Original Article

Study on the comparative activity of echinocandins on murine gut colonization by Candida albicans

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Abstract

Colonization of the gastrointestinal (GI) tract by Candida species is a principal pathogenic event for development of invasive candidiasis. Importantly, the effect of echinocandins, the preferred antifungal agents for treatment of invasive candidiasis, on GI tract colonization by Candida spp. is currently unknown. Herein, we used an established model of persistent murine GI tract colonization by Candida albicans to test the ability of different echinocandins to eradicate the yeast from murine gut. Adult male Crl:CD1 (ICR) BR mice were fed with chow containing C. albicans and subsequently treated with different echinocandins or normal saline via daily intraperitoneal injections for 10 days. Quantitative stool cultures were performed immediately before (week one), and weekly for three months after discontinuation of treatment. Notably, treatment with all three echinocandins used (caspofungin, anidulafungin, and micafungin) resulted in eradication of Candida albicans from the stools, as evidenced by the significant reduction of yeast cells from a mean of 4.2 log10 CFU/g of stool before treatment (week one of colonization) to undetectable (<2 log10 CFU/g of stool) levels (week 12, P < 0.0001). In contrast, there was no significant reduction of Candida yeast cells in the stools of control mice. Collectively, the ability of echinocandins to eradicate C. albicans from the stools could have important implications in prophylaxis of high-risk patients for development of invasive candidiasis originating from the GI tract.

Key words: Candida albicans, gastrointestinal tract, mice, caspofungin, anidulafungin, micafungin.
Introduction

Invasive fungal infections are important causes of morbidity and mortality in a large group of severely immunocompromised and debilitated patients [1, 2]. Candida species are the most common cause of invasive fungal infections in this group of patients [1, 3]. In addition, Candida is the fourth most common bloodstream pathogen in US hospitals accounting for 8–10% of cases of nosocomial sepsis [4, 5].

C. albicans is the species that accounts for most cases of invasive candidiasis although infections caused by non-albicans species, including C. glabrata, C. parapsilosis, C. tropicalis, and C. krusei, are on the rise [1]. As opposite to most other pathogenic fungi, Candida is a commensal of the human GI tract [6, 7]. Furthermore, the gastrointestinal (GI) tract is commonly the source of invasive candidiasis in patients being previously colonized by the yeast [6, 7]. In fact, if the yeast concentration increases beyond a threshold via massive oral administration, Candida organisms can translocate through the intact GI mucosa into the bloodstream and spread to internal organs and result in disseminated infection in an immunocompetent host [8]. In clinical practice, disruption of normal host defense homeostatic mechanisms as a result of aggressive chemotherapy, administration of immunosuppressive agents, exposure to broad-spectrum antimicrobial agents, indwelling central venous catheters, mechanical ventilation, prior surgery and transplantation are associated with increased risk of Candida dissemination from the GI tract [9].

The introduction of fluconazole in the early 1990s became the cornerstone for antifungal prophylaxis in high-risk patients with haematological malignancies [10]. Because colonization of the GI tract is the initiating event in pathogenesis of systemic candidiasis in immunocompromised patients with neutropenia, prophylactic use of an antifungal agent should ideally lead to eradication of Candida from the gut. To that end, we have previously reported that fluconazole treatment failed to eradicate Candida albicans from the GI tract in an established model of persistent Candida GI tract colonization in immunocompetent mice [11].

Echinocandins display fungicidal activity against most Candida species and have become the preferred treatment for invasive candidiasis [12–15]. However, there are no studies evaluating the efficacy of echinocandins on decolonization of the GI tract from Candida organisms. In the present study, we tested the effects of different echinocandins to decolonize the GI tract of mice from C. albicans in our established mouse model of Candida long-term GI colonization, which can imitate the human gastrointestinal conditions [16].

