Anti-metabolic activity of caspofungin against Candida albicans and Candida parapsilosis biofilms

Christophe Cocuaud, Marie-Hélène Rodier, Gyslaine Daniault and Christine Imbert*

Laboratoire de Parasitologie et Mycologie Médicales, Centre Hospitalier Universitaire La Milétrie, BP 577, 86021 Poitiers Cedex, France

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Objectives: Candidiasis can be associated with the formation of biofilms on bioprosthetic surfaces and the intrinsic resistance of Candida albicans biofilms to the most commonly used antifungal agents has been demonstrated. In this study, we report on the antifungal activity of caspofungin at two different concentrations, on C. albicans and Candida parapsilosis biofilms with different ages of maturation.

Methods: Fifteen strains of C. albicans (10 strains susceptible to fluconazole in vitro and five strains resistant to this antifungal agent) and six strains of C. parapsilosis (all were susceptible to fluconazole in vitro) were studied. The antifungal activity of caspofungin was assessed by looking for a significant inhibition of the metabolic activity of yeasts within biofilms. Biofilms of Candida were produced in vitro, on silicone catheters.

Results: Caspofungin used at MIC did not modify the metabolic activity of C. albicans, whatever the maturation age of the biofilms. The same concentration of caspofungin significantly reduced the metabolism (P≤0.001) of 25% (biofilms of 48 h) to 50% (biofilms of 2 h) of the C. parapsilosis yeasts. The use of a therapeutic concentration of caspofungin (2 mg/L) significantly decreased (P≤0.001) the metabolism of all the strains of C. albicans and C. parapsilosis tested, independently of the biofilm maturation age. This potent antifungal activity of caspofungin on C. albicans biofilms was observed independently of the yeast susceptibility to fluconazole.

Conclusions: This study demonstrated that caspofungin used at MIC was not sufficient to reduce C. albicans biofilms, but it suggested an activity on C. parapsilosis biofilms depending on their maturation age. This study also indicated that caspofungin used at 2 mg/L could be a good candidate in the prevention of candidiasis associated with silicone medical devices. Our results also suggested that fluconazole resistance of yeasts did not affect caspofungin activity.

Keywords: candidiasis, catheters, antifungals, metabolism, echinocandins

Introduction

Candida yeasts are increasingly important nosocomial fungal pathogens. Candida albicans still remains the most frequently reported even if other Candida species, such as Candida parapsilosis, are described.1–4 Candida species, especially C. albicans, are the most common fungi associated with biofilm-related infections;5 indwelling medical devices can act as substrates for fungal biofilm growth.6 Candida parapsilosis yeasts are also involved in nosocomial infections, notably in critically ill neonates, where it is known to be associated with central lines and parenteral nutrition.7,8 Candidiasis associated with indwelling medical devices can result in serious medical complications, expensive care and is noted as a frequent factor limiting the prolonged use of central venous catheters.9 Candida biofilm formation proceeds through three developmental phases: the early phase (0–11 h) involving adhesion of fungal cells to the substrate; the intermediate phase (~12–30 h) during which the blastospores coaggregate and proliferate, forming communities while producing a carbohydrate-rich extracellular matrix (ECM); and the maturation phase (~31–72 h) in which the fungal cells are completely encased in a thick ECM.10 A small number of antifungal drugs can be used to treat candidiasis associated with implanted medical devices, and infected devices generally need to be removed.11 Candida within biofilms are highly resistant to antimicrobial agents compared with their planktonically grown forms.12–14 Echinocandins, such as caspofungin, represent a new class of antifungal drug and act by inhibiting the synthesis of β-D-glucan in

*Corresponding author. Tel: +33-05-49-44-39-59; Fax: +33-05-49-44-39-08; E-mail: Christine.Imbert@univ-poitiers.fr

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fungal cell walls and show in vitro antifungal activity against *C. albicans*. The aim of this work was to compare the efficiency of caspofungin used at two different concentrations (MIC and therapeutic concentration), to reduce biofilms of *C. albicans* and *C. parapsilosis* of different maturation age obtained on silicone catheters. The efficiency of caspofungin was assessed by measuring the metabolic activity of yeasts with and without antifungal treatment. In this study, we also compared the activity of caspofungin on *C. albicans* biofilms, according to the susceptibility of individual strains to fluconazole in vitro. In a previous study, we have observed that caspofungin (MIC/2) induced a decrease in the adherence of *C. albicans* to polystyrene coated with ECM proteins, but this effect was reduced in the case of fluconazole-resistant yeast strains. Thus, to investigate further the usefulness of caspofungin for treating candidiasis associated with medical devices and potentially caused by fluconazole-resistant *C. albicans* strains, we used the biofilm model in the present work.

