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Antifungal therapies in murine disseminated phaeohyphomycoses caused by *Exophiala* species

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Objectives: We have evaluated the efficacy of posaconazole, itraconazole and amphotericin B in murine models of disseminated infection caused by *Exophiala* spp.

Methods: Immunosuppressed mice were treated with posaconazole at 10, 20 or 40 mg/kg/day orally (po), amphotericin B at 1.5 mg/kg/day intraperitoneally (ip) or itraconazole at 50 mg/kg/day po. Treatment began 1 day after infection and continued for 7 days post-infection. Two strains of each of the three most relevant clinical species, i.e. *Exophiala dermatitidis, Exophiala oligosperma* and *Exophiala xenobiotica*, were tested.

Results: Posaconazole showed the highest efficacy in mice infected with *E. dermatitidis*, the only species that showed a high neurotropism, while the three drugs showed a similarly good activity against *E. oligosperma* and *E. xenobiotica* infections.

Conclusions: The results suggest that posaconazole may have a clinical role in the treatment of disseminated infections caused by *Exophiala* species, especially in those with CNS invasion.

Keywords: posaconazole, itraconazole, amphotericin B, animal models

Introduction

The genus *Exophiala* comprises dematiaceous fungi widely distributed in nature.[1] In recent years, the incidence of these fungi has increased as a cause of human phaeohyphomycoses.[1] They are usually chronic infections, mainly involving cutaneous and subcutaneous tissues, but disseminated infections, especially in immunosuppressed patients, have also been described.[2–4] The main pathogenic species of *Exophiala* is *Exophiala dermatitidis*, which presents marked neurotropism, causing severe or lethal infections. However, recently described species such as *Exophiala oligosperma* and *Exophiala xenobiotica* have also been reported as agents of systemic phaeohyphomycosis.[4] The treatment of choice for these rare infections has not yet been established, since current antifungal drugs show poor efficacy or toxicity after long periods of administration, making it necessary to investigate more appropriate therapies. Amphotericin B, alone or in combination with flucytosine, or triazoles, such as voriconazole and itraconazole, have been used, but without completely satisfactory results.[4] Itraconazole has shown efficacy on some occasions, but its use is limited by its variable gastrointestinal absorption and alterations in cardiac conductivity.[5]

We have evaluated the efficacy of posaconazole, itraconazole and amphotericin B in murine infections caused by each of the three most common *Exophiala* species related to human systemic disease, i.e. *E. dermatitidis, E. oligosperma* and *E. xenobiotica*, using previously developed models of disseminated infection.[6]

Materials and methods

Six clinical isolates were used in this study, two isolates corresponding to each of the following species: *E. dermatitidis* (CBS 748.88 from the sputum of a cystic fibrosis patient, Norway; and FMR 10037 from a haemato-oncological patient, Argentina); *E. oligosperma* (CBS 725.88 from a tumour of the sphenoidal cavity, Germany; and CBS 109807 from a case of fungaemia, Brazil); and *E. xenobiotica* (CBS 117650 from an arm abscess, USA; and CBS 102455 from a keratitis case, Brazil). Their *in vitro* antifungal susceptibility to amphotericin B, itraconazole and posaconazole was tested by using a broth microdilution method following the CLSI guidelines for filamentous fungi.[7]

For the *in vivo* study, male OF1 mice (Charles River; Criffa SA, Barcelona, Spain) with a mean weight of 30 g were used. The animals were housed in standard boxes with corncob bedding and had free access to food and water. All animal procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Mice were immunosuppressed by a single intraperitoneal (ip) injection of 200 mg/kg cyclophosphamide (Genoxal; Laboratorios Funk SA, Barcelona, Spain) plus 5-fluorouracil (Fluorouracilo; Ferrer Farma SA, Barcelona, Spain) at 150 mg/kg intravenously 1 day prior to the infection.

In order to prevent bacterial infections, all mice received 5 mg/day ceftazidime subcutaneously from days 1 to 7 after infection.

On the day of infection, 10 day cultures on potato dextrose agar (PDA) of the *E. oligosperma* and *E. xenobiotica* strains and 2 day cultures of the *E. dermatitidis* strains were suspended in sterile normal saline and filtered through sterile gauze to remove clumps of cells or hyphae. The resulting suspensions were adjusted to the desired inoculum based on the haemocytometer counts. Dilutions of the original suspension were cultured on PDA plates to confirm the haemocytometer count.

