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In vitro Activity of Caspofungin against Planktonic and Sessile *Candida* sp. Cells

ANNA SEREFKO, BEATA CHUDZIK and ANNA MALM

Department of Pharmaceutical Microbiology, Medical University of Lublin,
Chodźki 1, 20-093 Lublin, Poland

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

Abstract

Candida sp. may be regarded as one of the leading etiologic agents of hospital-acquired infections, including those related with the indwelling medical devices, which become colonized by the yeasts, accompanied by biofilm formation. In this paper we assayed *in vitro* susceptibility to caspofungin of planktonic and sessile cells of nasopharyngeal isolates of *Candida* sp. Two types of biomaterials were used – silicone elastomer-coated latex urinary Foley catheter and PCV Thorax catheter. The minimal inhibitory concentrations (MIC) of caspofungin for planktonic *Candida* sp. cells ranged from 0.008 to 0.031 mg/l, while the minimal fungicidal concentrations (MFC) from 0.008 to 0.062 mg/l, with MFC/MIC ratios ≤2. The minimal concentration of caspofungin preventing adhesion process of *Candida* sp. on both biomaterials ranged from 0.004 to 0.031 mg/l, while preventing biofilm formation from 0.004 to 0.062 mg/l. In contrast, much higher minimal concentrations of caspofungin were needed to eradicate the mature biofilm (0.25 to >8 mg/l). In all cases, drug concentrations depended on the strain and the biomaterial used. Our preliminary data suggest that caspofungin, showing good anti-adherent activity *in vitro* against *Candida* sp., appears to be a potential agent rather for prophylaxis of the yeast infections associated with biomaterials but not for their treatment.

K e y w o r d s: caspofungin, *Candida* sp., biomaterials, adhesion, biofilm

Introduction

Recently, the yeasts belonging to the genus *Candida* may be regarded as one of the leading etiologic agents of hospital-acquired infections, including those related with the indwelling medical devices such as catheters, prosthetic joints and heart valves, dentures *etc.* These devices become colonized by the yeasts, accompanied by biofilm formation (Hawser and Douglas, 1994; Douglas, 2002; Jabra-Rizk *et al*., 2004). Treatment of candidiasis often presents a challenge for clinicians, since the number of effective antifungal agents at their disposal is limited, due to increasing resistance of the pathogens. In addition, sessile cells of *Candida* sp. within a biofilm are even more insensitive to current antimicrobial therapy in comparison to their planktonic (free-floating) counterparts. Therefore, the pharmaceutical industry is still working on the discovery of novel drugs which might reinforce conventional antifungal armamentarium (Chandra *et al*., 2001; Bachmann *et al*., 2002; Kuhn *et al*., 2002; Jabra-Rizk *et al*., 2004).

Caspofungin is the first antifungal compound of a new echinocandin class with a unique mode of action. It inhibits the synthesis of 1,3-β-D-glucan, a key component of the fungal cell wall, that is essential for osmotic stability, cell growth and cell division (Letscher-Bru *et al*., 2003). This antibiotic should be effective in combating both planktonic and biofilm-associated *Candida* sp. populations. However, the literature data regarding the effect of caspofungin *in vitro* on *Candida* sp. biofilm formation and its eradication are controversial (Bachmann *et al*., 2002; Kuhn *et al*., 2002; Ramage *et al*., 2002; Soustre *et al*., 2004).

Here we presented data on the *in vitro* susceptibility to caspofungin of planktonic and sessile cells of nasopharyngeal isolates of *Candida* sp.

Experimental

Materials and Methods

Microorganisms. A total of 7 clinical isolates of *Candida* sp. possessing hydrophilic or hydrophobic cell structure were studied. The hydrophobicity of cell surface was assessed using salt aggregation test (Lindahl *et al*., 1981). The collection included the following isolates: *C. albicans* (1 hydrophilic and 2 hydrophobic isolates), *C. famata* (1 hydrophilic and 1 hydrophobic isolates), *C. glabrata* (1 hydrophobic isolate), *C. krusei* (1 hydrophilic isolate). The isolates were obtained from nasopharynx of patients with lung cancer undergoing pulmonary resection and were stored on Sabouraud dextrose agar and before each experiment they were subcultured on Sabouraud glucose broth.

