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Postantifungal effect of caspofungin against the *Candida albicans* and *Candida parapsilosis* clades

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ABSTRACT

Killing and postantifungal effects could be relevant for the selection of optimal dosing schedules. This study aims to compare time-kill and postantifungal effects with caspofungin on *Candida albicans* (*C. albicans*, *Candida dubliniensis*, *Candida africana*) and *Candida parapsilosis* (*C. parapsilosis*, *Candida metapsilosis*, *Candida orthopsilosis*) clades. In the postantifungal effect experiments, strains were exposed to caspofungin for 1 h at concentrations 0.12–8 μg/mL. Time-kill experiments were conducted at the same concentrations. Caspofungin exhibited a significant and prolonged postantifungal effect (>37 h) with 2 μg/mL against the most strains of *C. albicans* clade. Against the *C. parapsilosis* clade, the postantifungal effect was <12 h at 8 μg/mL, except for two strains. Caspofungin was fungicidal against *C. albicans*, *C. dubliniensis* and *C. metapsilosis*.

1. Introduction

Nosocomial invasive candidiases are important causes of morbidity and mortality. Although *Candida albicans* is the most common cause of invasive candidiasis, the incidence of infections due to non-*C. albicans* species is rising. Among these species, *Candida parapsilosis* is emerging as a cause of nosocomial blood stream infection in both adult and children. In addition to *C. albicans sensu stricto*, two closely related species with differences in antifungal susceptibility, virulence and epidemiology have been described and added to the *C. albicans* clade: *Candida dubliniensis* and *Candida africana* (Pfaller and Diekema, 2007; Pfaller et al., 2010; Quindós, 2014). Similarly, the *C. parapsilosis* clade groups three different but closely related species: *C. parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis*. These species show differences in antifungal susceptibility and virulence, with their epidemiology and antifungal resistance patterns becoming matters of increasing interest (Cantón et al., 2010; Lockhart et al., 2008; Silva et al., 2009; Szabo et al., 2009; Tavanti et al., 2005). *C. parapsilosis* isolates with decreased susceptibility to echinocandins and azoles have been detected, but their importance in the clinical outcome is unknown (Moudgal et al., 2005; Pappas et al., 2016).

Caspofungin is an echinocandin that decreases fungal growth or kills the fungal cell by the non-competitive inhibition of 1,3-β-glucan synthase, an enzyme responsible for fungal cell wall synthesis. Caspofungin is being increasingly used as first line therapy for invasive candidiasis (Pfaller et al., 2006). Postantifungal effect (PAFE) is the continuation of the suppression of fungal growth after the drug is removed. The existence of a PAFE depends on both the fungal species and the class of the antifungal drug. Moreover, the PAFE may also be affected by the size of the fungal inoculum, the concentration of the drug and the time of exposure. There is an increasing need for understanding pharmacodynamic properties of antifungal agents, specially the new ones and their potential usefulness in treating these severe infectious diseases. For this reason, the PAFE may have a main clinical relevance in the design of dosing regimens for these new antifungal agents. Although killing and the PAFE could be relevant for the selection of optimal dosing schedules selection, there is limited information regarding both parameters for caspofungin against *Candida* (Clancy et al., 2006; Ernst et al., 2000; Louie et al., 2005; Smith et al., 2011).

The aim of this study was to compare the *in vitro* killing and PAFE of caspofungin against clinical isolates and reference strains of the *C. albicans* and *C. parapsilosis* clades. The interest of this study focuses on the current increasing use of echinocandins and on the influence of

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the PAFE on dose interval choice and infection outcome (Gumbo, 2007; Louie et al., 2005).

2. Materials and methods

2.1. Drugs

Thirteen clinical isolates and 8 reference strains of *C. albicans* and *C. parapsilosis* were included in this study: 5 *C. albicans* clinical bloodstream isolates, 2 *C. albicans* reference strains (NCPF 3153 and NCPF 3156), 4 *C. dubliniensis* clinical blood isolates, 1 *C. dubliniensis* reference strain (NCPF 3949), 1 *C. africana* vaginal isolate and 1 *C. africana* reference strain (ATCC 2669). Moreover, 1 clinical bloodstream isolate and 2 reference strains of *C. parapsilosis sensu stricto* (ATCC 22019 and ATCC 90018), 1 blood isolate and 1 reference strain of *C. metapsilosis* (ATCC 96143) and 1 blood isolate and 1 reference strain of *C. orthopsilosis* (ATCC 96139) were selected for study based upon extensive experience with these isolates in our laboratory. Isolate identification was performed as previously described (Miranda-Zapico et al., 2011; Pemán et al., 2012).

2.2. Test isolates

After preparing a stock solution of caspofungin (5120 μg/mL) (Merck Sharp & Dohme, Madrid, Spain) in dimethyl sulfoxide, further dilutions were made in RPMI 1640 medium with L-glutamine and without $NaHCO_2$ (Sigma-Aldrich, Madrid, Spain) and the solution was buffered to pH 7 with 0.165 M morpholinepropanesulfonic acid (Sigma-Aldrich). Stock solutions were stored at −80 °C until use.

2.3. Antifungal susceptibility testing

MICs (minimum concentrations that produce at least 50% growth reduction) were determined according to the M27-A3 document and M27-A3 S4 supplement (Clinical and Laboratory Standards Institute, 2008, 2012). All MICs were measured in the RPMI, and the results were read after 24 h of incubation.

