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ARTICLE

Paradoxical growth effects of the echinocandins caspofungin and micafungin, but not of anidulafungin, on clinical isolates of *Candida albicans* and *C. dubliniensis*

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Abstract *Objectives* To analyze the effects of a high concentration of three antifungal substances, the echinocandins anidulafungin, caspofungin, and micafungin, on the growth of *Candida spp*. *Methods* The growth of 127 *C. dubliniensis* isolates and 103 *C. albicans* isolates cultured in medium containing anidulafungin, caspofungin, or micafungin was analyzed using a broth microdilution test according to the guidelines of the CLSI M27-A2 [NCCLS (1997), Wayne, PA]. The final concentrations of all three echinocandins ranged from 0.125 to 64 μg/L. *Results* The different effects of these three antifungal substances on *C. albicans* cells in comparison to *C. dubliniensis* cells were quite distinct. When both *Candida species* were grown in the presence of anidulafungin only a trailing effect was observed. Micafungin induced an Eagle effect in *C. dubliniensis* only (63%), while caspofungin induced this effect in the majority of *C. dubliniensis* isolates (90%) and in only a few *C. albicans* isolates (14%). *Conclusions* Based on our observations, anidulafungin has effects that are different from the ones produced by micafungin and caspofungin. Whether this different response to high concentrations of echinocandins is based on genetic or phenotypic differences between *C. albicans* and *C. dubliniensis* has to be determined in future experiments.

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Introduction

There are currently three echinocandins available – anidulafungin, caspofungin, and micafungin. These antifungal substances exhibit a concentration-dependent activity against-*Candida* species [1, 2]. As they also demonstrate a limited toxicity profile and minimal drug–drug interactions, they are an attractive new option for the treatment of invasive fungal infections. In addition, a resistance to echinocandins seems to be a rare event. When *Candida spp.* are grown in media containing high concentrations of antifungal agents, such as caspofungin, the result can be a reduced activity of these agents against certain organisms. This phenomenon is called the Eagle effect or paradoxical growth effect, and there are several reports on the Eagle effect in fungi [3–8]. In addition to the Eagle effect, high concentrations of an antifungal agent can also result in a trailing growth effect (or trailing) during serial dilution testing in which there is a reduced but persistent growth of *Candida spp.* in medium containing high concentrations of an antifungal agent [9, 10]. Jacobsen et al. described a trailing effect in *Candida spp.* but only when the EUCAST protocol was used but not with the CLSI M27-A2 method [10]. To date, this trailing effect has only be found when*Candida spp.* have been cultivated in the presence of azoles – and not with other agents.

The MIC_{90} of anidulafungin for *C. albicans* and *C. dubliniensis* has been determined previously to be in the range of 0.03 (*C. albicans*) to 0.06 mg/L (*C. dubliniensis*), for micafungin, 0.03 mg/L (for both species), and for caspofungin, 0.5 mg/L for *C. albicans* and *C. dubliniensis* [12–14]. In order to analyze the paradoxical growth effect and trailing in greater detail, we examined a large number of *C. albicans* and *C. dubliniensis* isolates and determined their response to high concentrations of these three antifungal substances.

Materials and methods

Fungal strains

We included 127 *C. dubliniensis* isolates and 103 *C. albicans* isolates in this study. Of these, two *C. dubliniensis* strains and five *C. albicans* strains were reference strains (Tables 1, 2). All other isolates were collected in our laboratory from the oral cavities of patients with human immunodeficiency virus infection and recurrent oropharyngeal candidiasis (OPC); a few isolates have been described in an earlier publication [15]. The clinical isolates were identified by standard cultivation laboratory methods and by arbitrarily primed (AP)-PCR (data not shown).

