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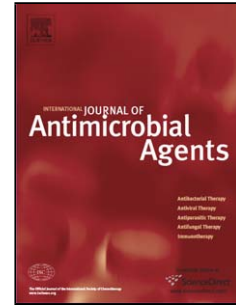


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Evaluation of antifungal therapy in a neutropenic murine model of *Neoscytalidium dimidiatum* infection

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ABSTRACT

We evaluated the efficacy of amphotericin B (1.5 mg/kg/day), voriconazole (60 mg/kg/day) and posaconazole (60 mg/kg/day) in a murine model of systemic infection caused by *Neoscytalidium dimidiatum*. All the treatments were able to prolong survival and to reduce the tissue burden in the spleen and kidneys of infected mice. Neither voriconazole nor posaconazole improved the results achieved with amphotericin B.

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1. Introduction

The dematiaceous fungus *Neoscytalidium dimidiatum* is the aetiological agent of chronic superficial infections in human skin and nail [1]. Cases of mycetoma, subcutaneous and cerebral infections, fungaemia and other deep-seated pathologies have been reported less frequently, mainly affecting patients with predisposing factors such as diabetes mellitus, solid organ transplant, corticosteroid therapy, systemic lupus erythematosus and trauma [2–9]. Optimal therapy has not been developed for diseases caused by *N. dimidiatum* and several cases of fatal invasive infections have been reported despite antifungal therapy [4,5,8]. The classic antifungal drugs show good in vitro activity against *N. dimidiatum*, including the most commonly used drug, amphotericin B (AmB) [10,11], which has induced clinical remission in a few cases of deep infection [12,13]. Newer antifungal agents and alternative therapeutic options need to be explored to improve the outcome of severe *Neoscytalidium* infections.

Given the scarcity of data on treatments for *Neoscytalidium* infections, we used a neutropenic murine model developed previously (unpublished) to evaluate the activity of AmB and the new triazoles voriconazole (VCZ) and posaconazole (PCZ) in systemic *N. dimidiatum* infections.

2. Materials and methods

2.1. Fungal strains

Two isolates of *N. dimidiatum* (FMR 9383 and FMR 9354) were used in this study. Fungi were stored at $-80\text{ }^{\circ}\text{C}$ in potato dextrose broth with glycerol and were subcultured on potato dextrose agar (PDA) at $35\text{ }^{\circ}\text{C}$ prior to testing. Inocula were prepared by flooding the surface of the agar plate with sterile saline, scraping the sporulating mycelium with a culture loop and drawing up the resultant suspension with a sterile Pasteur pipette. The suspensions were then filtered once through sterile gauze to remove hyphae. The resulting suspensions of conidia were adjusted to the desired inoculum size by counting with a haemocytometer. Dilutions of the original suspension were subcultured on PDA plates to confirm the haemocytometer counts.

2.2. In vitro studies

Minimum inhibitory concentrations (MICs) of the three antifungal drugs (AmB, PCZ and VCZ) for the strains used in this study were determined by the broth microdilution method according to Clinical and Laboratory Standards Institute guidelines for filamentous fungi [14]. In all cases, 100% inhibition of growth was used as the endpoint criterion. *Paecilomyces variotii* ATCC 36257 was used as the quality control strain.

2.3. *In vivo studies*

2.3.1. *Animals*

Male OF1 mice (Charles River, Criffa S.A., Barcelona, Spain) with a mean weight of 30 g were used. Animals were housed in standard boxes with corncob bedding and had free access to food and water.

2.3.2. *Immunosuppression*

Mice were immunosuppressed by a single intraperitoneal (i.p.) injection of 200 mg of cyclophosphamide per kg of body weight plus a single intravenous injection of 150 mg of 5-fluorouracil per kg 1 day before challenge [15].

2.4. *Drugs*

The drugs tested were AmB (Fungizona[®]; Squibb Industria Farmacéutica S.A., Barcelona, Spain), voriconazole (Vfend[®]; Pfizer Inc., Madrid, Spain) and posaconazole (Noxafil[®]; Schering-Plough Ltd., Welwyn Garden City, UK).

2.5. *Infection and therapy*

Mice were challenged with 1×10^5 colony-forming units (CFU)/mL of *N. dimidiatum* in 0.2 mL of sterile saline, injected into the lateral tail vein.