Materials and methods

The antifungal agents used were caspofungin (MSD Hellas, Athens, Greece), anidulafungin (Pfizer Hellas, Athens, Greece), and micafungin (Astellas Pharmaceuticals, Athens, Greece), supplied by their commercial manufacturers. For in vitro susceptibility testing broth microdilution testing was performed in accordance with the guidelines in Clinical and Laboratory Standards Institute (CLSI) document M27-A3 [17], using RPMI 1640 medium with 0.2% glucose, an inoculum of $0.5 \times 10^3$ to $2.5 \times 10^3$ cells/ml, and incubation in aerobic conditions at $35^\circ C$. MIC values were determined visually after 24 h of incubation, as the lowest concentration of drug that caused a significant diminution ($\geq 50\%$ inhibition) of growth relative to that of the growth control [17]. In all instances, minimal inhibitory concentration (MIC) trays were prepared using reagent-grade powders, as directed by CLSI. Two quality control (QC) isolates, C. parapsilosis ATCC 22019 and C. krusei ATCC 6258, were used, as recommended by CLSI [17, 18].

Three month-old male, healthy Crl:CD1 (ICR) BR specific-pathogen-free mice, of approximately 30 g weight were used in all of the experiments (Fig. 1). We used our established model of persistent GI tract colonization of immunocompetent mice by C. albicans [16]. Briefly, the GI tract of mice was colonized with C. albicans by feeding the animals with chow containing the yeast at a mean concentration of $1.5 \times 10^7$ colony forming units (CFU)/g for a period of two weeks. The C. albicans isolate (No. 268) was recovered from a patient with invasive candidiasis and has been previously used successfully in several experiments of Candida GI colonization [11, 19]. The preparation of this chow has been previously described [16]. Briefly, food for control animals consisted of a mixture of 300 g of powdered chow and 360 ml sterile water, which had been spread on sterile petri dishes and dried at $37^\circ C$ for 48h. Candida containing chow was prepared by inoculation of 0.9 ml of an overnight Candida suspension (without agitation) in 360 ml of sterile water that was subsequently mixed with 300g of food powder. The mean concentration of the Candida suspension was $6.0 \times 10^6$ CFU/ml. The mixture was then spread into 0.5 cm thick layers on sterile plastic petri dishes and left to dry at $37^\circ C$ for 48h and stored in sterile plastic containers at room temperature. No significant differences were found in the concentration of C. albicans per gram of chow [16].

Quantitative stool cultures performed one week after the end of the special diet, confirmed GI colonization of mice by C. albicans. Stool specimens from each group of five mice (one stool pellet from each mouse) were homogenized and inoculated directly onto Sabouraud dextrose agar
Figure 1. Diagram showing the flow of animals colonized with Candida albicans and treated with echinocandins and control groups. This Figure is reproduced in color in the online version of Medical Mycology.

Table 1. Equivalence of antifungal dosage schedules in humans and mice calculated by the method of Freireich et al. [20].

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>70 kg human</th>
<th>30 g mice</th>
</tr>
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<tbody>
<tr>
<td>Caspofungin (loading dose)</td>
<td>70 mg q 24 hours</td>
<td>0.36 mg q 24 hours</td>
</tr>
<tr>
<td>Caspofungin (maintenance dose)</td>
<td>50 mg q 24 hours</td>
<td>0.26 mg q 24 hours</td>
</tr>
<tr>
<td>Anidulafungin (loading dose)</td>
<td>200 mg q 24 hours</td>
<td>1 mg q 24 hours</td>
</tr>
<tr>
<td>Anidulafungin (maintenance dose)</td>
<td>100 mg q 24 hours</td>
<td>0.51 mg q 24 hours</td>
</tr>
<tr>
<td>Micafungin</td>
<td>100 mg q 24 hours</td>
<td>0.51 q 24 hours</td>
</tr>
</tbody>
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q, every.

(SDA) with gentamicin and SDA with cycloheximide and chloramphenicol. Each stool pellet was collected separately from each mouse immediately after fecal shedding. Quantitative fungal stool cultures were prepared by mixing each gram of stool with 9 ml of sterile isotonic saline (0.9%) and emulsifying in a vortex mixer. Tenfold serial dilutions were made and 100 μl of each dilution was inoculated into each of the previously mentioned agar plates [16]. The fungal burden that could be detected with these cultures is ≥10² CFU/g of stools.