Minimum inhibitory concentration and susceptibility tests

The broth microdilution test was performed strictly according to the guidelines of the CLSI M27-A2 document [16]. An inoculum of 10^3 cells per mL and RPMI 1640 medium buffered to pH 7.0 with 0.165 *M* morpholino-propanesulfonic acid (MOPS) (Sigma, Germany) were used. Yeast inocula (100 μL) were added to each well of U-shaped microdilution trays, with each well containing 100 μL of the antifungal agent at double strength. The final concentrations of anidulafungin, micafungin, and caspofungin ranged from 0.125 to 64 mg/L (ten reading points and twofold dilution steps). Drug-free and yeast-free controls were also included. The MIC was defined as the concentration of the drug that completely inhibited growth or produced an 80% reduction of turbidity compared with the drug-free control. The plates were incubated in air at 35°C for 2 days (i.e. 46–50 h), and the analysis was performed visually using a reading mirror for microtiter plates. Separate dilution series of all antifungal agents were made from the stock solution using the RPMI 1640 medium. For the statistical analysis, we usedGRAPHPAD PRISM, v 4.03 (GraphPad Software, San Diego, CA) software and considered a P value of ≤ 0.05 to be significant.

Results and discussion

The detection of the Eagle effect requires some skill and experience. We considered a reading mirror for the microtiter plates to be the most suitable instrument for this purpose. This mirror has an additional advantage of having a magnifying effect, thereby facilitating the reading. The results summarized in Tables 1 and 2 show a MIC_{90} of ≤ 0.125 mg/L for anidulafungin, micafungin, and caspofungin for both species. These antifungal substances also had a very distinct effect on *C. albicans* in comparison to *C. dubliniensis* cells. Both*Candida* species grown in the presence of anidulafungin produced a trailing effect exclusively. This trailing is the residual turbidity (as observed in liquid cultures in microtiter plates) observed at very high concentrations (i.e. above the MICs) of antifungal substances, indicating an incomplete growth inhibition. While only a few *C. albicans* isolates showed this effect (3/103), the majority of *C. dubliniensis* isolates (101/126) demonstrated a trailing, and this difference is statistically significant ($P \leq 0.0001$). Micafungin (1/72 in *C. albicans* and 3/126 in *C. dubliniensis*) and caspofungin (6/101 in*C. albicans* and 1/124 in *C. dubliniensis*) produced a trailing effect in only a few isolates in both species, and the difference between *C. albicans* and *C. dubliniensis* was not significant ($P=0.635$ and$P=0.224$, respectively). Interestingly, anidulafungin did not induce an Eagle effect in either*Candida* species, while micafungin (1/72 in *C. albicans* and 80/126 in *C. dubliniensis*) and caspofungin (14/101 in *C. albicans* and 112/124 in *C. dubliniensis*) were capable of inducing this effect. The cultures grew in the presence of low drug concentrations, showed no growth at intermediate concentrations, and again showed growth at

Table 1 Growth effects of anidulafungin, micafungin and caspofungin on *Candida albicans*

Growth effects	Anidulafungin – 103 strains tested	Micafungin – 72 strains tested	Caspofungin – 10 strains tested
MIC_{90}[a] of ≤0.125 μg/mL	102/103 (99%)	71/72 (99%)	99/101 (98%)
MIC_{90} of 0.25 μg/mL	1/103 (1%)	1/72 (1.4%)	2/101 (2%)
Trailing effect	3/103 (3%), ranging from 0.25 to 0.5 μg/mL	1/72 (1.4%), ranging from 1 to 32 μg/mL	3/101 (3%), up to 0.25 μg/mL; 3/101 (3%) up to 8 or 16 μg/mL
Eagle effect	None	None	14/101 (14%), ranging from 2 to 32 μg/mL

Five *C. albicans* reference strains were included, i.e. ATCC 76615, Y 0119, ATCC 36801, ATCC 44373 and ATCC 90028. The clinical isolates were collected in our laboratory from the oral cavities of patients with human immunodeficiency virus infection and recurrent oropharyngeal candidiasis (OPC)