Preliminary experiments with both strains tested demonstrated that this inoculum was the optimal dose for killing all of the animals within 7 days of infection (unpublished data). The efficacy of the different treatments was

evaluated through prolongation of survival, reduction of fungal tissue burden and histological studies of the organs of infected mice.

For survival studies, groups of 10 mice were randomly established for each treatment and control group. The different groups were treated as follows: AmB 1.5 mg/kg body weight/dose given i.p. once daily; PCZ 60 mg/kg body weight/dose given orally (p.o.) once daily; and VCZ 60 mg/kg body weight/dose p.o. once daily. All treatments began the day after challenge and lasted for 7 days. From 3 days prior to infection, the mice that received VCZ were given grapefruit juice 50% in place of water [16]. The animals were checked daily for 20 days.

For tissue burden studies, groups of 10 mice were also randomly established and the animals were sacrificed on Day 5 post infection, after 4 days of treatment. Spleen and kidneys were removed aseptically and approximately one-half of each organ was weighed and homogenised in 1 mL of sterile saline. Serial 10-fold dilutions of the homogenates were plated on PDA and incubated for 48 h at 35 °C. Data were expressed as log₁₀ CFU/g of tissue.

2.6. Histological studies

For the histopathology studies, one-half of each organ, obtained from the tissue burden studies, was placed in 10% buffered formalin. Samples were dehydrated, paraffin embedded and sliced into 2 µm sections, which were then stained with haematoxylin/eosin, Periodic acid–Schiff (PAS) or Gomori

methenamine silver (GMS) and examined in a blinded fashion by light microscopy.

2.7. Statistics

The mean survival time was estimated by the Kaplan–Meier method and was compared among groups using the log-rank test. In the tissue burden studies, colony counts were analysed by the Mann–Whitney *U*-test. Calculations were performed using SPSS version 15.0.1 (SPSS Inc., Chicago, IL) and GraphPad Prism version 4.0 for Windows (GraphPad Software Inc., La Jolla, CA). A *P*-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. *In vitro* results

Table 1 shows the results of the antifungal susceptibility tests. AmB was the most active drug and PCZ the least active.

3.2. *In vivo* results

Fig. 1 shows the results of the survival studies. AmB, PCZ and VCZ significantly prolonged survival with respect to the control group for both strains tested ($P < 0.05$). However, for strain FMR 9383, 50% of mice treated with AmB survived to the end of the experiment but all mice treated with VCZ and PCZ died within 2 weeks. AmB and VCZ significantly prolonged survival with respect to PCZ for

strain FMR 9383 ($P = 0.028$ and $P = 0.003$, respectively). There were no significant differences between AmB, PCZ and VCZ for strain FMR 9354.

Fig. 2 shows the effects of the different treatments on the fungal loads in the kidney and spleen. In general, AmB was the most effective treatment to significantly reduce the fungal load in all the organs tested. In addition, AmB was significantly more effective than PCZ in reducing the fungal load in both organs for strain FMR 9383 and in the kidneys for strain FMR 9354. AmB was also significantly more effective than VCZ in reducing the fungal load in the spleen and kidneys for strain FMR 9354 and in the spleen for strain FMR 9383 ($P < 0.05$). For strain FMR 9383, VCZ was more effective than PCZ in the kidney, whereas for strain FMR 9354 PCZ was more effective than VCZ in the spleen.

Fig. 3 shows the results of the histological studies. Kidneys of the control group infected with *N. dimidiatum* showed a massive infiltration of fungal elements with no inflammatory response. However, no fungal elements were observed in the kidneys of mice treated with AmB and VCZ, whilst in the kidneys of mice treated with PCZ few fungal elements were seen without an inflammatory response.

4. Discussion

This is the first time that antifungal treatments have been evaluated in vivo against *N. dimidiatum* infections. No previous data exist on the efficacy of AmB, PCZ or VCZ in experimental *Neoscytalidium* infections and there is little clinical