For comparative studies on the activity of the three different echinocandins on murine gut colonization by C. albicans, each group of 20 mice fed on C. albicans containing chow (for 14 days) that had been colonized with the yeast received caspofungin, anidulafungin, or micafungin by intraperitoneal administration on a daily basis for a total of 10 days (control). Additionally, 10 mice of the non-colonized group which were fed regular chow that did not contain yeast, were injected for 10 days with either the same antifungal agent or 300 μl of normal saline, served as control of C. albicans colonization as shown in Figure 1. For statistical comparisons on the effect of echinocandins on reduction of yeast CFUs in stool week 1 (day 0 of echinocandin treatment) and week 12 of colonization, we compared results from two different experiments with the use of the Mann–Whitney U test. For statistical comparisons of stool fungal burden across different groups before initiation of treatment (week 1) we used one-way analysis of variance, with Tukey’s post test for multiple comparisons. Statistical analyses were performed with Graph-Pad Prism software, version 4.0 (San Diego, CA). We considered P values of less than 0.05 to be statistically significant.

The dose schedules of all echinocandins were equivalent to those of humans and were calculated by the method of Freireich et al. [20] as shown in Table 1. All experiments were performed in duplicate.

Results

The C. albicans strain used for colonization of the GI tract of mice was found to be susceptible to all three echinocandins used. Specifically, the MICs of anidulafungin, caspofungin and micafungin were 0.03 μg/ml, 0.016 μg/ml, and 0.02 μg/ml, respectively.

Before initiation of antifungal treatment the mean concentration of C. albicans in the stools of mice fed chow containing the yeast was comparable across all groups of
C. albicans concentration (log$_{10}$ CFU/g of stool) in the stools of mice fed chow containing the yeast for 10 days before the initiation of treatment with three different echinocandins or saline (control), $P = \text{NS}$ for all statistical comparisons.

Figure 2. In addition, there was no evidence of Candida in the stools of control mice that received echinocandins or saline and were fed with regular food (without Candida-containing chow) at the end and weekly for three months after the end of treatment (data not shown).

No significant changes were observed in the concentration of Candida yeast cells in the stool of colonized mice that received treatment with normal saline at 12 weeks vs. 1 week after colonization (control) (Fig. 3). In contrast, when compared to week one of colonization (before initiation of treatment), we found a sharp ($>4 \log_{10}$) decrease in Candida CFU/g of stool in all groups of mice that received echinocandins at 12 weeks after colonization ($P < 0.0001$ for all treatment groups; Fig. 3). Hence, all three echinocandins tested resulted in persistent eradication of Candida from murine stools.

Figure 3. C. albicans concentration (log$_{10}$ CFU/g of stool) in the stools of mice before (week 1) and 12 weeks after treatment with echinocandins or normal saline (control group). Each symbol represents data obtained from pooled stool of a group of 5 mice, and horizontal bars represent the mean $\text{***}, P < 0.0001$, Mann–Whitney $U$ test.

No side effects related to the echinocandin treatment had been observed during and/or after discontinuation of the antifungal therapy.

**Discussion**

Antifungal prophylaxis is commonly used in high-risk patients for development of invasive candidiasis, including patients with malignancies, transplantation, immunosuppression and neutropenia, as a mean to decrease morbidity and mortality associated with this devastating fungal infection [21,22].

The GI tract serves as Candida reservoir and is often the source of systemic disease [23]. Therefore, decreasing the burden of GI colonization, may result in reduced risk of invasive candidiasis. Because of their favorable pharmacokinetics and pharmacodynamics, including good oral bioavailability, reliable concentrations at most sites of infection and broad spectrum of activity, oral azoles, including fluconazole and the newer triazoles voriconazole and posaconazole, have become the agents of choice for antifungal prophylaxis in patients at high risk for development of invasive fungal infections [10]. Nonetheless, in a previous study on our established model of GI tract colonization in immunocompetent mice, we found that treatment with three different azoles, including ketoconazole, itraconazole, and fluconazole, had minimal effect on the reduction of yeast cells in the murine stools of anazole susceptible C. albicans clinical isolate [11].

