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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Caspofungin affects adhesion of *Candida* to a human cell line

La caspofungine modifie l'adhésion des *Candida* sur une lignée des cellules humaines

G. Kyselgof [a,1], H. Sandovsky-Losica [a], I. Berdicevsky [b], E. Segal [a,*]

[a] *Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel Aviv 69978, Israel*
[b] *Faculty of Molecular Microbiology, The Rappoport School of Medicine, Technion, Haifa*

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KEYWORDS
Caspofungin;
Adhesion;
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Abstract
Objective. — Caspofungin is a novel antifungal drug that acts on the fungal cell wall by inhibiting the synthesis of a major component of the cell wall, glucan. Adhesion of *Candida* to host tissues is an initial prerequisite of evolution of infection. Interception of adhesion may lead to prevention of infection. Thus, the goal of the present study was to assess the effect of caspofungin at subinhibitory concentrations on the adhesion of *C. albicans* to a human cell line, HaCat (Human keratinocytic cell line).
Materials and methods. — The adhesion to HaCat cells was carried out in a microtiter system using fluorescent labeled (Fluorescein isothiocyanate -FITC) *C. albicans* and fluorometric measurements to quantitate the level of adherence. The effect of caspofungin was assessed at sub-MIC (Minimal inhibitory concentration) concentrations of 1/4, 1/3, 1/2, and 3/4 MIC in two models: a) as a supplement to the adherence mixture and b) following exposure of the yeasts to the drug for 2 and 3 h prior to the adherence assay.
Results. — Caspofungin reduced the level of adherence by 40 to 90% in comparison to nontreated controls, in all tested conditions, in dependence of drug concentration and time of exposure. The effect seems not to be associated with the cidal activity of the drug, as the XTT assay and Trypan blue staining showed only a small percentage of nonviable organisms among the caspofungin treated *C. albicans*. Scanning electron microscopy of *C. albicans* cultures exposed to caspofungin revealed morphological changes in the treated yeasts that might be associated with the decrease in the adherence level.

* Corresponding author.
E-mail address: segale@post.tau.ac.il (E. Segal).
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Conclusion. — The findings of this study reveal that caspofungin at sub-MIC concentrations affects adhesion of *C. albicans* to human cells, a finding that may be a basis for further investigations, towards the concept of using sub-MIC drug concentrations for prophylaxis in clinical trials.

MOTS CLÉS
Caspofungine ;
Adhérence ;
Lignée cellulaire humaine ;
Candida

Résumé
Objectif. — La caspofungine est un nouvel antifongique qui agit au niveau de la paroi cellulaire en inhibant la synthèse du composant principal de la paroi, le glucane. L'adhésion des *Candida* aux tissus de l'hôte est une condition préalable à l'évolution de l'infection. L'inhibition de l'adhésion peut conduire à la prévention de l'infection. Le but de ce travail consiste à étudier l'effet de la caspofungine à des doses subinhibitrices sur l'adhésion de *C. albicans* sur une lignée cellulaire humaine, HaCat.
Matériels et méthodes. — L'adhésion aux cellules HaCat est mesurée par un système de microtitration en utilisant une souche de *C. albicans* marquée à l'aide d'un marqueur fluorescent (l'isothiocyanate de fluorescéine) et par la quantification des niveaux d'adhésion effectuée par des mesures fluorimétriques. L'effet de la caspofungine est determiné à des concentrations subinhibitrices minimales (sub-MIC) 1/4, 1/3, 1/2 et 3/4 MIC sur deux modèles : a) en tant que supplément au mélange d'adhérence et b) à la suite du traitement des levures par la drogue deux et trois heures avant l'essai d'adhésion.
Résultats. — Comparée aux contrôles non traités, la caspofungine réduit d'environ 40 à 90 % le niveau d'adhérence dans toutes les conditions testées, de façon proportionnée à la dose utilisée et du temps d'exposition. L'effet inhibiteur ne parait pas être lié à l'activité fongicide de la drogue, puisque l'essai XTT et le marquage au bleu Trypan ne montrent qu'un faible pourcentage de cellules non viables parmi les levures traitées à la caspofungine. L'étude en microscopie électronique à balayage des cultures de *Candida* traitées par la caspofungine révèle des changements morphologiques dans les levures qui pourraient être associés à une diminution de l'adhérence.
Conclusion. — Les résultats du présent travail révèlent que la caspofungine affecte l'adhésion de *C. albicans* sur cellules humaines à des concentrations sub-MIC. Ce résultat peut constituer la base d'études ultérieures en vue de l'utilisation de doses sub-MIC à titre prophylactique dans des essais cliniques.

