>≤0.25</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>SC5314 (WT)</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>1</td>
</tr>
</tbody>
</table>

The MIC<sub>80</sub> represents the lowest echinocandin concentration that inhibited 80% of the metabolic activity of the cells. Abbreviations: MIC, minimum inhibitory concentration; WT, wild-type FKS1.

Table 2. Comparison of paradoxical effect concentrations of planktonic and sessile cells of *Candida albicans* fks1 mutants.

<table>
<thead>
<tr>
<th><em>C. albicans</em> strain (mutation)</th>
<th>Anidulafungin (µg/ml)</th>
<th>Caspofungin (µg/ml)</th>
<th>Micafungin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Planktonic</td>
<td>Biofilm</td>
<td>Planktonic</td>
</tr>
<tr>
<td>2762 (F641S)</td>
<td>≥128</td>
<td>≥128</td>
<td>—</td>
</tr>
<tr>
<td>32746 (F641S)</td>
<td>≥128</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4254 (F641S)</td>
<td>64</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4715 (F641S)</td>
<td>≥128</td>
<td>—</td>
<td>—</td>
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<tr>
<td>41509 (F641S)</td>
<td>—</td>
<td>—</td>
<td>≥128</td>
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<tr>
<td>42996 (F641S)</td>
<td>—</td>
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<tr>
<td>43001 (F641S)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>41301 (F641S)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>42379 (S645P)</td>
<td>≥128</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>53264 (S645P)</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>42286 (S645P/S)</td>
<td>≥128</td>
<td>64</td>
<td>≥128</td>
</tr>
<tr>
<td>5415 (S645P/H)</td>
<td>—</td>
<td>32</td>
<td>—</td>
</tr>
<tr>
<td>SC5314 (WT)</td>
<td>≥128</td>
<td>—</td>
<td>≥128</td>
</tr>
</tbody>
</table>

The paradoxical effect concentration was defined as the highest concentration at which the metabolic activity of the cells was observed to increase at two dilutions above the MIC<sub>80</sub>. A dash denotes that no paradoxical effect was observed. Abbreviations: MIC, minimum inhibitory concentration; WT, wild-type FKS1.

Double orbital shaking at fast speed and 2 mm frequency, with OD<sub>600 nm</sub> readings taken at 15-min intervals. Filamentation was assessed on solid RPMI-1640 media with L-glutamine and buffered with 0.165M 4-morpholinepropanesulfonic acid at pH 7.0. Overnight cultures were washed with 1× phosphate-buffered saline (PBS) and portions spotted to agar plates that were then incubated at 37°C for 5 d. Colonies were visualized, and the degree of filamentation was assessed based on presence/absence and density of hyphal structures.

**Determination of planktonic MICs using XTT**

We determined planktonic MICs using the Clinical and Laboratory Standards Institute M27-A3 broth microdilution method [25], incubating at 35°C for 24 h. However, rather than reading turbidity, we determined the MICs using the XTT-reduction assay [26], where the reduction of the tetrazolium salt, (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) (XTT), is followed spectrophotometrically.
effects of the antifungal agents were then expressed as the percent of metabolic activity relative to the metabolic activity of the untreated planktonic cells.

Biofilm formation and XTT assay
For biofilm studies, cells were washed twice in 1× PBS and resuspended at a density of 1 × 10⁶ cells/ml in RPMI-1640 supplemented with L-glutamine (US Biological, Swampscott, MA, USA) and buffered to pH 7.0 with 0.165M MOPS (Sigma, St. Louis, MO, USA; buffered RPMI-1640). The methods used for C. albicans biofilm formation in polystyrene 96-well microtiter plates and the XTT-reduction assay to determine the metabolic activities of the biofilms were performed as previously described [26]. Serial, twofold dilutions indexed to base 2 of the echinocandin (from 0.25 to 128 µg/ml) were added to preformed (24 h) biofilms formed in buffered RPMI-1640 and incubated at 37°C for 24 h. Each experiment was independently performed three times, in quadruplicate. Drug-free biofilm wells containing only buffered RPMI-1640 were used as untreated controls. The production of formazan was measured spectrophotometrically at an optical density of 490 nm on a microplate reader (BioTek ELx808; BioTek Instruments, Inc., Winooski, VT, USA). The effects of the antifungal agents were then expressed as the percent of metabolic activity relative to the metabolic activity of the untreated biofilms (controls).