Caspofungin. Standard antifungal powder of caspofungin (caspofungin acetate) was examined (Merck & Co., Inc., USA). Stock solution containing 16 mg/ml was prepared in distilled water and was stored frozen at –20°C.

Determination of minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of caspofungin. Determination of MIC of caspofungin for planktonic *Candida* sp. cells was performed by a broth microdilution method in accordance with the guidelines recommended by CLSI (Clinical Laboratory Standards Institute), using serial two-fold dilutions of caspofungin in Sabouraud glucose broth. Final concentrations of caspofungin ranged from 0.0002 to 16 mg/l. Stock inoculum suspensions of yeasts were prepared in Sabouraud medium and adjusted to optical density corresponding with 0.5 Mc Farland standard *i.e.* 150×10^6 CFU (colony forming units). After 48 h of incubation at 35°C, the MICs were assessed visually as the lowest drug concentration showing complete growth inhibition. In order to determine the MFC of caspofungin for the planktonic cells of the yeasts, 10 µl from each tube that showed thorough growth inhibition, from the last positive one and from the growth control was streaked onto Sabouraud dextrose agar plates. After 48 h of incubation at 35°C, the MFCs were assessed visually as the lowest drug concentration at which there was no growth. All experiments were done in triplicates. The representative data are presented.

Biomaterials. All assays were carried out on two types of catheters that differed in unevenness of surface from each other – silicone elastomer-coated latex urinary Foley catheter and PCV Thorax catheter.

The effect of caspofungin on adhesion of *Candida* sp. and biofilm formation on the biomaterials. The adhesion process and biofilm formation were determined by using the MTT (tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay (Levitz *et al*., 1985). The catheters used were cut aseptically into *ca* 0.5 cm^2 fragments and placed into Petri dishes. The standardized yeast suspensions (optical density of 0.5 Mc Farland standard) were prepared. Various concentrations of caspofungin (0.004 – 8 mg/l) were used. (i) In order to assay the effect of caspofungin on adhesion process, the yeast suspensions in sterile PBS (phosphate-buffered saline) containing various concentrations of caspofungin were incubated with biomaterials for 1 h at 35°C. Nonadherent cells were removed by careful rinsing catheter discs with sterile PBS and then resuspended in Sabouraud glucose broth, followed by overnight incubation at 35°C. (ii) In order to assay the effect of caspofungin on biofilm formation, the yeast suspensions in Sabouraud glucose broth containing various concentrations of caspofungin were incubated with biomaterials for 24 h at 35°C. Nonadherent cells were removed by careful rinsing catheter discs with sterile PBS and then resuspended in fresh Sabouraud glucose broth. Medium changing and catheters washing procedures after overnight incubation at 35°C were repeated thrice (total incubation period lasted 72 h). (iii) In order to assay the effect of caspofungin on biofilm eradication, the mature biofilms were incubated in the presence of various concentrations of caspofungin for 24 h. In all assays a drop of 1% MTT solution was added to each dish. After incubation for 24 h at 35°C, in the presence of *Candida* sp. viable cells tetrazolium salt was reduced to the violet tetrazolium formazan product, accompanied by violet colour of the medium. In each experiment control free-drug assays were carried out. All experiments were done in triplicates. The representative data are presented.

Results

The MICs of caspofungin for planktonic *Candida* sp. cells ranged from 0.008 to 0.031 mg/l (Table I). Paradoxical turbidity at the highest drug concentrations, imitating fungal growth was observed for 3 isolates. However, this so-called "eagle" effect was ignored for the MIC determinations according to the literature data (Bartizal and Odds, 2003; Stevens *et al*., 2004). The MFCs of caspofungin for planktonic *Candida* sp. cells was 0.008 – 0.062 mg/l (Table I). For 6 strains tested MFC/MIC ratios were = 2, while for one strain MFC/MIC = 1. It is worth notifying that the turbidity observed at the highest drug concentrations during the MIC readings was accompanied by the lack of fungicidal effect of caspofungin, which was revealed during MFC determinations.