[a] Minimum inhibitory concentration required to inhibit the growth of 90% of organisms

Table 2 Growth effects of anidulafungin, micafungin and caspofungin on *Candida dubliniensis*

Growth effects	Anidulafungin – 127 strains tested	Micafungin – 126 strains tested	Caspofungin – 124 strains tested
MIC_{90}[a] of ≤ 0.125 μg/mL	127/127 (100%)	126/126 (100%)	112/124 (90%)
MIC_{90} of 0.25 μg/mL	None	None	11/124 (9%)
MIC_{90} of 0.5 μg/mL	None	None	1/124 (0.8%)
Trailing effect	101/127 (80%) ranging from 0.25 to 8 μg/mL (in one case up to 16 μg/mL)	3/126 (2%) up to 64 μg/mL	1/124 (0.8%) up to 0.5 μg/mL
Eagle effect	None	80/126 (63%) ranging from 0.5 up to 64 μg/mL	1/124 (0.8%) up 4 μg/mL; 6/124 (5%) from 0.5 to 8 μg/mL; 103/124 (83%) from 1 to 16 μg/mL; 2/124 (1.6%) from 1 to 32 μg/mL

Two *C. dubliniensis* reference strains were included, i.e. CBS 8500 and CBS 8501. The clinical isolates were collected in our laboratory from the oral cavities of patients with human immunodeficiency virus infection and recurrent oropharyngeal candidiasis (OPC)

[a] Minimum inhibitory concentration required to inhibit the growth of 90% of organisms

high concentrations of two of the echinocandins, i.e. micafungin and caspofungin. While micafungin induced an Eagle effect in the majority of *C. dubliniensis* isolates, only a few *C. albicans* isolates showed this effect. With caspofungin, we observed an Eagle effect in all *C. dubliniensis* isolates, in contrast to *C. albicans* in which only a few isolates demonstrated this effect. The difference in the numbers of isolates showing an Eagle effect was significant for both agents ($P \leq 0.0001$). Since we did not aim at determining the MIC, the minimum drug concentration used in some of our experiments was too high to establish this value correctly.

The Eagle effect in fungi has been reported by a number of researchers. Hall et al. reported that cilofungin, a semisynthetic antifungal agent, showed both inhibitory and fungicidal activity against some members of the genus*Candida* [17]. When Sabouraud dextrose broth and yeast nitrogen base broth were used instead of antibiotic medium no. 3, the isolates of *C. albicans* and *C. tropicalis* demonstrated an Eagle effect in that growth was partially inhibited at MICs equivalent to those in antibiotic medium no. 3, but growth continued, in many instances, throughout all concentrations tested. An Eagle effect with a number of *C. albicans* strains against aculaecin A was also described by Iwata et al. [18]. Pfaller and coworkers found that 58% of the *C. albicans* and 27% of the *C. tropicalis* isolates demonstrated an Eagle effect when the cells were grown in the presence of cilofungin (LY121019, an analog of echinocandin B) at higher concentrations, i.e. 10–40 mg/L [19]. An Eagle effect has previously been reported for caspofungin with several *Candida spp* [12], for all echinocandins with four different *Candida spp.* [20], and for itraconazole with *C. albicans* [21]. Arikan et al. [7] analyzed *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. kefyr*, *C. krusei*, *C. lusitaniae*, *C. norvegensis*, *C. guilliermondii* and *C. lipolytica* cells and observed an Eagle effect in 31 and 8% of the isolates at highest concentrations of caspofungin and itraconazole, respectively. Interestingly, caspofungin produced an Eagle effect for various *Candida species*, while itraconazole showed an Eagle effect for isolates of *C. albicans* and *C. tropicalis* only. While in all of these studies the Eagle effect was analyzed in vitro, there are two studies in which this effect was examined in vivo as well. Clemons et al. observed a paradoxical growth effect in vivo when *C. albicans* cells were inoculated into mice who were subsequently treated with various dosages of caspofungin. These researchers found that a paradoxical fungal response occurred with some*Candida* isolates but not with others and that it could not be reproducibly demonstrated in vivo [22]. Gumbo et al. did not observe a paradoxical increase in fungal burden when up to 100 mg/kg of micafungin was administered to mice, but these researchers only examined one*Candida* isolate [23]. These observations led us to conclude that the Eagle effect is more an in vitro phenomenon and may be less relevant in the clinical setting.