Introduction

Candida species are etiological agents of muco-cutaneous and deep-seated/disseminated infections in man and are among the most common human mycoses [2,9,13,18]. Deep-seated and disseminated candidiasis is of particular significance in immuno-compromised and debilitated patients, being the 4th most common blood borne nosocomial infection [2,9,13]. The drug armament for treatment of systemic candidiasis includes primarily, the polyene amphotericin B in its conventional or lipid formulations, the triazoles fluconazole and voriconazole and recently also the echinocandins, mostly, caspofungin [3,8,14,26]. Despite the considerable improvement in the outcome of patients with systemic candidiasis, as a result of the introduction of a greater variety of therapeutic agents, management of the infection leaves still place for further advances. Particular attention is warranted to the prevention of development of the infection in the risk population.

Adhesion of *Candida* to host tissues is considered a major requirement in the development of infection and an initial step in the infectious process. Hence, inhibition of this step is believed to contribute to prevention of development of infection, as was shown in previous studies in various experimental models [7,20—22]. Modulation of adhesion can be achieved by competitive binding of analogs of the microbial adhesins or of the host cell receptors, or by physical and chemical manipulations that could interfere in the interaction of the microbe with the host [16,17]. Antimicrobial drugs may be included as possible agents that could affect the adhesion process by alterations of the microbial cell surface involved in the interaction.

In vitro exposure of microbes to antimicrobials at inhibitory and subinhibitory concentrations has indeed been shown to affect adhesion [6,28]. Several investigators [1,5,10,11], as well as the authors [23,24], have shown such effects on adhesion of *Candida* to epithelial cells.

The recently introduced echinocandins are the only group of commercially available, clinically usable antifungals that acts on the fungal cell wall by interfering in glucan synthesis [8], and not on the cell membrane, as do most of the commercially usable antifungals [4].

These agents act by inhibiting 1,3-beta-d-glucan synthase, an enzyme involved in production of glucan, an essential component of the fungal cell wall. Echinocandins demonstrate activity in vitro against a variety of yeasts (including both *C. albicans* and non-*albicans* species) as well as select moulds (including *Aspergillus* spp.) [4,8]. In view of the need for means of prophylaxis of development of candidiasis in high risk hosts, and based on the significance of adhesion in the infectious process and the possibility to interfere in this process, we initiated the present study to investigate the effect of subinhibitory concentrations of caspofungin on the adhesion of *Candida* to a human cell line.

The following manuscript is, to the best of our knowledge, the first report describing an inhibitory effect of caspofungin on the adhesion of *Candida* to human cells.

Materials and methods

Strains and growth

C. albicans CBS 562 was used throughout the study. Clinical *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. krusei* strains isolated from human subjects were provided by Sheba Medical Center and from other clinical laboratories. All strains were grown on Sabouraud dextrose agar (SDA), supplemented with chloramphenicol at 28 °C. For Fluorescein isothiocyanate (FITC) labeling and for adherence reactions the *Candida* were grown on yeast extract (YE) broth at 28 °C for 18 h under constant shaking, then the cultures were washed (×3) with PBS and suspended to the desired concentration.

Determination of MIC of caspofungin against *Candida*

Caspofungin acetate (powder) was a gift from Merck & Co., Inc, Whitehouse Station, N.J. USA. Minimal inhibitory concentration (MIC) of caspofungin was determined using a modification of the CLSI micro-dilution test using yeast nitrogen base (YNB) [15]. One hundred microliters of *Candida* suspension at a concentration of $1-5\times10^3$ ml was added to microtiter wells containing 100 μl caspofungin double diluted to concentrations ranging from 0.1 to 50 μg ml. MIC was determined after 48 h incubation at 37 °C, as the minimal concentration that inhibits visible growth of the fungus, by spectrophotometry at 530 nm in an ELISA reader.

Fluorescein isothiocyanate (FITC) labeling of *Candida* cells

Candida labeling with FITC was performed as previously described [19] and as used in other microbial systems [12]. Briefly, washed *Candida* cells (1×10^8) suspended in carbonate buffer were incubated overnight at room temperature with FITC, then the labeled *Candida* cells were washed with carbonate buffer and suspended to the desired concentration.

