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In vitro pharmacodynamics and in vivo efficacy of fluconazole, amphotericin B and caspofungin in a murine infection by *Candida lusitaniae*

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ABSTRACT

The in vitro activities of fluconazole (FLC), amphotericin B (AmB) and caspofungin (CSP) were evaluated against three isolates of *Candida lusitaniae* using time–kill curves. AmB showed in vitro fungicidal activity, whilst FLC and CSP exerted mainly strain-dependent fungistatic activity. The in vivo efficacies of the three drugs were evaluated in a murine model of disseminated infection. The doses administered were FLC 50 mg/kg/day, AmB 0.8 mg/kg/day and CSP 5 mg/kg/day. All three drugs were able to reduce the fungal burden in the kidneys of infected mice, with AmB showing the highest efficacy, followed by CSP. At least in this model, FLC, AmB and CSP are good candidates for treating invasive infections by *C. lusitaniae*.

1. Introduction

Invasive fungal infections by *Candida* spp. are a cause of death, especially in intensive care units, owing to the difficulty in making a quick diagnosis and initiating treatment [1]. Several *Candida* spp., *Candida lusitaniae* among them, have emerged as human pathogens in the last decade. Approximately 1–2% of candidaemia cases in adults, mainly in patients with haematological malignancies, are attributed to this yeast, which has also been reported as an agent of nosocomial outbreaks [2,3]. Optimal treatment of infections caused by this species has not been fully established. The recommended drugs for invasive infections by *Candida* currently involve echinocandins as the first choice and liposomal formulations of amphotericin B (AmB) as an alternative, with fluconazole (FLC) being considered as a step-down treatment option [4]. *Candida lusitaniae* shows a particular susceptibility pattern. Although it usually appears susceptible to all antifungal agents in vitro [5,6], in vivo resistance to AmB has been observed in several clinical cases during the course of therapy, which is frequently associated with treatment failure. Hence, several authors recommend avoiding the use of this drug for treating *C. lusitaniae* infections [7,8]. Resistance to FLC [6] and caspofungin (CSP) [9] has also been reported. However, to better understand the clinical significance of resistance to these drugs, data are needed regarding antifungal treatment against *C. lusitaniae* in experimental models under controlled conditions or clinical assays [10]. It is unknown whether therapeutic failure in the reported clinical cases was due to the poor underlying clinical condition of the patients or to the particular resistance or virulence of the fungi. Consequently, we have evaluated the in vitro activity and in vivo efficacy of FLC, AmB and CSP by means of time–kill assays and in an experimental murine model of systemic infection using three clinical strains of *C. lusitaniae*.

2. Materials and methods

Three clinical isolates of *C. lusitaniae* (FMR 9474, UTHSC 11-586 and UTHSC 11-537) with different minimum inhibitory concentrations (MICs) were included in the study. Their identification was confirmed by comparing the sequences of the large-subunit ribosomal RNA gene of the three isolates with that of the type strain of the species. The MICs of FLC, AmB and CSP determined by the reference broth microdilution method [11] are shown in Table 1. The fungal isolates were stored at −80 °C and were subcultured on potato dextrose agar (PDA) at 35 °C for 48 h prior to testing.

Time–kill curves were performed according to Klepser et al. with some modifications [12]. In brief, each drug concentration ranged from 0.25× to 32× the MIC for the corresponding isolate using serial doubling dilutions, i.e. 0.063–512 μg/mL for FLC, 0.063–32 μg/mL

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Table 1
Minimum inhibitory concentrations (MICs) of three antifungal agents against three clinical isolates of *Candida lusitaniae*.

Isolate	MIC (μg/mL)		
	FLC	AmB	CSP
FMR 9474	0.25	0.25	0.25
UTHSC 11-586	16	1	2
UTHSC 11-537	1	1	0.5

FLC, fluconazole; AmB, amphotericin B; CSP, caspofungin.

for AmB and 0.063–64 μg/mL for CSP. At predetermined time points (4, 8, 24 and 48 h), an aliquot of 0.1 mL was removed from each drug dilution tube and was serially diluted in sterile water. Dilutions were placed onto PDA and were incubated at 35 °C for 24–48 h to determine the colony count as CFU/mL. This procedure allowed a quantification limit of 33 CFU/mL. All assays were carried out in duplicate, with two replicates for every dilution at each time point, and the geometric mean and standard deviation were calculated for mismatched results.

For animal inoculation, cultures on PDA were suspended in sterile saline. The resulting suspensions were adjusted to the desired concentration based on haemocytometer counts and the viability of the inocula was checked by serial plating onto PDA plates.

Male OF-1 mice (Charles River, Criffa S.A., Barcelona, Spain) (4 weeks old, weight ca. 30 g) were used. Animals were housed under standard conditions and the care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee (Reus, Spain). Mice were immunosuppressed 1 day prior to infection by administration of a single dose of 200 mg/kg cyclophosphamide given intraperitoneally followed by a single dose of 150 mg/kg 5-fluorouracil administered intravenously [13]. Animals were inoculated intravenously by the lateral tail vein with a suspension of 1×10^4 (UTHSC 11-586 and UTHSC 11-537) or 1×10^8 (FMR 9474) CFU per animal in 0.2 mL of sterile saline solution. No mortality was observed with the inocula tested (data not shown). The experimental model used for isolate FMR 9474 required a higher inoculum since previous assays showed that the tissue fungal load of controls was notably lower than the values obtained for the other isolates (ca. 3 log lower), suggesting a markedly lower virulence of this strain.