To date, the occurrence of a trailing effect with *Candida spp.* has mainly been described for drugs belonging to the azole group [24]. Our results clearly demonstrate that a trailing phenomenon can also be seen when *C. dubliniensis* is grown in the presence of echinocandins in general and anidulafungin specifically; caspofungin, and micafungin were much less effective in inducing a trailing effect. Jacobsen et al. recently reported on a different response of *C. albicans* and *C. dubliniensis* to echinocandins and compared the CLSI method with the EUCAST test [11]. The results obtained by these researchers with*C. albicans* are comparable to ours. In contrast, we observed a trailing

of *C. dubliniensis* isolates almost exclusively with anidulafungin, while Jacobsen et al. found that micafungin and caspofungin induced the same effect in their experiments. Most interesting is the observation that Jacobsen and coworkers found their effects exclusively using the EUCAST method, finding no effect at all using the CLSI method. Both methods are known to yield different results when *Candida spp.* were tested for susceptibilities to different azole agents, but whether this difference is the explanation for the variable results is unknown as yet [25].

The principal mechanism of action of the echinocandins had been described as a non-competitive inhibition of β-(1,3)-D-glucan synthase, an essential component of the cell wall of many fungi [2]. A reduction of the ergosterol content has been demonstrated as well [26]. Another antifungal drug, aculaecin A, which is able to induce a paradoxical growth effect, has been shown to inhibit the ß-1,3-glucan synthase reaction [27]. The question which emerges from our results is why the three antifungal substances, which are thought of having the same target, produce these different effects (i.e. trailing vs. Eagle effect) on *C. albicans* and *C. dubliniensis*, and this cannot be answered as yet. The second question which comes up is why the frequency of the Eagle effect seen in *C. dubliniensis* is much higher than that in*C. albicans*. This result is even more surprising since a very high degree of phenotypic similarity between *C. albicans* and *C. dubliniensis* has been described [28, 29].

Stevens et al. [3, 4] did not demonstrate an Eagle effect in a smaller group of *C. albicans* isolates, but we found that 14% of our *C. albicans* isolates showed this growth pattern in the presence of high concentrations of caspofungin, but not micafungin. We also observed an Eagle effect in the majority of *C. dubliniensis* isolates with both micafungin and caspofungin, which is in contrast to published observations [30]. Stevens et al. demonstrated that the growth of one particular *C. albicans* strain in a high Caspofungin concentration resulted in the concurrent decline of ß-1,3-glucan and ß-1,6-glucan content and a greatly increased chitin content [31]. These results suggest that changes in the expression of the genes involved in this metabolic pathway play an important role. We do not yet know whether growth in the presence of high concentrations of micafungin and caspofungin affects the same genes. In addition it is not clear whether the growth of *C. albicans* and *C. dubliniensis* at high concentrations of echinocandins has the same genetic basis. The mean peak serum concentration of caspofungin was determined to be 12.1 μg/L after a single 70-mg dose, but concentrations in some tissues appeared to greatly exceed this value [3, 4]. Whether the Eagle effect might have any biological or clinical meaning (so far there has no clinical correlate been described) is unknown to date. Based on our results, we hypothesize that the mode of action of anidulafungin is different from that of micafungin and caspofungin. In addition, we assume that the genetic differences between *C. albicans* and *C. dubliniensis* are larger than one might expect given that these two species are phenotypically very similar.

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