**Materials and methods**

*Organisms and growth conditions*

Fifteen strains of *C. albicans* were studied: seven strains (92, 109, 163, 165, 182, 240 and 444) were isolated in our laboratory from patients with candidaemia. The identification of these clinical isolates was performed by using conventional physiological and morphological methods such as the germ-tube test in serum, agglutination (Bio-Rad, Marnes-La-Coquette, France) and metabolic properties (API 20C, bioMérieux, Marcy-L’Étoile, France). The strain 1066 of *C. albicans*, originally isolated from a patient with septicaemia, was kindly provided by Pr. R. Robert (Laboratory of Immunology, Parasitology and Mycology, Angers, France). The strain IP 2091 was obtained from Pasteur Institute (Paris, France) and ATCC 3153 was purchased from the American Type Culture Collection. These 10 strains were susceptible to fluconazole (MIC < 8 mg/L, Etest method). Five other strains of *C. albicans* were obtained from IHEM (Biomedical Fungi and Yeasts Collection, Brussels, Belgium) and were originally isolated from human mouth (IHEM-9581, IHEM-9582, IHEM-9584, IHEM-9586) or human blood (IHEM-10266); these strains showed high MIC values of fluconazole by the Etest method. These MICS have also been determined by the microlitigation method (Table 1).

Six strains of *C. parapsilosis* were also studied: five strains (28, 249, 359, 360 and 363) were isolated in our laboratory from patients with candidaemia and identified using the same methodologies described above for *C. albicans* isolates. The strain ATCC 22019 was purchased from the American Type Culture Collection.

Yeasts were first grown for 48 h at 28°C on Sabouraud agar slants (Sano Diagnostics Pasteur, Marnes-La-Coquette, France) to obtain a culture of synchronous stationary yeasts of *Candida*. A loopful of this culture was transferred to 25 mL of Yeast Nitrogen Base medium (YNB; Difco, Detroit, MI, USA) supplemented with 30 mM glucose (Sigma, St Louis, MO, USA; YNB-glu), and incubated for 16 h at 37°C without shaking.

Prior to use in the biofilm experiments, blastospores were harvested, washed twice in 0.1 M phosphate-buffered saline (PBS, pH 7.2, BioMérieux) and adjusted to 5 × 10⁶ blastospores/mL in YNB-glu.

Standard antifungal powders of caspofungin (caspofungin acetate, Merck, New Jersey, USA) and fluconazole (Pfizer, Orsay, France) were kindly provided by the manufacturers. Caspofungin was prepared as a stock solution of 10 mg/mL in dimethyl sulphoxide, aliquotted and stored at –80°C. Fluconazole was prepared as a stock solution of 10 mg/mL in water, aliquotted and stored at –80°C.

The MICS were determined after incubation for 24 h and 48 h at 37°C without shaking.

<table>
<thead>
<tr>
<th>Strains</th>
<th>MIC&lt;sub&gt;YNB-Glu&lt;/sub&gt; (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>caspofungin</td>
</tr>
<tr>
<td>Ca92</td>
<td>0.008</td>
</tr>
<tr>
<td>Ca109</td>
<td>0.015</td>
</tr>
<tr>
<td>Ca163</td>
<td>0.030</td>
</tr>
<tr>
<td>Ca165</td>
<td>0.015</td>
</tr>
<tr>
<td>Ca182</td>
<td>0.015</td>
</tr>
<tr>
<td>Ca240</td>
<td>0.060</td>
</tr>
<tr>
<td>Ca444</td>
<td>0.015</td>
</tr>
<tr>
<td>Ca1066</td>
<td>0.015</td>
</tr>
<tr>
<td>ATCC 3153</td>
<td>0.015</td>
</tr>
<tr>
<td>IP 2091</td>
<td>0.060</td>
</tr>
<tr>
<td>IHEM 9581</td>
<td>0.008</td>
</tr>
<tr>
<td>IHEM 9582</td>
<td>0.001</td>
</tr>
<tr>
<td>IHEM 9584</td>
<td>0.001</td>
</tr>
<tr>
<td>IHEM 9586</td>
<td>0.001</td>
</tr>
<tr>
<td>IHEM 10266</td>
<td>0.015</td>
</tr>
<tr>
<td><em>C. parapsilosis</em></td>
<td></td>
</tr>
<tr>
<td>Cp28</td>
<td>0.24</td>
</tr>
<tr>
<td>Cp249</td>
<td>0.24</td>
</tr>
<tr>
<td>Cp359</td>
<td>0.24</td>
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<tr>
<td>Cp360</td>
<td>0.24</td>
</tr>
<tr>
<td>Cp363</td>
<td>0.12</td>
</tr>
<tr>
<td>ATCC 22019</td>
<td>0.24</td>
</tr>
</tbody>
</table>

ND, not determined.

