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Efficacy of echinocandins against murine infections by *Diutina (Candida) rugosa*

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ABSTRACT

Echinocandins are recommended as a first-line therapy for invasive candidiasis. *Candida rugosa* was recently transferred to the new genus *Diutina*. We have determined the *in vitro* killing kinetics of two echinocandins, anidulafungin, and caspofungin and their *in vivo* efficacy, administering doses of 5 or 10 mg/kg, and 1 or 5 mg/kg, respectively against 2 clinical strains of *D. rugosa*. Both drugs showed a fungicidal concentration-dependent activity and, in a neutropenic murine model of disseminated infection, were able to reduce tissue burden and to prolong survival of mice. These results suggest that both echinocandins could be useful to treat infections by this fungus when isolates show minimal inhibitory concentrations within the range of susceptibility for both drugs.

1. Introduction

Diutina (formerly *Candida*) *rugosa* is an emerging pathogen isolated from clinical samples such are traumatic lesions, oral mucosa, or sputum and that causes nosocomial fungemia in immunocompromised population, mainly associated with invasive medical practices such as the use of catheters (Behera et al., 2010; Colombo et al., 2003). It is estimated that the incidence of invasive candidiasis (IC) by this specie is around 0.2% being higher in India (18.4%) (Paredes et al., 2012). Recently, the phylogeny of *Candida rugosa* species complex has been reviewed and eight species belonging to this complex were redefined and re-accommodated in the new genus *Diutina* (Khunnamwong et al., 2015). The habitat of this fungus remains uncertain however, it has been isolated from soil, water forest human feces and human oral cavities (Pires-Gonçalves et al., 2007). *D. rugosa* has been reported causing mastitis in cattle being commonly isolated from milk from mastitic cows (Crawshaw et al., 2005) and also its lipases are widely used in the biotechnology industry in different processes such as production of many food products (i.e. ice cream, fermented foods, fats and oils) (Benjamin and Pandey, 1998). Thus, this infection may be linked to dietary habits such as consuming contaminated milk or food product. Current recommendations for the treatment of IC in neutropenic patients include echinocandins as a first-line treatment and fluconazole (FLC) or amphotericin B (AmB) as alternatives (Arendrup et al., 2014; Nucci et al., 2013). There is little information available on the *in vitro* susceptibility of *D. rugosa* species to echinocandins and is considered azole resistant like *C. glabrata* or *C. krusei*. A recent multilaboratory study suggest that this species has a low susceptibility to polyenes, azoles, and echinocandins (Espinel-Ingroff et al., 2014) but some activity of the latter has been reported (Diekema et al., 2009). Clinical experience in the management of such infections is very limited and does not allow therapeutic recommendations to be established. AmB should probably not be used due to its lack of efficacy, even among strains that have *in vitro* susceptibility to this drug (Behera et al., 2010; Diekema et al., 2009). FLC has been reported as effective for two patients with candidemia produced by FLC-susceptible *D. rugosa* strains that displayed negative cultures after therapy (Minces et al., 2009). There has been only one experimental study conducted in mice on the efficacy of azoles that has shown FLC, voriconazole and posaconazole to be effective against a disseminated infection by two clinical isolates of *D. rugosa* (Hernandez et al., 2004). However, the efficacy of echinocandins has not

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been experimentally explored in animals. In order to explore the role that echinocandins can play in the treatment of disseminated infection by *D. rugosa*, *in vitro* kinetics of anidulafungin (AFG) and caspofungin (CFG) using time-kill curves and their *in vivo* efficacy in a neutropenic murine model of disseminated infection by this fungus were evaluated.

2. Materials and methods

2.1. Fungal isolates

Two clinical isolates of *D. rugosa* from the Fungus Testing Laboratory University of Texas Health Science Center at San Antonio (UTHSCSA), (UTHSCSA 06–3976 isolated from sputum and UTHSCSA 05–1919 from a lesion) previously identified by analysis of the D1/D2 domains and the internal transcribed spacer sequences of the rRNA genes, were used (Paredes et al., 2012). Antifungal profile susceptibility was previously determined by following the CLSI guidelines, document M27-A3 (CLSI, 2008) both strains showing susceptibility to azoles (Paredes et al., 2012). MICs of AFG and CFG were 0.5 μg/mL and 1 μg/mL against the strain UTHSCSA 06–3976 and 0.06 μg/mL of both drugs against the strain UTHSCSA 05–1919, as previously reported (Paredes et al., 2012). Both isolated were included in the study due to their echinocandins MICs differences consisting on 3 to 4 dilutions.

2.2. Inocula preparation

Isolates were cultured for 24 h at 35 °C on potato dextrose agar (PDA) plates. On the day of infection, cultures were scraped off with a sterile loop, suspended in sterile saline and yeast cell suspensions were adjusted to the desired concentration by hemocytometer count. Viability of the inocula was confirmed by placing 10-fold dilutions onto PDA plates.