HaCaT cell line

HaCaT (Human keratinocytic cell line) cells were grown in tissue culture plates in DMEM supplemented with 10% FCS at 37 °C in 7% CO_2. The cells were transferred to new plates at three days intervals or when the culture was confluent. For adherence experiments the cells (2.5×10^6 ml) were inoculated onto microtiter plates (0.1 ml/well), and were incubated for 48 h or until the culture in the microtiter wells was confluent.

Adherence assay

Adherence reaction of FITC labeled *Candida* (each strain was tested in triplicate) to HaCaT cells was performed in 96 wells microplates [19]. The microplates with HaCaT cells were washed three times with PBS, then 100 μl of FITC labelled *C. albicans* yeasts (10^8 ml PBS) was added to each well, and the plates were incubated for 2 h at 37 °C in a CO_2 incubator.

Two adherence protocols were used:

I. Pre treatment assay: treatment of *Candida* with sub-MIC of caspofungin, FITC labeling of the treated *Candida*, interaction of the labeled *Candida* with HaCat cells,

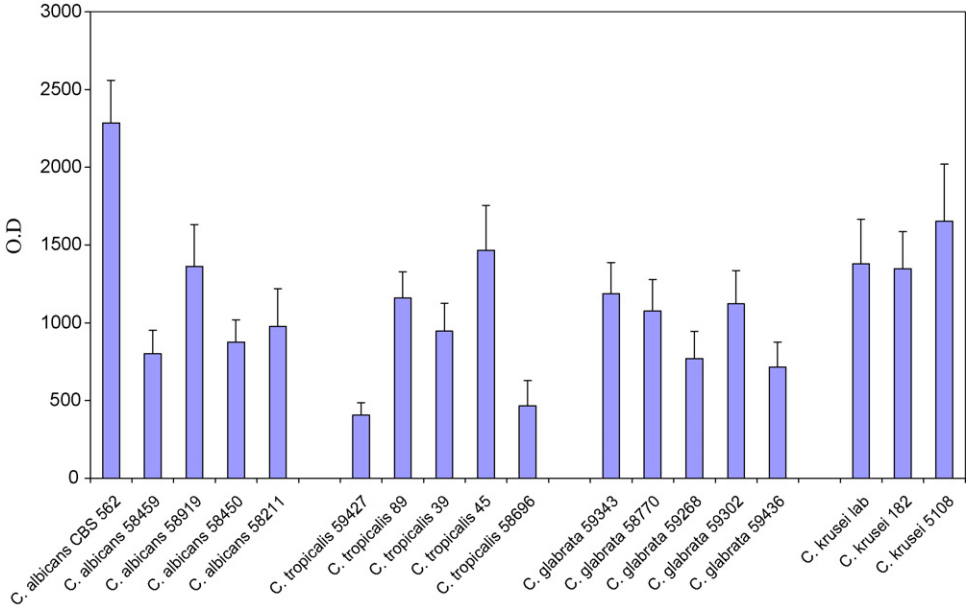

Figure 1 Adherence values of *Candida* species to HaCat cell line.
Figure 1 Valeurs de l'adhérence des *Candida* testés sur la lignée cellulaire HaCat.

washing and reading by fluorometry at O.D 530/20 and 485/20 using FL-600 reader (Bio Tek Instruments Inc. USA) to determine the adherence level;

II. FITC labeling of *Candida*, interaction of labeled *Candida* with HaCaT cells in the presence of sub-MIC of caspofungin, washing and evaluating by the fluometry.

Effect of caspofungin on *Candida*

C. albicans suspension (1×10^8 cells/ml) was incubated with caspofungin at MIC and sub-MIC concentrations: 1/4, 1/3, 1/2, and 3/4 MIC, for 2 and 3 h, (the same exposure times as in the adherence assays) then the *Candida* suspension was washed ($\times 3$). The effect of the treatment on *C. albicans* viability was evaluated using XTT test in 96 wells microtiter plates, and microscopically by Trypan blue staining [27]. As a control we used amphotericin B at a concentration corresponding to its MIC and untreated *C. albicans*.