Echinocandins are a novel class of parenterally administered antifungal agents, that mediate their antifungal activity by noncompetitive inhibition of (1,3)-$\beta$-D-glucan synthase, a fungus-specific enzyme, essential for the synthesis of the cell wall glucan [24]. All three echinocandins, caspofungin, anidulafungin, and micafungin, display potent fungicidal activity against most Candida species and have become the preferred antifungal agents in treatment of invasive candidiasis [24].
Notably, in vivo studies in humans and murine models of invasive candidiasis demonstrate that the 24-h AUC/MIC is the variable that best describes the pharmacodynamic properties of echinocandins [25]. Of interest, all these antifungal agents are highly protein bound and the AUC/MIC relationships of caspofungin, anidulafungin and micafungin are similar between humans and mice [25]. Specifically, Andes et al. have recently shown that all three echinocandins administered at a dose of 5 mg/kg–20 mg/kg in mice, similar to that used in our study, resulted in total drug AUC values of 96 to 520 mg h/l that are similar or well above to the total AUC values in healthy volunteers upon administration of approved echinocandin doses for the treatment of invasive candidiasis (100 mg/day of anidulafungin and micafungin and 50 mg/day of caspofungin) [25].

In addition, echinocandins have been successfully used as prophylactic antifungal therapy in high-risk patients [13–15]. Specifically, caspofungin has been previously given for prevention of intra-abdominal candidiasis in high-risk surgical patients and was proved effective in reducing *Candida* colonization of the oropharynx, stools, lower respiratory tract, peritoneal fluid, and urine [26]. Micafungin has been also used for prophylaxis against invasive fungal infections during neutropenia in patients undergoing hematopoietic stem cell transplantation and has been proven more effective than fluconazole in reducing *C. glabrata* colonization [15,27,28]. The drug is also effective in preventing invasive fungal infections in high-risk transplant patients [29]. Anidulafungin has given favorable results when was used prophylactically for prevention of fungal infections in solid organ transplant recipients [30,31]. However, there are no studies evaluating the efficacy of these compounds on the colonization of the GI tract by *Candida* organisms.

In the present study we investigated the effects of all three echinocandins, caspofungin, anidulafungin and micafungin on the decolonization of the mouse GI tract by *Candida* organisms, using a murine model that has been previously shown to mimic the human conditions and predict the effects of several pharmaceutical agents on the GI *Candida* colonization [11,32].

In agreement with our previous studies [11,16,19], there were no significant changes in the concentration of *Candida* yeast cells in the stool of colonized mice that received treatment with normal saline at 12 weeks versus 1 week after colonization (control). In contrast, all three echinocandins used essentially eliminated completely *Candida* from the GI tract of mice. Additionally, decolonization was sustained for three months after the end of the echinocandin treatment. This is probably due to the pharmacokinetic properties of these drugs, since all three echinocandins are mainly excreted in the feces [33] and their rapid fungicidal activity against *Candida* [24].

Importantly, the lack of assessment of yeast fungal burden in upper GI tract of mice in our study is an inherent limitation of our model. In addition, because our study was performed in immunocompetent adult mice, our findings may not directly apply on *Candida* colonization in immunocompromised hosts. Finally, we cannot exclude the possibility that the immunomodulatory properties of echinocandins could be implicated in eradication of *Candida* from the stools of mice [34]. A concern associated with prolonged and extensive use of antifungal agents is the development of secondary drug resistance in *Candida*. In fact, resistance to echinocandins is an emerging problem specifically among *C. glabrata* [35]. Nonetheless, the eradication of *Candida* from the stool of mice prevented susceptibility testing for assessment of development of secondary drug resistance. Future studies with the use of sub-inhibitory concentrations of echinocandins in our model of *Candida* GI tract colonization could address questions on the molecular mechanisms of *in vivo* evolution of antifungal drug resistance.

Collectively, our study demonstrates a unique property of the echinocandins to eradicate *Candida* from murine stools. Given the significance of *Candida* gut colonization in the pathogenesis of invasive candidiasis, our findings could have important implications in antifungal prophylaxis strategies in high-risk patients.

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### Declaration of interest

No conflicts of interest for Sofia Maraki, Dimitra Dimopoulou, Angeliki M. Andrianaki, Alexander Steven Karageorgiadis, Andreas Kyvernitakis, Stelios Lionakis, and Diamantis P. Kofteridis. Dr. George Samonis and Dr. George Hamilos have received research grants from pharmaceutical companies Pfizer, Astellas, MSD, and Novartis.

### References