2.3. Time-kill studies

Time-kill curves were performed according to previous studies (Cantón et al., 2013) with some modifications. A range of concentrations of each drug (0.03, 0.12, 0.5, 1, 2, 8, and 32 μg/mL) and controls (drug-free) were prepared in RPMI with final volumes of 9 mL. Then, each tube was inoculated with 1 mL of a yeast suspension containing 5×10^6 colony-forming units (CFU)/mL, and incubated at 35 °C. At predetermined time points (0, 2, 4, 6, 8, 24, and 48 h), an aliquot from each tube was collected to determine the number of CFU/mL by preparing 10-fold dilutions of the aliquots and placing onto PDA plates. A CFU decrease of ≥99.9% or ≥3 log10 units compared to the starting inoculum was considered fungicidal, while a reduction of <99.9% or <3 log10 units was considered fungistatic (Cantón et al., 2013). The limit of detection was 50 CFU/mL. All time-kill curve studies were performed in duplicate.

2.4. Animals

Four-week-old OF-1 male mice weighing approximately 30 g (Charles River, Criffa S.A., Barcelona, Spain) were used. Mice were immunosuppressed 2 days prior to the infection by intraperitoneal (i.p.) administration of 200 mg/kg of body weight of cyclophosphamide and once every 5 days thereafter (Clemons et al., 2005). All animals were housed under standard conditions and care procedures were supervised and approved under the procedure number 8249, by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

2.5. Infection

Mice were challenged intravenously (i.v.) via the lateral tail vein with 0.2 mL of a yeast suspension containing 1×10^6 CFU of each strain. This inoculum size has previously been shown to be adequate for producing an acute infection with a high mortality in 14–15 days post-infection (Hernandez et al., 2004).

2.6. Treatments

Groups of 20 mice were randomly established, 10 for survival and 10 for tissue burden studies. One day after infection, animals received AFG (Ecalta; Pfizer Inc., Madrid, Spain) at 5 or 10 mg/kg or CFG at 1 or 5 mg/kg (Cancidas; Merck & Co. Inc., Whitehouse Station, NJ, USA), both drugs administered i.p. once daily for 7 days. Control animals received no treatment. Doses of CFG and AFG were selected based on previous experimental (Paredes et al., 2015; Salas et al., 2013) and pharmacokinetic studies (Andes et al., 2010). To prevent bacterial infections, all animals received ceftazidime at 5 mg/kg subcutaneously once daily during the experimental period. The efficacy of the drugs was evaluated by prolongation of survival over 30 days and reduction of tissue burden in kidneys.

2.7. Tissue burden

Animals were euthanized by CO_2 anoxia 7 days post infection, 4 h after the last dose was administered. Kidneys were aseptically removed, weighed, and mechanically homogenized in 1 mL of sterile saline. Ten-fold dilutions of the homogenates were placed onto PDA plates and incubated for 24 h at 35 °C for CFU/g determination (Capilla et al., 2007).

2.8. Statistics

Mean survival time was estimated by the Kaplan-Meier analysis, and the long-rank test was used. Tissue burden data were analysed using the Mann–Whitney *U* test, using Graph Pad Prism 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. In vitro studies

Time-kill studies showed fungicidal activity of both drugs against the two isolates evaluated (Fig. 1). AFG required reaching the fungicidal endpoint between 30 h at 8 μg/mL for UTHSC 05–1919 and 46 h at 16 μg/mL for UTHSC 06–3976. However, for CFG fungicidal activity was only achieved at the highest concentration tested, 32 μg/mL, after 8 h against UTHSC 05–1919 and after 20 h against UTHSC 06–3976.

3.2. In vivo studies

Control animals began to die from 5 to 6 days post-infection and all had succumbed 9 days after challenge (Fig. 2). Administering high doses of either drug, i.e. AFG 10 mg/kg and CFG 5 mg/kg, significantly prolonged the survival of the mice infected with either of the two strains with respect to the control group ($P \leq 0.040$), with no statistical differences between AFG and CFG ($P \geq 0.308$); however, the lower doses of both drugs only prolonged survival respect to the control group against the strain UTHSC 05–1919 ($P \leq 0.014$) but not against UTHSCSA 06–3976, which showed higher MICs for both drugs ($P \geq 0.284$).

The tissue burden study shows that both drugs at both doses were able to significantly reduce the fungal load in kidneys of mice infected by either strain in comparison to the control group ($P \leq 0.0001$) (Fig. 2), showing a dose-dependent response. For UTHSCSA 05–1919, the high doses of CFG and AFG were significantly more effective in reducing the kidney fungal burden than the low doses of each drug ($P \leq 0.003$). However, against strain UTHSC 06–3976, only the high dose of AFG was more effective than the lower dose ($P \leq 0.035$). Also, significant differences were found between AFG and CFG were administered at