Scanning electron microscopy of caspofungin treated *C. albicans*

C. albicans was grown for 24 h on SDA, then $1.5 - 2 \times 10^6$ cells/ml were suspended in yeast nitrogen base containing diluted caspofungin (1/2, 1/4 MIC) for 24—48 h. After incubation the cells were fixed in 1% glutaraldehyde overnight at 4 °C, post fixed with 1% OsO_4 solution for 2 h at room temperature and then re-hydrated in an alcohol series, air dried, coated with gold (15 nm) and examined with a Joel 840 SEM at 20 kV.

Statistical analysis

The results were analyzed by ANOVA using SPSS and Excel.

Results

Adherence of *Candida* species to HaCat cells

The initial stage of our research was to establish a baseline of the adherence level as a comparison for the following experiments to evaluate the effect of caspofungin on adherence. We therefore carried out assays using FITC labelled *Candida* and a fluorometric evaluation method. The adherence of five strains of *C. albicans*, five strains of *C. tropicalis*, five strains of *C. glabrata*, and three strains of *C. krusei* to HaCat cells was evaluated, using FITC labeled *Candida* cells. The comparison between the adherence values of the tested *Candida* strains is represented in Figure 1 with no obvious differences.

Determination of MIC values of caspofungin for the different *Candida* species

The next step of the study consisted of determination of MIC values (Figure 2) again as a baseline for further experiments. It can be noted that *C. krusei* strains have higher MICs than strains of the other species.

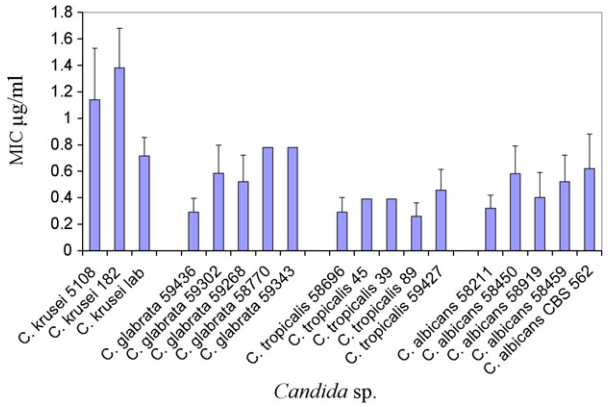

Figure 2 Caspofungin MIC of *Candida* species.
Figure 2 CMI de la caspofungine vis-à-vis des *Candida* testés.

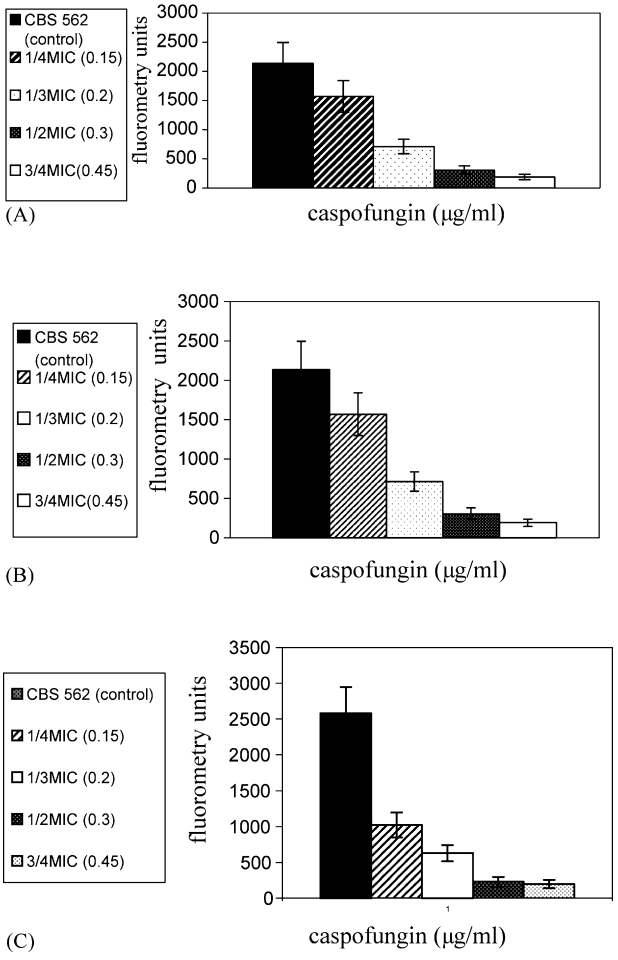

Figure 3 Effect of subinhibitory MIC concentrations of caspofungin on adherence of *C. albicans* CBS 562 to HaCat cell line: (A) Caspofungin in adherence mixture; (B) 2 h caspofungin pretreatment; (C) 3 h caspofungin pretreatment.
Figure 3 Effet de concentrations subinhibitrices minimales de la caspofungine sur l'adhésion de *C. albicans* CBS 562 sur les cellules de la lignée HaCat : (A) Caspofungine et mélange d'adhésion ; (B) Deux heures de prétraitement à la caspofungine ; (C) Trois heures de prétraitement à la caspofungine.