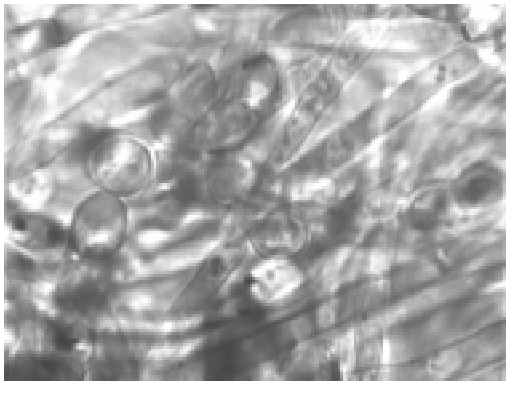

Fig. 1. The structure of the mature biofilm of hydrophobic strain of *C. albicans* on the surface of Thorax catheter (after 72 h incubation). Magnification 1200x.

All assayed *Candida* sp. isolates were able to adhere both to Thorax and Foley catheters, following by biofilm formation on both biomaterials. This was monitored by formation of violet tetrazolium formazan product inside and outside of the catheters and violet coloured medium after addition of MTT solution. The structure of the biofilm of hydrophobic strain of *C. albicans* on Thorax catheter was presented in Fig. 1.

Table I
In vitro activity of caspofungin against planktonic cells of *Candida* sp.

Strain	MIC (mg/l)	MFC (mg/l)	MFC/MIC ratio
C. albicans hydrophilic*	0.015	0.031	2
C. albicans hydrophobic*	0.031	0.062	2
C. albicans hydrophobic	0.008	0.008	1
C. famata hydrophobic	0.008	0.015	2
C. famata hydrophilic*	0.008	0.015	2
C.glabrata hydrophobic	0.031	0.062	2
C. krusei hydrophilic	0.015	0.031	2

* In these cases the "eagle" effect was observed.

Table II
In vitro effect of caspofungin on adhesion process of *Candida* sp. to biomaterials

Strain	Minimal concentration preventing adhesion process (mg/l)	
	Thorax catheter	Foley catheter
C. albicans hydrophilic	0.008	0.008
C. albicans hydrophobic	0.031	0.031
C. albicans hydrophobic	0.004	0.004
C. famata hydrophobic	0.008	0.008
C. famata hydrophilic	0.008	0.004
C.glabrata hydrophobic	0.031	0.031
C. krusei hydrophilic	0.008	0.008

Table III
In vitro effect of caspofungin on biofilm-embedded cells of *Candida* sp.

Strain	Minimal concentration preventing biofilm formation (mg/l)		Minimal concentration eradicating biofilm (mg/l)	
	Thorax catheter	Foley catheter	Thorax catheter	Foley catheter
C. albicans hydrophilic	0.031	0.008	>8	8
C. albicans hydrophobic	0.062	0.015	>8	8
C. albicans hydrophobic	0.015	0.004	>8	>8
C. famata hydrophobic	0.015	0.015	>8	>8
C. famata hydrophilic	0.015	0.004	8	8
C. glabrata hydrophobic	0.062	0.062	2	0.25
C. krusei hydrophilic	0.015	0.015	2	0.25

The minimal concentration of caspofungin preventing adhesion process on both biomaterials ranged from 0.004 to 0.031 mg/l (Table II). The above data indicate that caspofungin significantly inhibited *in vitro* the adherence capacity of the yeasts to the biomaterials assayed at concentrations ranged from 0.5×MIC or 1×MIC.

The minimal concentration of caspofungin preventing biofilm formation ranged from 0.004 to 0.062 mg/l. In contrast, much higher minimal concentrations of caspofungin (0.25 to >8 mg/l) were needed to eradicate the mature biofilm of *Candida* sp., depending also on the strain and the biomaterial used (Table III). The total eradication of *Candida* sp. biofilms with the drug concentrations below 8 mg/l was obtained for 2 strains only (Table III). The above data indicate that caspofungin significantly inhibited *in vitro* the biofilm formation capacity of the yeasts to the biomaterials assayed at concentrations ranged from 0.5×MIC to 2×MIC, while eradication of the mature biofilm required concentrations from 4×MIC to >1032×MIC.

Generally, using the Thorax catheter higher concentrations of caspofungin were needed in order to achieve the same effects on adhesion process of *Candida* sp. and the yeast biofilm formation as were obtained with the Foley catheter (Table II and III).

Discussion

Candida sp. infections can be regarded as an important medical problem, especially in the immunocompromised patients, *e.g.* in lung cancer patients. Additionally, according to our unpublished data, *Candida* sp. isolates have shown to possess a potential ability to colonize pleural drains in patients undergoing pulmonary resection. Although *C. albicans* remains a predominant etiologic agent of candidiasis, other species that tend to be less susceptible to the commonly used antifungal agents such as *C. krusei*, *C. glabrata* or *C. famata* have emerged as opportunistic patogens (Chandra *et al*., 2001; Jabra-Rizk *et al*., 2004).