experience of their use in human infections. In this study, we demonstrated the efficacy of AmB, and to a lesser extent both azoles, in a murine model of disseminated infection. The low MICs of AmB are consistent with in vitro data reported previously [10,11] and it was the most effective treatment, which correlates with in vitro results. The low MICs of AmB, the efficacy in terms of survival, the reduction in tissue burden and the lack of noticeable fungal elements in the histological sections agrees with the favourable clinical outcomes observed in infections with *N. dimidiatum* in immunosuppressed patients treated with this antifungal agent [12,13]. However, this drug has not always shown favourable effects in vivo, since treatment has been reported to have failed in several patients receiving this drug [4,8]. VCZ also showed some efficacy in our experimental model, in agreement with the results obtained in other cases [3,7]. PCZ showed the highest MICs in this study, which together with the poor improvement in survival of mice and the fact that it was the only drug with which fungal elements were observed in the histological sections, appears to indicate that this drug is less recommendable for treating *N. dimidiatum* infections. Although clinical data on the use of PCZ in these infections are scarce, the outcome was reported to be unfavourable in a case where the patient was treated with PCZ following failure of the AmB therapy [4].

Taking into account the results obtained in the current study, and although VCZ was not superior to AmB, this azole could become a therapeutic option in the treatment of disseminated infections caused by *Neoscytalidium* in those cases where AmB cannot be used. Further studies are needed to ascertain its clinical relevance.

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Competing interests

None declared.

Ethical approval

All animal experiments were approved by the Animal Welfare Committee of the Universitat Rovira i Virgili (Reus, Spain).

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Fig. 1. Cumulative mortality of neutropenic mice infected with *Neoscytalidium dimidiatum* (A) strain FMR 9383 or (B) strain FMR 9354 and treated with 1.5 mg/kg amphotericin B (AMB), 60 mg/kg posaconazole (PSC) or 60 mg/kg voriconazole (VRZ). Treatments started on Day 1 post infection and continued for 7 days. ^a $P < 0.05$ versus control; ^b $P < 0.05$ versus PSC.

Fig. 2. Effects of antifungal treatments on colony counts of *Neoscytalidium dimidiatum* (A,B) strain FMR 9383 and (C,D) strain FMR 9354 in the kidneys (A,C) and spleen (B,D) of infected mice: 1.5 mg/kg amphotericin B (AMB); 60 mg/kg posaconazole (PSC); and 60 mg/kg voriconazole (VRC). ^a $P < 0.05$ versus control; ^b $P < 0.05$ versus PSC; ^c $P < 0.05$ versus VRC. The horizontal lines of scatter plots indicate mean values.

Fig. 3. Histopathology of kidneys from neutropenic mice infected with *Neoscytalidium dimidiatum* and treated with amphotericin B 1.5 mg/kg/day (AMB), posaconazole 60 mg/kg/day (PCZ) or voriconazole 60 mg/kg/day (VCZ): (A) control group showing minimal acute perivascular haemorrhage with numerous fungal elements [Gomori methenamine silver (GMS) stain, magnification $\times 400$]; (B) AmB- and VCZ-treated groups. Normal tissue and no fungal elements were observed (GMS stain, magnification $\times 400$); and (C) PCZ-treated group. Presence of fungal hyphae without necrosis or inflammatory response (GMS stain, magnification $\times 400$).

Table 1

In vitro activities of antifungal drugs against two clinical strains of

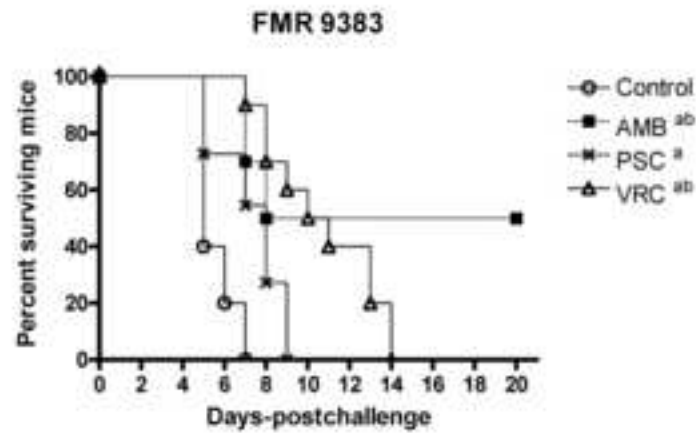
Neoscytalidium dimidiatum

Strain	MIC ($\mu\text{g/mL}$)		
	AmB	VCZ	PCZ
FMR 9383	0.5	2	4
FMR 9354	0.125	1	4

MIC, minimum inhibitory concentration; AmB, amphotericin B; VCZ, voriconazole; PCZ, posaconazole.

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A)



B)