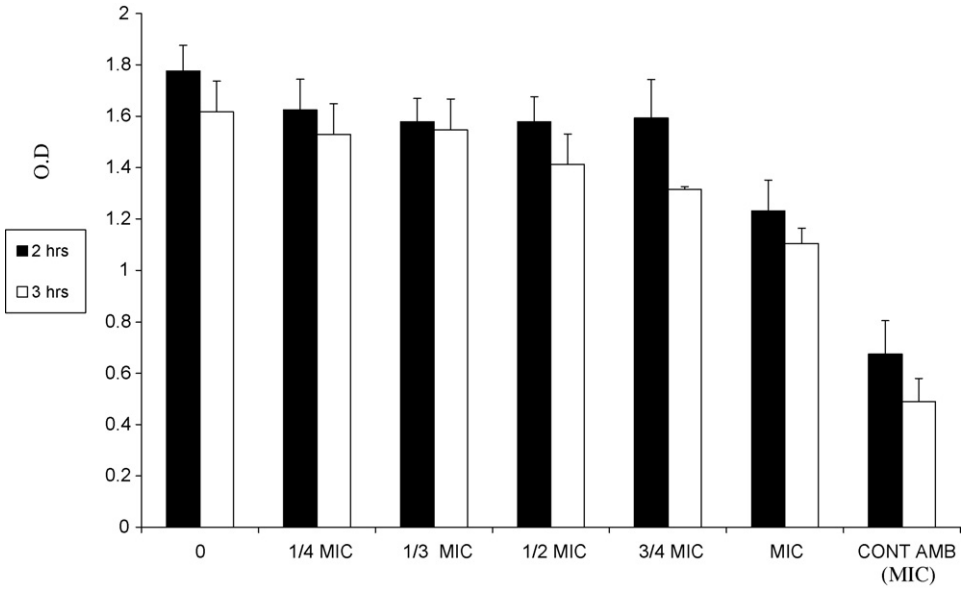

Figure 4 Effect of caspofungin on *C. albicans* viability as measured by XTT.
Figure 4 Effet de la caspofungine sur la viabilité de *C. albicans* mesurée par XTT.

Effect of subinhibitory MIC concentrations of caspofungin on adherence of *C. albicans* CBS 562 to HaCat cell line

Since *C. albicans* CBS 562 was used throughout the study as a reference strain we used this strain as a representative to study the effect of caspofungin on adherence of the fungus to the HaCat cell line.

We assessed the effect of caspofungin using 1/4, 1/3, 1/2, and 3/4 MIC concentrations (MIC of CBS 562 = 0.62 μg ml) in two different experimental models: a) exposure during the adhesion reaction, b) exposure of the yeasts to the drug for 2 and 3 h prior to the adherence reaction.

Figure 3(A) summarizes the results of adherence of *C. albicans* CBS 562 to HaCat cell line in the presence of caspofungin (various sub-MIC concentrations), and Figure 3(B) and (C) summarize, respectively, the adherence of *C. albicans* exposed to the drug for 2 and 3 h prior to the assay.

The data demonstrate that caspofungin reduces the adherence of *C. albicans* and that the reduction is concentration dependent. It can be calculated that even low doses of caspofungin (1/4 MIC) inhibit adherence by 40% compared to nontreated fungi, when present in the adherence mixture (Figure 3(A)). Higher concentrations of caspofungin (3/4 MIC) reduce adherence by over 80%.

The data show that pretreatment of *C. albicans* with caspofungin (Figure 3(B) and (C)) also affects its adherence capacity. The effect is dose and time related, specifically, treatment of *C. albicans* with low concentrations of caspofungin (1/4 MIC) resulted in a significant inhibition of adherence ($p < 0.005$), and the 1/2 or 3/4 MIC concentrations resulted in inhibition of up to 90%.