2.4. Time-kill curve studies

Time-kill studies were performed as previously described (Cantón et al., 2010, 2013; Gil-Alonso et al., 2015a, 2015b). Previously stored isolates and strains were subcultured on Sabouraud dextrose agar (SDA) plates prior to testing. After picking several colonies of *Candida* from a previously prepared 24 h culture, suspensions were adjusted to 1 McFarland ($\approx 10^6$CFU/mL). One milliliter of the fungal suspension was added to vials containing 9 mL of RPMI. Time-kill studies were performed on microtiter plates in a microbiological incubator (BioScreen C MBR, LabSystems, Helsinki, Finland) in RPMI (final volume 200 μL) by using an inoculum size of 1–5 × 10^5CFU/mL. For the *C. albicans* clade, the tested concentrations of caspofungin were 0.12, 0.5, and 2 μg/mL, and for the *C. parapsilosis* clade, the tested concentrations were 0.25, 2, and 8 μg/mL. The studied concentrations of caspofungin are clinically achieved in plasma after usual dosing schedules (Cappelletty and Eiselstein-McKitrick, 2007; Catalán González and Montejo Gonzalez, 2008). Inoculated plates were incubated for 48 h at 36 ± 1 °C. At predetermined time points 0, 2, 4, 6, 24, and 48 h, 10 μl (0–6 h)or 6 μl (24–48 h) were collected from each culture well (control and test solution wells), serially diluted in phosphate buffered saline (PBS) and plated onto SDA. After the plates were incubated at 36 ± 1 °C for 48 h, *Candida* colonies were counted. The lower limit of accurate and reproducible detectable colony counts was 200 CFU/mL.

The assays were performed in duplicate. Plots of averaged colony counts ($\log_{10}$ CFU/mL) *versus* time were constructed and compared against a growth control (in the absence of drug). The antifungal carryover effect was determined as previously described (Cantón et al., 2004).

2.5. Postantifungal effect

PAFE studies were performed as previously described (Ernst et al., 2000; Gil-Alonso et al., 2015c). Standard 1 McFarland turbidity fungal suspensions were prepared in sterile distilled water: 1 mL from these suspensions was added to 9 mL of RPMI. Caspofungin concentrations were the same as described for the time-kill procedures. After an incubation of 1 h, caspofungin was removed by three repeated cycles of repeated centrifugation (2000 rpm, 10 minutes) and washing with PBS. Following a final centrifugation, the fungal pellet was suspended in 600 μL of RPMI. All samples, with a final volume of 200 μL, were incubated on microtiter plates in BioScreen C incubator at 36 ± 1 °C. At the same predetermined time points described in the time-kill experiments, the samples were serially diluted in PBS and inoculated onto a SDA plate for CFU counting.

The PAFE was determined for each isolate as the difference in time required for control (in the absence of drug) and treated isolates to grow 1 × $\log_{10}$ following drug removal. The PAFE was calculated using the equation: PAFE = T − C, where T is the time needed for counts to increase by 1 $\log_{10}$ unit above that obtained following caspofungin removal in the treated wells, and C is the time required for counts to increase by 1 $\log_{10}$ unit above that after the final wash in the control wells (Ernst et al., 2002; Smith et al., 2011).

2.6. PAFE and time-kill comparison

Fungicidal activity was described as a greater than or equal 3 $\log_{10}$ (99.9%) reduction, and fungistatic activity was defined as a less than 99.9% reduction in CFU from the starting inoculum size (Lewis et al., 2002). Plots of the averaged colony counts ($\log_{10}$ CFU per mL) *versus* the time obtained in the PAFE experiments were constructed and compared against a growth control. The ratios of the log killing during the PAFE experiments to the log killing during the time-kill experiments were calculated (Clancy et al., 2006; Nguyen et al., 2010). The time-kill and PAFE experiments were simultaneously performed.

2.7. Statistical analysis

Differences in the PAFE (in h) were determined by an analysis of variance among species and concentrations using GraphPad Prism™ 5.01 (GraphPad Software, San Diego, *CA*, USA). The considered significance level was less than 0.05.

3. Results

No antifungal carryover effect was detected in the time-kill studies. Caspofungin MICs for isolates from the *C. albicans* and *C. parapsilosis* clades are shown in Table 1.

The results of the time-kill and the PAFE experiments for *C. albicans*, *C. dubliniensis* and *C. africana* are summarized in Table 2. Caspofungin exhibited a significant and prolonged PAFE (greater than 37 h) at the highest tested concentration (2 μg/mL) against 12 out of 14 (86%) strains from the *C. albicans* clade, except for one strain of *C. dubliniensis* and one strain of *C. africana*, with PAFEs of 20 and 13.5 h, respectively. Significant differences were detected when compared with the controls (without drug) ($p < 0.005$). In the time-kill experiments, caspofungin was fungicidal against 3 out of 14 (21%) strains of the *C. albicans* clade. A fungicidal endpoint was achieved against *C. albicans* UPV/EHU 99–101 at the three tested concentrations and at 2 μg/mL against *C. dubliniensis* UPV/EHU 00–131 and *C. dubliniensis* UPV/EHU 00–135. A fungicidal endpoint was also attained after caspofungin removal in the PAFE experiments against *C. albicans* UPV/EHU 99–101. The mean value of the PAFE/time-kill ratio was 55.27 with 2 μg/mL for the *C. albicans* clade; moreover, against 8