Sensitivity of *Candida albicans* isolates to caspofungin – comparison of microdilution method and E-test procedure

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Abstract

Introduction: *Candida albicans* still remains the prevailing fungal opportunistic pathogen responsible for serious mucosal and invasive infections. The efficacy of conventional antifungal drugs is becoming limited since several mechanisms of resistance developed by *C. albicans* have been described. We assessed *in vitro* activity of caspofungin – an inhibitor of (1,3)-β-glucan synthase – against *C. albicans* isolates and compared the results obtained by two different methods.
Material and methods: The *in vitro* activity of caspofungin against 96 *C. albicans* isolates was obtained from the nasopharynx of hospitalised and ambulatory patients assessed using both the reference Clinical Laboratory Standards Institute (CLSI) microdilution method and the E-test procedure. The Kappa test was used in order to calculate the degree of agreement between the outcomes obtained by two different methods.
Results: The microdilution and E-test minimal inhibitory concentration values of caspofungin for 90% *C. albicans* isolates (MIC_{90}) were 0.5 and 0.12 mg/l, respectively. The minimal fungicidal concentration value of caspofungin for 90% *C. albicans* isolates (MFC_{90}) was 1 mg/l. The outcomes obtained by the E-test procedure exhibited a very good correlation with those achieved by the recommended method as the agreement between MICs within ±2 dilutions read by these two techniques was 88% and the Kappa coefficient was 0.88.
Conclusions: Caspofungin demonstrated an excellent potency against *C. albicans* isolates and the E-test method appears to be useful for determination of *in vitro* caspofungin activity against these pathogens.

Key words: caspofungin, *Candida albicans*, E-test, minimal inhibitory concentration, minimal fungicidal concentration.

Introduction

The rate of serious fungal infections caused by *Candida* species, i.e. candidaemia and candidiasis, has soared lately as a consequence of the increasing number of patients whose immunity is compromised. This problem affects HIV/AIDS people, patients undergoing aggressive therapies for cancer and autoimmune diseases, organ or tissues recipients, and patients in neonatal intensive care units. Although yeasts belonging to non-*albicans* species have emerged recently as frequent aetiological agents of fungal infections, the main pathogen remains *Candida albicans*; it can cause either mucosal or systemic diseases [1-3].

Caspofungin is an echinocandin with a novel mode of action – inhibition of (1,3)-β-glucan synthesis. This drug is indicated for treatment of invasive aspergillosis and invasive candidiasis in cases of peritonitis, candidaemia in neutropenic patients, intra-abdominal ulcer, infection of the pleural cavity, oesophageal candidiasis, as well as empirical treatment of patients with fever and neutropenia if fungus infection (*Aspergillus* or *Candida*) is highly suspected [1].

Caspofungin exhibits concentration-dependent fungicidal or fungistatic activity against *Candida* spp. [4]. The broth microdilution method recommended by CLSI (Clinical Laboratory Standards Institute) for assessing *in vitro* activity of antifungals is the one widely used in laboratories for estimating the potency of caspofungin. However, this procedure is laborious and time-consuming, so an alternative, more practical but reliable method is in demand, e.g. E-test [5-7].

The aim of our study was to (i) estimate *in vitro* activity of caspofungin against yeasts of *C. albicans*, (ii) evaluate the agreement between minimal inhibitory concentrations (MICs) of caspofungin obtained by the CLSI reference broth microdilution method and the E-test procedure.

Material and methods

Microorganisms

A collection of 96 *C. albicans* isolates was tested. They were obtained from the nasopharynx of hospitalised and ambulatory adults and children between 2004 and 2006. The number of patients participating in the study was 565, including 188 adults and 447 children. The throat and nasal swabs were streaked onto Sabouraud dextrose agar with or without chloramphenicol in order to isolate the yeast-like fungi. Identification was carried out using microscopic methods (Gram stain), biochemical microtests API 20C Aux (bioMerieux) and by testing the formation of chlamydospores. All yeast isolates were stored on Sabouraud dextrose agar until the study was performed. Before the experiment, each isolate was passaged onto fresh agar to ensure purity and optimal growth characteristics.

Caspofungin

Standard antifungal powder of caspofungin acetate was examined (Merck & Co., Inc., USA). Stock solution containing 16 mg/ml was prepared in sterile distilled water and stored frozen at –20°C until use. E-test strips with caspofungin were purchased from AB Biodisk, Sweden. Susceptibility testing *in vitro*.