Studying the effect of caspofungin on *C. albicans*

In order to assess whether the decrease in adherence level due to caspofungin treatment damaged the fungal cells in a way that affected their viability, we evaluated the effect of caspofungin on *C. albicans* using the XTT test and Trypan blue staining as well as electron scanning microscopy (SEM).

a. Effect of caspofungin on *C. albicans* measured by the XTT method:

The XTT tests (Figure 4) revealed that only high concentrations of caspofungin (3/4 MIC or higher) affect *Candida* viability and cause a decrease of up to a 32% (calculated) in viability as compared to a 5% decrease in *Candida* viability post exposure to lower concentrations. While exposure to the MIC concentration of caspofungin causes a 32% decrease in *Candida* viability, MIC concentration of AMB causes a 70% decrease in *Candida* viability.

b. The Trypan blue staining method of *C. albicans* organisms exposed to MIC concentrations of caspofungin treatment (for 2 or 3 h) revealed that 76.8 or 64.9%, respectively, of the cells were alive (stained in light blue compared to dead cells stained in dark blue). Incubation of the fungus in sub-MIC concentrations (1/4—3/4 MIC) for 2 h, resulted in 90—94% live cells compared to 76.9—83.5% after 3 h incubation (data not shown).
c. Scanning electron microscopy analysis of caspofungin (1/4 and 1/2 MIC) treated *C. albicans* versus nontreated control is shown in Figure 5.

As shown in the figure, caspofungin-treated cells for 2 h (B- 1/2 MIC, C- 1/4 MIC) demonstrate wrinkled surface and abnormal structure as compared to control cells (A) which have smooth surface and round structure, indicating morphological changes in the treated organisms.

Discussion

The aim of this research was to study the effect of the novel antifungal drug, caspofungin, on adherence of *C. albicans* to