FLC (Diflucan®; Vinci Farma, Madrid, Spain) was administered orally at 25 mg/kg twice daily, amphotericin B deoxycholate (Farmacia Xalabarder, Barcelona, Spain) was administered intravenously at 0.8 mg/kg once daily and CSP (Cancidas®; Merck & Co. Inc., Whitehouse Station, NJ) was administered intraperitoneally at 5 mg/kg once daily. These doses were selected based on time–kill results and previous drug pharmacokinetic studies in order to obtain high serum concentrations without exceeding the antifungal doses that have usually been used in similar mice experiments with other *Candida* spp. [14–16]. To prevent bacterial infection, all animals received ceftazidime at 5 mg/kg/day subcutaneously. Control groups received no antifungal treatment. All treatments began 24 h after challenge and lasted for 7 days.

The efficacy of the different drugs was evaluated by tissue burden reduction in the kidneys. Groups of 10 animals were established for each treatment. Mice were euthanised by anoxia using a carbon dioxide chamber on day 8 after infection 24 h after the last dose of treatment. The kidneys were aseptically removed and were homogenised in 2 mL of sterile saline solution. Homogenates were serially diluted (1:10) and were plated onto PDA agar plates to determine fungal load (CFU/g of tissue).

Colony counts were analysed using the Mann–Whitney *U*-test. Statistical analyses were performed using GraphPad Prism v.4.00 for MS Windows (GraphPad Software Inc., La Jolla, CA).

3. Results

Fig. 1 shows time–kill plots for each strain–drug combination. CSP activity was strain-dependent, showing fungistatic activity against isolates UTHSC 11-586 and UTHSC 11-537 and fungicidal activity against isolate FMR 9474 at high drug concentrations (16–32× MIC). FLC exhibited only fungistatic activity against the three isolates for drug concentrations above 2× MIC. AmB showed fungicidal activity against the three strains for drug concentrations above 4× MIC.

The three drugs tested significantly reduced the fungal burden in comparison with the control group for the three isolates tested. AmB and CSP showed higher efficacy than FLC against isolates FMR 9474 and UTHSC 11-586, whilst the three treatments showed similar efficacy for isolate UTHSC 11-537. AmB showed the highest overall in vivo efficacy (Fig. 2).

4. Discussion

In this study, the in vitro and in vivo activities of the antifungal drugs recommended for the treatment of *C. lusitaniae* infections were assessed. AmB and CSP were the most active both in vitro and in vivo against all of the isolates tested.

Although few isolates were used, only AmB showed a correlation between in vitro activity and in vivo response, with higher efficacy against the isolate that had a lower MIC to this drug. By contrast, although the MICs of FLC and CSP were related to the in vitro pharmacodynamic results, the in vivo efficacy was shown to be strain-dependent. These results showed that MIC values might not be a good predictor of the result of antifungal treatment for infections by this species, although it is necessary to test a considerably larger number of strains with a wider MIC distribution in order to get a final conclusion.

AmB and FLC have been the most commonly used antifungals in the reported clinical cases of invasive infections by *C. lusitaniae* [17], but studies of in vitro kinetics and susceptibility patterns of these drugs in *C. lusitaniae* are scarce. In a previous study involving 11 *C. lusitaniae* isolates, FLC primarily showed fungistatic activity, although fungicidal activity was noted against two isolates [18]. In the current study, we used a small number of isolates that represented a wide FLC MIC range and observed only fungistatic activity. The in vivo results showed a good response to treatment with this drug, although the results were never as good as those achieved with AmB or CSP in terms of kidney fungal burden reduction.

Despite several studies showing that AmB has higher in vitro activity than FLC against *C. lusitaniae* [19], this fungus has traditionally showed clinical resistance to AmB [18,20]. This has been attributed to a phenotype switching phenomenon similar to that shown in *Candida glabrata* and *Candida albicans*, in which the fungal response to antifungal agents may change as an environmental adaptation by selective gene expression [8,21]. Although the mechanism for this resistance in *C. lusitaniae* is not clearly understood, its occurrence has been related to morphological alterations in colony size and colour or cell filamentation changes [8,21,22]. In the present study, AmB not only showed good in vitro activity but also the highest in vivo efficacy of the three antifungals tested, whilst no colony changes were observed. This can be explained by the short duration of the experimental treatment, since clinical resistance has been documented mainly in persistent infections with long-term treatments [8,23].

Several studies have described the in vitro activity of echinocandins as fungistatic against *Candida* spp. [19,24]. However, in a recent study, high CSP concentrations exerted fungicidal activity against *C. lusitaniae* [25]. The antifungal behaviour of CSP against the isolates in the current study was very similar to results seen in the