Broth microdilution method

Broth microdilution testing was performed according to CLSI directions [6]. Serial two-fold dilutions of caspofungin were made in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). Final concentrations of caspofungin ranged from 0.03 to 32 mg/l. Stock inoculum suspensions of yeasts were adjusted to optical density corresponding with 0.5 Mc Farland standard, i.e. 1 to 5 × 10^6 cells/ml in sterile 0.85% NaCl, and then suitably diluted at first 1 : 100 and then 1 : 20 with RPMI 1640 medium, according to the procedures accepted by CLSI. After 48 h of incubation at 35°C, the MICs were visually evaluated and defined as the lowest drug concentration that showed complete growth inhibition. Drug-free and yeast-free controls were included. All experiments were done in triplicate. Representative data are presented.

E-test procedure

The E-test method was performed according to the manufacturer's guidelines (AB Biodisk). The strips of caspofungin possess continuous concentration gradients of 0.002 to 32 mg/l. The yeast inocula were prepared in sterile 0.85% NaCl to match the turbidity of 0.5 Mc Farland standard, i.e. 1 to 5 × 10^6 cells/ml. 90 mm-diameter plates containing RPMI 1640 agar medium supplemented with 2% glucose and MOPS were used. The RPMI agar was inoculated by using a sterile swab dipped in a yeast suspension and the plates were left at room temperature for about 15 min in order to allow moisture to be absorbed completely. Then, an E-test strip was applied onto each plate. The plates were incubated at 35°C for 48 h. Minimal inhibitory concentrations values were read at the point of intersection between inhibition ellipse edge and the scale on the E-test strip. Micro-organisms inside the inhibition zone were not taken into account. All experiments were done in triplicate. Representative data are presented.

Minimal fungicidal concentration

In order to determine the minimal fungicidal concentration (MFC) of caspofungin, 10 µl from each tube from the broth microdilution method 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 triplicate. Representative data are presented.

Correlation coefficient

The Kappa test was used to calculate the degree of agreement. We converted our results for

statistical analysis requirements and treated the MIC values obtained by both methods within two dilutions as compatible. Kappa coefficients greater than 0.75 represent excellent agreement, coefficients below 0.4 represent poor agreement and coefficients between 0.4 and 0.75 represent fair to good agreement [8].

Quality control

Quality control was performed for broth microdilution and E-test methods by using the CLSI reference strain *C. parapsilosis* ATCC 22019. The MICs of caspofungin obtained for the quality control isolate (*C. parapsilosis* ATCC 22019) were 1 mg/l evaluated by the microdilution method and 0.5 mg/l assessed by the E-test strips, i.e. within the control range (0.5-2 mg/l) established by Barry *et al*. [9].

Results

The MICs of caspofungin for *C. albicans* isolates evaluated by the microdilution method ranged from 0.06 to 4 mg/l and from 0.047 to 0.5 mg/l when the E-test procedure was applied. Caspofungin inhibited 50 and 90% of the isolates at 0.25 mg/l (MIC_{50}) and 0.5 mg/l (MIC_{90}), respectively, when the CLSI reference method was used. Both MIC_{50} and MIC_{90} assessed by the E-test strips were 0.12 mg/l. All examined strains were susceptible to caspofungin. Determining the MIC of caspofungin by means of the microdilution method, the increased turbidity of the fungal inoculum at the highest drug concentrations was recorded for 26 *C. albicans* strains (27.1% of all isolates tested). This "Eagle effect" in accordance with the literature data was neglected for the MIC readings [10].

The MFC of caspofungin for *C. albicans* isolates ranged from 0.06 to >16 mg/l with the MFC_{90} of 1 mg/l. In the course of MFCs determination the correlation between the turbidity at the highest drug concentrations during the MIC reading and the lack of fungicidal effect of caspofungin at these high concentrations was observed. This phenomenon was ignored where MFCs were estimated. Activity of caspofungin for 2 isolates tested (2.1%) was fungistatic. For 53 *C. albicans* strains (55.2%) the MFC/MIC ratio was 1, for 30 strains (31.2%) it was 2, and for another 11 isolates (11.5%) MFC = 4 × MIC.