For each strain and each treatment, groups of 20 mice were established and challenged with a conidial suspension of 2×10^5 cfu of *E. dermatitidis* strains, 1.2×10^6 cfu of *E. oligosperma* strains or 1.6×10^6 cfu of *E. xenobiotica* strains in 0.2 mL of sterile normal saline into the lateral tail vein. Previous studies performed with each one of the six strains tested demonstrated that these inocula produced an acute infection, with all the animals dying within 15 days (data not shown).

The drugs assayed were: amphotericin B (Fungizone; Squibb Industrial Farmacéutica SA, Barcelona, Spain), posaconazole (Noxafil; Schering-Plough Ltd, Welwyn Garden City, UK) and itraconazole (Canadiol; Laboratorios Dr. Esteve S.A., Barcelona, Spain), administered as follows: amphotericin B given at doses of 1.5 mg/kg ip once daily; posaconazole given at doses of 10, 20 or 40 mg/kg orally once daily; and itraconazole given at doses of 25 mg/kg orally twice daily. Control animals received no treatment. All treatments began 1 day after challenge and the therapy lasted for 7 days. The efficacy of the different drugs was evaluated through prolongation of survival and reduction of fungal tissue burden.

Ten mice of each group were randomly assigned to the survival study and checked daily for 30 days. At the end of the experiment, survivors were sacrificed by carbon dioxide inhalation. For tissue burden studies, 10 mice of each group identified before the study started were sacrificed on day 6 post-infection. Kidneys, brain and spleen were aseptically removed and homogenized in 1 mL of sterile normal saline. Serial 10-fold dilutions of the homogenates were plated on PDA, incubated at 30°C and examined daily for 7 days. The numbers of cfu/g of tissue were calculated.

Mean survival time was estimated by the Kaplan–Meier method and compared between groups by using the log rank test. Colony counts in tissue burden studies were analysed using the Kruskal–Wallis test. When the Kruskal–Wallis test was significant, we used the Mann–Whitney *U*-test to compare pairs of strains. The Bonferroni correction was used to avoid an increase in type I error due to multiple comparisons. When $P<0.05$, the observed differences were considered statistically significant.

Results

The three drugs tested showed low MICs for all the isolates tested in this study (Table 1). All treatments, with a few exceptions, significantly prolonged survival with respect to the control group (Figure 1). Posaconazole at 40 mg/kg performed significantly better than amphotericin B for both *E. dermatitidis* strains, and significantly better than itraconazole in mice infected with the strain FMR 10037.

Table 2 shows the results of the fungal load study. In mice infected with *E. dermatitidis* strains, in general all the treatments significantly reduced the fungal load in the three organs analysed. In brain, posaconazole at 20 and 40 mg/kg performed better than the other two drugs against the two strains tested. In mice infected with *E. oligosperma*, posaconazole at 20 mg/kg and at 40 mg/kg and amphotericin B were able to significantly reduce the fungal load in spleens of mice infected with both strains. In such tissue, posaconazole at 40 mg/kg for the strain CBS 109807 and amphotericin B for both *E. oligosperma* strains also significantly reduced the fungal burden with respect to itraconazole. In kidneys, only amphotericin B was unable to reduce the tissue burden. Although the fungal load in brain tissue was generally very low, it could be reduced significantly by posaconazole at 40 mg/kg.

Table 1. *In vitro* activity of amphotericin B, posaconazole and itraconazole against three species of *Exophiala*

		MIC (mg/L)		
Species	Strain	AMB	ITC	PSC
E. dermatitidis	CBS 478.88	1	0.25	0.5
	FMR 10037	0.5	0.5	0.25
E. oligosperma	CBS 725.88	0.5	0.5	0.12
	CBS 109807	0.5	0.5	0.12
E. xenobiotica	CBS 117650	0.25	0.5	0.25
	CBS 102455	0.25	0.5	0.12

AMB, amphotericin B; PSC, posaconazole; ITC, itraconazole.

In the case of *E. xenobiotica*, only amphotericin B showed efficacy against strain CBS 117650 in spleen. All other treatments were effective apart from posaconazole at 10 mg/kg against one strain in spleen, and itraconazole and amphotericin B against one strain each in kidneys. On several occasions, posaconazole was able to further improve the results that had been obtained with the other drugs.

In summary, posaconazole was the best drug for reducing the fungal burden in kidneys for the three species tested, and in brains of mice infected with *E. dermatitidis* strains. Amphotericin B showed better efficacy in reducing the fungal load in spleen tissue, especially in comparison with itraconazole.