The MICS were obtained with a broth microdilution method performed in YNB-glu medium (MIC<sub>YNB-Glu</sub>). Results are representative of two separate experiments.

**Substrate material**

Catheters in silicone 100% (2 mm inside and 3.2 mm outside diameters) were obtained from A-M systems (USA). Calibrated sections of 5 mm obtained by cutting these catheters were autoclaved, incubated overnight in fetal bovine serum (FBS) and washed twice with YNB-glu prior to use to produce biofilms.

**Biofilm formation**

The sections of silicone catheters were incubated in 96-well tissue culture plates (polystyrene, Evergreen Scientific, USA) for 1 h at 37°C with 200 µL of a suspension of *C. albicans* or *C. parapsilosis* blastospores (5 × 10⁶ blastospores/mL). Each section was then washed twice with YNB-glu to remove the non-adherent yeasts, moved into a new well of the culture plate, and incubated for 2, 24 or 48 h in YNB-glu to obtain biofilms with different maturation stages. Biofilms of 48 h
were tested with only eight representative strains (four C. albicans and four C. parapsilosis) chosen for their behaviour with biofilms of 24 h.

**Treatment of Candida biofilms with caspofungin**

Each section of catheter coated with Candida biofilm of 2, 24 or 48 h of maturation was incubated in a new well of the culture plate for 24 h at 37°C with YNB-glu + caspofungin used at two concentrations: a low concentration corresponding to the MIC for each tested strain and a therapeutic concentration of 2 mg/L. Controls without antifungal were included in each experiment.

**Metabolic activity of yeast included in biofilm**

The metabolic activity was assessed using the tetrazolium (XTT) assay: the principle is based upon the reduction of XTT tetrazolium to tetrabromophenol blue (TB) by mitochondrially active yeasts in the presence of a coenzyme, an electron-coupling agent. Briefly, each silicone section coated with biofilm and treated or not with caspofungin for 24 h was incubated in a new well of the culture plate for 3 h at 37°C with XTT (300 mg/L) and menadione (0.13 mM) in 200 μL of PBS. The absorbance of XTT formazan was then measured at 492 nm (microplate reader LP400, Sanofi Diagnostics Pasteur) and correlated with the yeast metabolic activity within the biofilms. All experiments were performed twice with six replicates.

**Statistical analyses**

An analysis of variance (ANOVA) and Scheffe’s test were conducted to determine statistical differences between groups.

**Results and discussion**

Some authors have shown that the MIC values are dependent on the experimental conditions. Microdilution and Etest methods were used together in our study. Both tests are different, but in our case all the strains that showed high MICs of flucloxacilin with the microdilution method were determined as resistant to this antifungal with the Etest method, and all the strains that were susceptible to flucloxacilin showed MICs below 3.12 μg/mL.

The MIC values of caspofungin obtained with YNB-glu medium were similar for the different strains of C. albicans ranging between 0.001 and 0.060 μg/mL (Table 1). This result is in accordance with other studies suggesting an in vitro broad-spectrum antifungal activity of caspofungin against C. albicans. MICs of caspofungin were determined using the YNB-glu method to correspond to the experimental conditions related to the biofilm studies. Microdilution methodology usually implements RPMI as the culture medium. In a previous work, caspofungin MIC values for C. albicans strains in YNB-glu medium were compared with the corresponding MICs obtained in RPMI medium in order to permit a best understanding of the efficiency of caspofungin in vitro: results showed slight differences, corresponding to a deviation of three or four dilutions, and only for two strains (IHEM-9584 and IHEM-9586).

Among the Candida yeasts growing as biofilms, C. albicans has been the most frequently studied due to its incidence. We first investigated the temporal development of C. albicans and C. parapsilosis biofilms using one strain of each Candida species (Figure 1). Our results confirmed the significant development of the two biofilms in a time-dependent manner, and were consistent with previous studies. These results also confirmed the usefulness of the metabolic activity assay to study the growth of yeast biofilms.

Caspofungin is the first representative of a new antifungal class, and its influence on fungal colonization is not yet well characterized. Some authors have suggested that caspofungin could affect the cellular morphology and the metabolic status of C. albicans cells within biofilms. Our paper deals with the efficiency of caspofungin on metabolic activity of C. albicans yeasts included in biofilms with different ages of maturation.