Crystal violet assay
Biofilm mass was quantified using the crystal violet assay as previously described [27]. Biofilms were grown in triplicate at a concentration of 1 × 10⁶ cells/ml in buffered RPMI-1640 at 37°C for 24 h. Biofilms and controls (SC5314 and empty wells) were washed three times with 200 µl of PBS. Next, 50 µl of crystal violet solution (0.6 g crystal violet, 10 ml isopropanol, 10 ml methanol, and 180 ml water) was added to each well and allowed to stand at room temperature for 5 min, after which any excess crystal violet dye was removed by washing the biofilms three times with 200 µl of water. Then 100 µl of ethanol was added to each well and mixed thoroughly by gently pipetting up and down until the crystal violet solution was uniform in color; 75 µl of the ethanol/crystal violet solution was then transferred to a new 96-well microtiter plate and the light absorbance of the crystal violet solution read at 630 nm.

Light and scanning electron microscopy
Light micrographs of the biofilms formed in the presence of each test reagent were acquired using an inverted microscope (Micromaster Digital Inverted Microscope with Infinity Optics; Fisher Scientific, Pittsburgh, PA, USA) and data acquisition software (Micron 2.0.0; Westover Scientific, Mill Creek, WA, USA). Sample preparation for scanning electron microscopy (SEM) was performed on biofilm samples formed on a coverslip (Thermanox; Nalge Nunc International, Rochester, NY, USA) after 24 h incubation of a 0.5-ml inoculum containing 1 × 10⁶ cells/ml in buffered RPMI-1640 medium as previously described [28]. Scanning electron micrographs were obtained using a scanning electron microscope (Hitachi S-800; Hitachi High Technologies America, Inc., Pleasanton, CA, USA) set at 20 kV with images acquired using Quartz PCI software (Quartz Imaging Corporation, Vancouver, BC, Canada).

Definitions
Antifungal activity was defined as a statistically significant reduction in the metabolic activity of C. albicans planktonic cells or biofilms treated with an echinocandin compared with the metabolic activity of the untreated cells. MIC₈₀ represents the lowest echinocandin concentration that inhibited 80% of metabolic activity of either planktonic cells (pMIC₈₀) or biofilms (sMIC₈₀). A PE was defined as regrowth at two dilutions above the MIC₈₀ [29]. For strains exhibiting a PE, the concentration at which the metabolic activity was the greatest was termed the PE concentration (PEC).

Statistical analyses
The metabolic activities of the treatment groups were compared to the controls using one-way analysis of variance and Tukey multiple comparisons post-test. Differences were considered significant at P < 0.05. The relationship between the crystal violet and XTT assay values was calculated using the Pearson product correlation. Statistical analyses were performed, as well as graphs produced, with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

Results
Analyses of biofilm mass and metabolic activity
Biofilm mass and metabolic activity were assessed and categorized as follows: poor (≤25% mass/metabolic activity relative to SC5314), moderate (between 25% and 75% mass/metabolic activity relative to SC5314), and strong (≥75% mass/metabolic activity relative to SC5314). There were variable biofilm masses, as measured by crystal violet assay, among the fks1 mutant strains in comparison...
Figure 1. Assessment of biofilm mass of Candida albicans reference strains and fks1 clinical isolates. Biofilms were grown in triplicate at a concentration of $1 \times 10^6$ cells/ml in buffered RPMI-1640 at 37°C for 24 h. Biofilm mass was quantified using the crystal violet assay. Light absorbance was measured in a plate reader at OD 630 nm. Each experiment was performed independently three times.

Figure 2. Assessment of biofilm metabolic activity of Candida albicans reference strains and fks1 clinical isolates. Biofilms were grown in quadruplicate at a concentration of $1 \times 10^6$ cells/ml in buffered RPMI-1640 at 37°C for 24 h. The XTT assay was used to assay sessile metabolic activity. Formation of the colored formazan was subsequently measured at OD 490 nm. Each experiment was performed independently three times.

with SC5314 (Fig. 1). Two fks1 mutant strains (32746 and 5415) were categorized as poor biofilm producers, four (2762, 4254, 4715, and 42286) and the three ATCC reference strains were moderate biofilm producers, and six fks1 mutant strains (41509, 42379, 42996, 43001, 53264, and 41301) were categorized as strong biofilm producers. In addition to biofilm mass, the metabolic activities within the biofilms were ascertained using the XTT assay (Fig. 2), which indicated that, with the exception of strains 53264 and 41301, all of the fks1 strains had poor to moderate metabolic activity compared with wild-type SC5314. Two of the ATCC reference strains had strong metabolic activity similar to that found in SC5314, but ATCC 10231 had little metabolic activity even though its biofilm mass was comparable to that of the other ATCC strains. Overall correlation between the XTT-reduction assay and crystal violet assay was positive and highly statistically significant (correlation coefficient 0.47; $P < 0.0001$). However, it should be noted that results of the crystal violet and XTT assays from several of the individual strains were discordant in terms of biofilm mass and biofilm activity. We were unable to identify a clear association between FKS1 hot-spot mutation in these strains and degree of biofilm formation.