The results presented in this paper confirmed the good inhibitory activity of caspofungin at low concentrations against planktonic cells of *Candida* sp., observed also by other authors (Bachmann *et al*., 2002;

Ramage *et al*., 2002; Bartizal and Odds, 2003; Stevens *et al*., 2004). The MICs and MFCs determined for the nasopharyngeal *Candida* sp. isolates were lower than those reported in the literature (Bartizal *et al*., 2003; Stevens *et al*., 2004). However, such discrepancies are possible since the type of medium used in antifungal susceptibility testing is of the utmost importance. The standard medium for testing drug susceptibility of *Candida* sp. was RPMI 1640 medium, whereas Sabouraud glucose broth was used for studies on *Candida* sp. biofilm formation (Chryssanthou and Cuenca-Estrella, 2002; Bartizal *et al*., 2003). The MFC/MIC ratio, not higher than 2 for the tested strains, confirmed the excellent *in vitro* fungicidal activity of caspofungin against planktonic cells of *Candida* sp.

Our data demonstrating *in vitro* anti-adherent activity of caspofungin are in accordance with those from literature (Soustre *et al*., 2004). The drug concentrations as low as the 0.5 × MIC and 1 × MIC were sufficient to deprive biofilm-precursor planktonic cells of *Candida* sp. of adherence capacity and ability to form a mature biofilm. It's worth mentioning that these values are within the range of mean through levels of caspofungin in serum in humans after standard dosing – 1–2 mg/l (Letscher-Bru and Herbrecht, 2003). As shown by other authors (Bachmann *et al*., 2002; Kuhn *et al*., 2002; Ramage *et al*., 2002; Soustre *et al*., 2004), exposure of planktonic *Candida* sp. cells even to subinhibitory concentrations of caspofungin inhibited adhesion process and subsequent biofilm formation. On the other hand, all examined strains of *Candida* sp. became much more resistant to caspofungin when adhered to the biomaterials and embedded in the mature biofilm. Eradication of sessile *Candida* sp. populations required much higher concentrations of the antibiotic in comparison with their free-floating counterparts. Analogous investigations also have shown that the complete sterility of the mature *Candida* sp. biofilms as well as the thorough eradication of adherent cells was hard to attain (Bachmann *et al*., 2002; Ramage *et al.*, 2002). Nevertheless, a significant reduction in metabolic activity in caspofungin-treated sessile yeast cells was detected by other authors (Bachmann *et al*., 2002; Ramage *et al.*, 2002; Soustre *et al*., 2004). In addition, caspofungin-treated yeast biofilms appeared to be less hyphal and showed minor defects in the overall biofilm architecture (Bachmann *et al*., 2002; Ramage *et al.*, 2002; Soustre *et al*., 2004). However, Ramage *et al*. (2002) found that caspofungin killed >99% of sessile cells of some strains of *C. albicans* within the mature biofilm at therapeutically attainable concentrations (0.125 mg/l and 1 mg/l). The highest concentration of caspofungin (8 mg/l) was less efficaciuos due to paradoxical lack of fungicidal effect of caspofungin at this concentration. Therefore, in studies on the effect of caspofungin on removal of *Candida* sp. cells adhered to the biomaterials or embedded in the biofilm, the "eagle" effect observed in case of some yeast strains should be taken into account (Bartizal and Odds, 2003; Stevens *et al*., 2004).

The observed in this paper differences in antifungal activity of caspofungin against catheter-associated cells of *Candida* sp. depended on the type of biomaterial used. Using the Thorax catheter higher concentrations of the drug were needed in order to achieve the same outcomes as were obtained with the Foley catheter.

To conclude, the controversial literature data (Bachmann *et al*., 2002; Kuhn *et al*., 2002; Ramage *et al.*, 2002; Soustre *et al*., 2004) and our studies concerning *in vitro* effect of caspofungin against sessile *Candida* sp. cells suggest that this antibiotic, showing good anti-adherent activity *in vitro*, appears to be a potentially effective agent rather for prophylaxis of the yeast infections associated with biomaterials but not for their treatment.

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