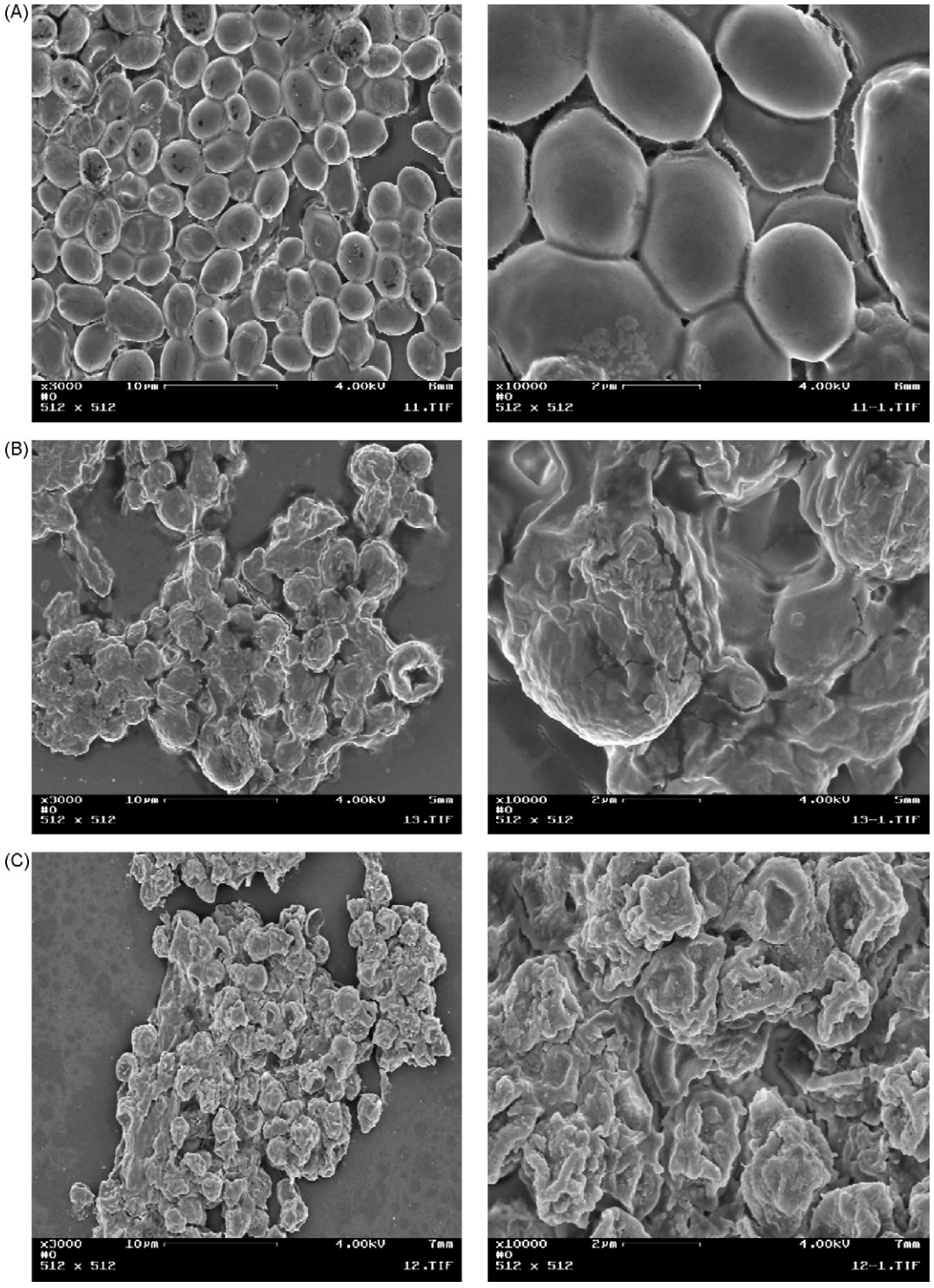

Figure 5 SEM analysis of caspofungin treated *C. albicans*: (A) Untreated control; (B) *C. albicans* treated with 1/2 MIC of caspofungin; (C) *C. albicans* treated with 1/4 MIC of caspofungin.

Figure 5 Analyse par microscope électronique à balayage de *C. albicans* traité par la caspofungine : (A) Témoin non traité ; (B) *C. albicans* traité par 1/2 CMI de caspofungine ; (C) *C. albicans* traité par 1/4 CMI de caspofungine.

human cells. The model target cell used, the human keratinocytes cell line (HaCat), has the advantage of consisting of cells that can undergo numerous passages in vitro and hence enable experimental conditions that are subjected to a lesser degree of variability.

The method for assessment of the level of adherence used in this study, was based on fluorometric measurements using fluorescently labeled yeasts, a method less prone to subjectivity, contrary to many other in vitro adhesion systems, in which adhesion is evaluated microscopically [20,22]. This

method was shown previously to be compatible with adherence evaluated microscopically, using FITC labeled or non-labeled *Candida*, and was used by other investigators [12] to study microbe-host cell interactions. The results obtained have shown that caspofungin affects the level of adherence of *C. albicans* to the HaCat cell line under the two different experimental conditions of the study. Direct exposure to caspofungin at sub-MIC values during the interaction period (when added to the adhesion mixture) reduced the adhesion level by about 40—80% in dependence of the concentration (1/4—3/4 MIC) (Figure 4). The effect of pretreatment of the *Candida* with caspofungin prior to the interaction with the HaCat cells was time and concentration dependent. Pretreatment at 3/4 MIC concentration for 3 h, revealed a reduction of adhesion by over 90%.

The effect of caspofungin on adherence cannot be attributed to its cidal activity since all concentrations were at the sub-MIC level. Moreover, as revealed by the XTT assays and Trypan blue staining there was only a relatively small percentage of nonviable organisms among the caspofungin exposed *C. albicans*. The SEM analysis demonstrating morphological changes in the *Candida* cells exposed to caspofungin, may suggest that the decrease in the level of adhesion could possibly be associated with the morphological changes of the yeasts. The observation that the changes were noticeable already at low concentrations (1/4 MIC) and after a relatively short exposure time (2 h), as was also the reduction in adherence level, may be meaningful for the applicability of the concept of prophylactic use of sub-MIC concentrations in a risk population. It should, however, be emphasized that validation of such a concept would necessitate further experimentations.

Although caspofungin has been shown to modulate adherence to plastic [25], our study is, to the best of our knowledge, the first involving human cells.

In summary, the present study demonstrated that caspofungin at sub-MIC concentration, reduces significantly adhesion of *C. albicans* to human cells, a finding that could be a basis for further research aiming to explore the feasibility of prophylactic use of low dose antifungals in risk populations.

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