Discussion

Disseminated phaeohyphomycosis is a severe but relatively rare infection with poor response to antifungal drugs.[3] In the clinical setting, posaconazole has shown encouraging results since it was successfully used in a case of disseminated phaeohyphomycosis caused by *Exophiala spinifera*.[2] Despite these promising results, clinical experience with posaconazole in this kind of infection is still limited, and animal models can be helpful in predicting its efficacy against rare or poorly known dematiaceous fungi. In the case of *Exophiala* spp., few data are available about the experimental activity of posaconazole, although *in vitro* studies have demonstrated good activity of this drug against clinical isolates of these species.[4,8] Additionally, posaconazole was effective in a murine systemic infection caused by *E. dermatitidis*,[9] suggesting that it could be a therapeutic option in the treatment of systemic phaeohyphomycosis. In general, our results agree with the mentioned *in vitro* and *in vivo* studies.

In our murine model, posaconazole showed better results than itraconazole and amphotericin B in prolonging the survival of animals infected with *E. dermatitidis* strains, the only species

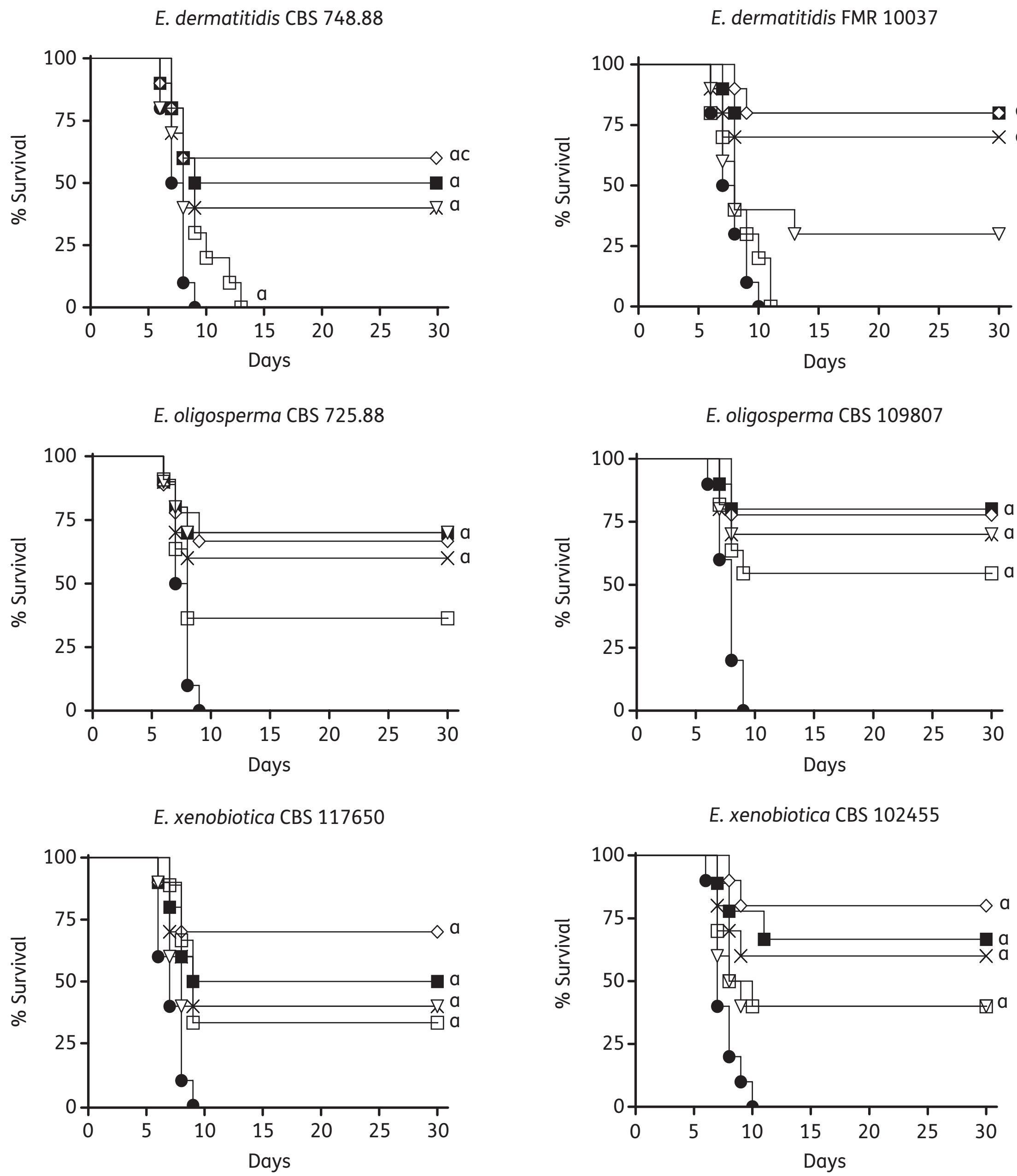

Figure 1. Cumulative mortality of mice infected with various *Exophiala* strains and species. Filled circles, control; crosses, posaconazole at 10 mg/kg; filled squares, posaconazole at 20 mg/kg; open diamonds, posaconazole at 40 mg/kg; open triangles, itraconazole at 50 mg/kg; open squares, amphotericin B at 1.5 mg/kg. [a]$P < 0.05$ versus control. [b]$P < 0.05$ versus itraconazole at 50 mg/kg. [c]$P < 0.05$ versus amphotericin B at 1.5 mg/kg.

that showed clear neurotropic behaviour. On the other hand, the limited fungal invasion in brains of mice infected with *E. oligosperma* and *E. xenobiotica* makes it difficult to detect significant differences between therapies. These good posaconazole results were also observed in a previous study in which we obtained similar results when administering the same drugs and doses against a murine infection caused by *Fonsecaea monophora*, a dematiaceous fungus causing subcutaneous infections.[10]

To our knowledge, this is the first study that has explored the *in vivo* efficacy of antifungal therapy against the novel species *E. oligosperma* and *E. xenobiotica* in experimental infections. Both species seem to have lower virulence than *E. dermatitidis*,[6] infecting mainly cutaneous and subcutaneous tissues. However, the real incidence of such species in disseminated phaeohyphomycosis could be underestimated due to their so recent description.[4] In our study, all the drugs tested were effective against the systemic infections provoked by these species.