Assessment of in vitro growth and filamentation

We assessed both in vitro growth and filamentation capacity of the strains, but found there was no correlation between in vitro growth rates (doubling times) and the degree of biofilm formation in these strains (data not shown). The degree of filamentation of each strain was also assayed on solid RPMI-1640 agar. There was no apparent correlation in the degree of filamentation and the mutations in FKS1 or in the degree of biofilm formation (data not shown).

Structural analyses using light and scanning electron microscopy

Direct visualization of SC5314 biofilm morphology revealed a dense, filamentous growth with minimal yeast forms present (Fig. 3a). Similar filamentous growth was observed for the strong biofilm-producing fks1 mutant strains 42379, 42996, and 53264, but there were more yeast forms observed in the preformed (24 h) biofilms in comparison with SC5314 (Fig. 3a). The fks1 mutant strains that produced poor to moderate biofilm growth demonstrated less dense filamentous growth with many yeast forms present (Fig. 3a).

In order to assess biofilm ultrastructure in detail, SEM studies were conducted with C. albicans strains SC5314 and a representative fks1 mutant with poor (4254), moderate (42286), and strong (53264) biofilm growth (Fig. 3b). SC5314 formed a dense, filamentous biofilm with minimal yeast forms present in the mature biofilm. While the fks1 mutant produced a strong biofilm (53264) with filamentous growth, there was a marked increase of yeast forms present that was more clearly seen using SEM. The other fks1 mutants similarly produced biofilms with a predominance of yeast forms and pseudohyphae. Interestingly, we observed pits on the cell surface of fks1 mutants 42286 and 4254, both of which formed less robust biofilms (Fig. 3b). These pit-like structures were consistent throughout the biofilms formed by these fks1 mutants, suggesting a structural defect in the cell surface of these strains, although the specific origin of these defects is not clear. Strain 53264 also had some pit-like structures, but the structures appeared less frequently than in the other two fks1 mutant strains (Fig. 3b).
**Figure 3.** Ultrastructural assessment of biofilm morphology using scanning electron microscopy. (A) Scanning electron microscopy of representative *Candida albicans fks1* mutants that form poor (4254), moderate (42286), and strong (53264) biofilms compared with reference strain SC5314. (B) Scanning electron microscopy view of pit-like cell surface structures identified on select *C. albicans fks1* mutants.

**Effect of echinocandins on biofilm metabolic activity**

The effect of echinocandins on preformed *C. albicans fks1* biofilms was assayed using the XTT-reduction assay. Notably, among all *C. albicans fks1* strains, ANID demonstrated the greatest antifungal activity against preformed biofilms with the lowest sMIC. In 11 of 12 *C. albicans fks1* strains (Table 1), sMICs of *C. albicans* biofilms to ANID, MICA, and CAS were at least two- to three-fold higher than the MICs of their planktonic counterpart (pMIC). In some strains, there was a dramatic increase of sMIC compared with pMIC for CAS; in contrast, in strain 41509, the pMIC was higher than in the sMIC to CAS (Table 1).
Characterization of the PE

A PE was observed within most fks1 biofilms at higher echinocandin concentrations (Table 2). CAS was associated with a PE in 7 of the 12 (58.3%) fks1 strains in our in vitro biofilm assay, with the PE occurring at concentrations of 64 µg/ml or higher. ANID produced a PE in 3 of 12 (25.0%) fks1 strains, and MICA produced a PE in 3 of 12 (25.0%) fks1 strains (Table 2). A PE also occurred in a number of strains in the planktonic form, depending on the echinocandin used (Table 2). Although some strains had a PE in planktonic form, this did not necessarily correlate with a PE in biofilm form. Thus, the planktonic PE is not predictive of a biofilm PE.

Discussion

In this study, we demonstrated in vitro differences in echinocandin activity against biofilms formed by mutant clinical isolates of C. albicans fks1, but without any clear correlation with the type of fks1 hot-spot mutation carried by each strain. Overall, ANID was found to have the lowest sMIC₈₀ within biofilms of the three echinocandins tested. In vivo differences in echinocandin efficacy have recently been described in C. glabrata, which could be correlated with specific hot-spot mutations [5]. In this mouse model of disseminated C. glabrata infection, MICA had wild-type activity against a C. glabrata Fks2p-S663F mutant strain, in contrast to ANID and CAS. Thus, there appears to be increasing evidence of echinocandin-, species-, strain-, and mutation-specific differences in antifungal activity.