We have recently shown that the growth of C. albicans in medium containing a sub-inhibitory concentration of caspofungin induced a decrease of subsequent fungal adherence to plastic coated with ECM proteins. Furthermore, the adherence process of C. albicans to implanted medical devices corresponds to the early phase of the biofilm formation.

In the present work, yeasts were grown as biofilms with different maturation ages to study the early and later adherence phases in the formation of a fungal biofilm. The efficiency of caspofungin in inhibiting the metabolism of yeasts at the different stages of the biofilm maturation was then evaluated. Under the experimental conditions of this study, the activity of caspofungin could be dependent on three parameters: (i) the Candida species (i.e. C. albicans or C. parapsilosis); (ii) the maturation age of the biofilms (i.e. 2, 24 or 48 h); and (iii) the concentration of caspofungin (MIC or 2 μg/mL).

Our results confirmed the importance of the first parameter: in the case of the biofilms with a maturation age of 2 h, caspofungin used at MIC significantly inhibited (P < 0.001) the metabolism of 67% of the C. parapsilosis yeasts tested (four out of six strains), but this efficiency was observed only for 7% of the C. albicans yeasts (Figure 2). This species specificity was also observed for the biofilms with 24 h of maturation where caspofungin (MIC) inhibited (P < 0.001) the metabolic activity of 33% of the C. parapsilosis yeasts but had no effect on the metabolism of C. albicans yeasts (Figure 3).

Our results also suggested that when caspofungin was used at low concentration (MIC), the maturation age of the biofilm modulated the activity of this antifungal. For example, caspofungin...
(MIC) inhibited the metabolic activity of 50% of the *C. parapsilosis* yeasts grown in a 2 h maturation biofilm, 33% of *C. parapsilosis* yeasts grown in a 24 h maturation biofilm and 25% of the *C. parapsilosis* yeasts grown in a biofilm of 48 h of maturation (Figures 2, 3 and 4). This phenomenon was less obvious with *C. albicans* biofilms because of the lack of efficiency of caspofungin at MIC (Figure 2).

Finally, the efficiency of caspofungin in inhibiting yeasts embedded in biofilms was dependent on the concentration of antifungal used: caspofungin used at MIC could reduce the metabolic activity of only a small number of *C. albicans* yeasts (a significant activity was obtained for 0–7% of *C. albicans*), whatever the maturation age of the biofilms, whereas the same antifungal used at the therapeutic concentration of 2 mg/L significantly reduced (*P* ≤ 0.001) the metabolic activity of all the yeasts tested (Figures 2, 3 and 4). This effect was also observed with *C. parapsilosis* biofilms: caspofungin significantly reduced (*P* ≤ 0.001) the metabolism of 25–67% of the strains when it was used at MIC and this reduction was obtained for 100% of the strains when the antifungal was used at a concentration of 2 mg/L.

The results obtained with *C. albicans* strains showed that the efficiency of caspofungin in inhibiting the metabolism of yeasts within a biofilm was not dependent on the yeast’s *in vitro* susceptibility to fluconazole (Figures 2–4). Caspofungin (MIC) inhibited...
the metabolic activity of 7% of C. albicans yeasts (biofilm of 2 h), distributed as 10% fluconazole susceptible and 0% resistant yeasts (Figure 2); the efficiency of caspofungin used at a therapeutic concentration was observed with all C. albicans yeasts, whatever their susceptibility to fluconazole in vitro. A similar result was obtained with older biofilms (Figures 3 and 4).

In a recent study, Pfaller et al. identified 351 Candida isolates that were resistant to fluconazole in vitro (MIC ≥ 64 mg/L) and all these isolates were susceptible to caspofungin (99% of the MICs were ≤ 2 mg/L). Other authors demonstrated that caspofungin was efficient on azole-resistant Candida infections. Taken together, our results confirmed that caspofungin could be a good candidate in the treatment of candidiasis related to silicone medical devices infected with C. parapsilosis and C. albicans fluconazole-resistant strains or with fluconazole-resistant strains of C. albicans.

In conclusion, this study demonstrated that caspofungin used at 2 mg/L inhibited all the Candida isolates tested, whatever the species, C. albicans or C. parapsilosis, and whatever the state of maturation of the biofilms (2, 24 or 48 h). The efficiency of caspofungin on C. albicans biofilms was not affected by the yeast’s resistance to fluconazole. Our results confirmed the real potential of caspofungin as an inhibitor of Candida within biofilms on bioprosthetic surfaces.

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