Table 2. Effects of the antifungal treatments on colony counts of three species of *Exophiala* in spleen, kidneys and brain of mice

		Mean $\log_{10}$ cfu/g of (95% CI)		
Isolate	Drug dose in mg/kg/day	spleen	kidneys	brain
E. dermatitidis				
CBS 748.88	none	4.80 (4.72–4.86)	4.34 (4.10–4.49)	5.45 (5.27–5.58)
	PSC (10)	4.09 (3.93–4.21)[a]	3.08 (2.92–3.20)[a]	3.69 (3.54–3.79)[a]
	PSC (20)	4.25 (4.13–4.34)[a]	2.88 (2.78–2.96)[a]	3.53 (3.24–3.71)[a,b,c]
	PSC (40)	4.03 (3.87–4.14)[a]	2.64 (2.54–2.71)[a,c,d,e]	3.43 (3.17–3.58)[a,b,c]
	ITC (50)	4.01 (3.91–4.09)[a]	2.77 (2.63–2.87)[a,d]	4.36 (3.98–4.56)[a]
	AMB (1.5)	3.84 (3.68–3.95)[a,e]	2.88 (2.77–2.97)[a]	4.20 (3.92–4.38)[a]
FMR 10037	none	4.38 (4.24–4.49)	4.00 (3.94–4.06)	4.70 (4.60–4.49)
	PSC (10)	4.04 (3.91–4.13)[a,b]	3.33 (3.22–3.41)[a,b,c]	4.32 (4.21–4.40)[a]
	PSC (20)	4.11 (3.92–4.24)[a]	3.37 (3.29–3.44)[a,b,c]	3.92 (3.84–3.98)[a,b,c,d]
	PSC (40)	4.00 (3.83–4.12)[a,b]	2.85 (2.56–3.02)[a,b,c,d,e]	3.86 (3.79–3.91)[a,b,c,d]
	ITC (50)	4.24 (4.15–4.32)	3.73 (3.62–3.82)[a]	4.28 (4.18–4.36)[a]
	AMB (1.5)	3.84 (3.69–3.95)[a,b]	3.61 (3.52–3.69)[a]	4.20 (4.09–4.28)[a]
E. oligosperma				
CBS 725.88	none	4.73 (4.55–4.86)	3.54 (3.34–3.68)	2.16 (2.05–2.27)
	PSC (10)	4.49 (4.31–4.61)	2.96 (2.78–3.09)[a,c]	2.02 (1.88–2.15)
	PSC (20)	4.34 (4.17–4.46)[a]	2.83 (2.65–2.96)[a,c]	1.97 (1.77–2.17)
	PSC (40)	4.22 (4.05–4.34)[a]	2.61 (2.28–2.80)[a,c,d]	1.94 (1.79–2.09)
	ITC (50)	4.46 (4.32–4.57)	2.96 (2.76–3.10)[a,c]	2.13 (2.03–2.24)
	AMB (1.5)	4.19 (3.96–4.34)[a,b]	3.34 (3.13–3.48)	2.17 (2.04–2.97)
CBS 109807	none	5.72 (5.62–5.80)	3.43 (3.31–3.52)	2.22 (1.98–2.38)
	PSC (10)	5.34 (5.19–5.45)[a]	2.83 (2.68–2.94)[a,c]	1.99 (1.75–2.14)
	PSC (20)	5.32 (5.20–5.42)[a]	2.82 (2.67–2.93)[a,c]	2.00 (1.81–2.12)
	PSC (40)	5.16 (5.01–5.27)[a,b]	2.65 (2.26–2.86)[a,b,c]	1.82 (1.64–1.95)[a]
	ITC (50)	5.43 (5.31–5.53)[a]	3.01 (2.87–3.11)[a,c]	2.10 (1.94–2.22)
	AMB (1.5)	5.11 (4.85–5.27)[a,b]	3.26 (3.16–3.35)	2.08 (1.90–2.21)
E. xenobiotica				
CBS 117650	none	5.90 (5.72–6.04)	3.20 (2.96–3.35)	2.03 (1.81–2.07)
	PSC (10)	5.90 (5.64–6.06)	1.34 (0.11–1.65)[a,b]	1.42 (0.23–1.71)[a]
	PSC (20)	5.68 (5.49–5.81)	ND[a,b,c]	1.33 (0.16–1.65)[a]
	PSC (40)	5.55 (5.29–5.72)	ND[a,b,c]	1.39 (0.50–1.66)[a]
	ITC (50)	5.61 (5.36–5.77)	2.93 (2.69–3.08)	1.16 (1.10–1.49)[a]
	AMB (1.5)	5.15 (4.78–5.35)[a,d,e]	2.12 (0.29–2.45)[a,b]	1.03 (0.10–1.35)[a]
CBS 102455	none	5.61 (5.45–5.73)	2.99 (2.83–3.11)	1.98 (1.68–2.16)
	PSC (10)	5.26 (4.93–5.44)	2.22 (1.79–2.43)[a,c]	1.64 (1.28–1.83)[a]
	PSC (20)	5.23 (4.85–5.43)[a]	0.94 (0.10–1.28)[a,b,c,d]	1.10 (0.20–1.46)[a]
	PSC (40)	5.09 (4.70–5.29)[a]	ND[a,b,c,d]	0.75 (0.11–1.12)[a]
	ITC (50)	5.11 (4.91–5.25)[a]	2.07 (1.55–2.30)[a,c]	0.84 (0.16–1.21)[a]
	AMB (1.5)	4.61 (4.38–4.76)[a,b,d,e]	2.84 (2.64–2.98)	ND[a]