Although the echinocandins as a class retain excellent in vitro activity against Candida biofilms [30–32], paradoxical activity within biofilms and of the planktonic cells of Candida species has been described [6]. In a series of 60 Candida bloodstream isolates grown in planktonic form, the prevalence of paradoxical growth in the presence of CAS, ANID, and MICA was 60%, 40%, and 0%, respectively [10]. Of 127 C. dubliniensis and 103 C. albicans isolates, a PE was observed in 63% of C. dubliniensis strains with MICA; in 90% of C. dubliniensis, and in 14% of C. albicans isolates with CAS. Note that no PE was reported with ANID [11]. Within biofilms, Melo et al. described a PE in 30 clinical Candida spp. isolates in an in vitro biofilm model with CAS [13]. When compared with these strains grown in planktonic form, the PE was overall seen more frequently within biofilms, although the presence of a PE within individual strains in planktonic form did not correlate with occurrence of a PE when grown as a biofilm. In our in vitro studies, we also saw a PE in both planktonic and sessile forms of the strains tested. As with these previously published studies, there was no direct correlation between the PE within planktonic or sessile forms or the echinocandin used. Specifically, the PE profile observed in the planktonic form is not predictive of a PE in the biofilm form. In a neutropenic mouse model of invasive pulmonary aspergillosis, a modest PE (increased fungal burden) occurred with CAS but not MICA [12]. However, in a murine model of disseminated candidiasis, C. albicans strains that demonstrated an in vitro PE of CAS did not display a consistent PE in vivo [33]. It should be noted that the PE can be eliminated in the presence of 50% serum in vitro [34,35], and thus the findings of this study must be interpreted cautiously from a clinical standpoint.

There are limited data on the mechanisms responsible for the PE. Potential mechanisms responsible for this phenomenon include an increase in cell wall chitin content [36], upregulation of the protein kinase C cell wall integrity pathway, and involvement of the calcineurin pathway [37]. Interestingly, brief exposures to caspofungin resulted in killing of C. albicans in vitro due to a prolonged antifungal effect but did not result in a PE, suggesting a compensatory response occurs with prolonged exposure to drug [34]. Of note, the PE has not been associated with mutations in known resistance-related regions or with increased expression of FKS1 in the yeast form [38]. In the biofilm-related experiments presented here, a PE occurred in the presence of the echinocandins with no clear association with the type of FKS1 hot-spot mutation. However, it should be noted that these clinical isolates are not fully characterized and other, as yet unknown, genetic factors may contribute to the resulting PE profile.

There is a paucity of data regarding the ability of echinocandin-resistant strains to form biofilms. Given the importance of glucan synthase in cell wall biogenesis and maintenance, we postulated that these fks1 clinical isolates may be less able to form biofilms. Overall, the fks1 mutants tended to form biofilms of reduced metabolic activity and density than the robust biofilm-forming reference strain SC5314. These results were less pronounced when compared with additional ATCC clinical isolates, as they also formed less dense biofilms. In the strains we examined, there was no clear association between the type of hot-spot mutation and the degree of biofilm formation. It should be noted that these strains are not of the same genetic background, and thus a direct comparison between strains is not formally possible. Differences in each strain’s biofilm-forming capacity is likely due to multiple factors and not simply differences in FKS1 activity. While many of these strains have the same FKS1 hot-spot mutation, they have differing biofilm-forming phenotypes.
In contrast, from a structural standpoint, the \textit{fks1} mutant biofilms clearly had a much greater proportion of yeast and pseudohyphal structures than those of the reference \textit{C. albicans} strain SC5314, which was characterized by biofilms composed of dense hyphae. Of further interest, at least two of the three selected \textit{fks1} mutants studied with SEM demonstrated pit-like structures on their cell surfaces, suggestive of a substantial cell surface defect. The third \textit{fks1} mutant strain also had some surface pits, but these pits were less consistent than the mutants that formed biofilms of reduced mass. While the significance of these pits is unknown, they do not appear to be consistent with bud scars, and it is unlikely that they represent artifacts since they were observed throughout the biofilms of the \textit{fks1} mutants and not in wild-type control strain SC5314. These pits bear a resemblance to the cell surface defects characterized by SEM in wild-type \textit{C. albicans} biofilms treated with CAS, which inhibit glucan synthase [39,40].

In summary, we compared the \textit{in vitro} activity of ANID, CAS, and MICA against biofilms formed by a set of clinically derived \textit{fks1} mutant strains in order to characterize antifungal and paradoxical activity of each echinocandin. We identified the occurrence of a strain- and drug-dependent PE in these strains, but there was no correlation between the PE observed in the planktonic form of the \textit{fks1} mutant strains and the PE observed in their corresponding biofilm. The \textit{fks1} mutant strains demonstrated the lowest sMICs in wild-type \textit{C. albicans} species-related differences. We also demonstrated that biofilm growth was altered among the \textit{fks1} mutants, with a predominance of yeast and pseudohyphae and of pit-like structures on the cell surface of several representative strains.

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Declaration of interest

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