The E-test MIC values were compared with the MICs obtained by the microdilution method (Table I). As the scale of the E-test strip possesses a continuous gradient of the drug concentrations, the MICs in-between twofold dilutions were raised to the next twofold level in order to make them correspondent with the dilution scheme of the CLSI recommended procedure. The percentages of agreement within ±1 and ±2 dilutions between the broth microdilution MICs and MICs assessed by use of E-tests were 49 and 88%, respectively. All the MICs values were read after 48 h of incubation. Kappa coefficient calculated for the MICs outcomes provided by both techniques was 0.88.

Table I. Comparison of the MICs of caspofungin for *C. albicans* isolates determined by the microdilution method and the E-test procedure

Number of *C. albicans* isolates	MIC determined by the microdilution method	MIC determined by the E-test procedure
28	0.5	0.12
22	0.25	0.12
12	0.12	0.12
7	0.12	0.06
7	0.25	0.06
6	0.5	0.06
3	0.25	0.25
3	0.5	0.25
3	1	0.12
2	1	0.25
1	1	0.06
1	1	0.12
1	1	0.5

Discussion

Candida albicans is still notorious for being a causative agent of fungal infections associated with high morbidity and mortality rates, especially in immunosuppressed patients. Although modern medicine employs a wide range of antifungal agents, mucosal and invasive candidiasis are not always treated successfully due to emerging resistance of the yeasts belonging to *Candida* spp. [1, 2, 4, 11]. It is known that the isolates of *C. albicans* obtained from carriers may be useful in predicting drug resistant patterns of invading isolates, since candidiases are usually endogenous in origin [12].

Caspofungin, a member of a novel echinocandin family, appears to be a potent fungicidal or rarely fungistatic antibiotic against all strains of *C. albicans* tested in our *in vitro* study. The MICs (MIC_{90} 0.5 and 0.12 mg/l) and MFCs (MFC_{90} 1 mg/l) obtained in this research were comparable to the values presented by other authors [10, 13-15]. For comparison, according to our published [16] and unpublished data, $MICs_{90}$ of the routinely used antifungal reagents amphotericin B, flucytosine, and fluconazole, for *C. albicans* isolates were 1, 0.25-0.5, 2-3 mg/l, respectively. These values are

in accordance with the outcomes achieved by Marco *et al*. [17]. A remarkably low MFC/MIC ratio ≤ 2 and ≤ 4 for successively 83 (86%) and 94 (98%) examined isolates confirms excellent anti-candidal *in vitro* activity of caspofungin. According to the literature, similar low MFC/MIC ratios were noted for other echinocandins – micafungin and anidulafungin [18, 19]. Taking into account that the mean trough plasma concentration of caspofungin when administered at clinical doses is greater than 1 mg/l [4], 99% of our isolates, i.e. 95 strains, were inhibited and 92%, i.e. 88 strains, were killed within this therapeutically attainable concentration. After all, the clinical correlation between the *in vitro* and *in vivo* responses cannot be unmistakably evaluated, because neither the echinocandin breakpoints nor the complete standardisation of the susceptibility testing procedure has been fully established yet [7, 20, 21].

The paradoxical growth of *C. albicans* isolates in broth at some supra-MIC and supra-MFC concentrations of caspofungin noted during our investigation is known in the literature as the "Eagle effect". This phenomenon has not been reported for other echinocandins and according to the literature [18, 19] and our unpublished data, it is rare for *Candida* non-*albicans* species. Notably, the concentrations of caspofungin at which this paradoxical growth is observed *in vitro* are within the range achieved in human serum after the recommended dosage. The peak plasma concentration of caspofungin after a single intravenous infusion of 70 mg amounts even to 16 mg/l and the concentrations in some tissues appear to greatly exceed the serum values. Although the clinical relevance of the "Eagle effect" is unknown, a paradoxical effect was not reproducibly demonstrated *in vivo* in murine systemic models of candidiasis [22-25].

Comparing caspofungin MIC values for our isolates determined by the CLSI reference method with those obtained by E-tests a high similarity of the outcomes within 2 dilutions was noted. The correlation between readings provided by these two procedures was statistically significant and confirmed by Kappa coefficient of 0.88. In general, when a discrepancy was reported between both techniques the E-test MICs were the lower ones. Our data are consistent with the outcomes of other authors [5, 7, 19].

In conclusion, caspofungin seems to be a highly effective antifungal agent against *C. albicans*, with potent *in vitro* fungicidal activity towards the majority of the isolates. Therefore, it may constitute an interesting therapeutic option for the treatment of *C. albicans* infections. In addition, the E-test method proved to be a reliable technique for testing *in vitro* susceptibility of *C. albicans* to caspofungin.

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