AMB, amphotericin B; PSC, posaconazole; ITC, itraconazole; ND, not detected.
[a]$P<0.003$ versus control.
[b]$P<0.003$ versus 50 mg/kg ITC.
[c]$P<0.003$ versus 1.5 mg/kg AMB.
[d]$P<0.003$ versus 10 mg/kg PSC.
[e]$P<0.003$ versus 20 mg/kg PSC.

In conclusion, our results confirm previous studies that suggest posaconazole as an alternative in the treatment of invasive infections caused by melanized fungi, especially in those with CNS involvement. But in general, all the antifungal therapies tested showed good efficacy in the treatment of the systemic murine infections caused by *Exophiala*, including the recently described species *E. oligosperma* and *E. xenobiotica*.

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Transparency declarations
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References
1 Horré R, de Hoog GS. Primary cerebral infections by melanized fungi: a review. *Stud Mycol* 1999; **43**: 176–93.

2 Negroni R, Helou SH, Petri N *et al*. Case study: posaconazole treatment of disseminated phaeohyphomycosis due to *Exophiala spinifera*. *Clin Infect Dis* 2004; **38**: 15–20.

3 Revankar SG. Phaeohyphomycosis. *Infect Clin Dis North Am* 2006; **20**: 609–20.

4 Zeng JS, Sutton DA, Fothergill AW *et al*. Spectrum of clinically relevant *Exophiala* species in the United States. *J Clin Microbiol* 2007; **45**: 3713–20.

5 Okamoto J, Fukunami M, Kioka H. Frequent premature ventricular contractions induced by itraconazole. *Circ J* 2007; **71**: 1323–5.

6 Calvo E, Rodríguez MM, Mariné M *et al*. Comparative virulence of three species of *Exophiala* in mice. *Med Mycol* 2010; doi:10.3109/13693780903582481.

7 Clinical and Laboratory Standards Institute. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi—Second Edition: Approved Standard M38-A2*. CLSI, Wayne, PA, USA, 2008.

8 Fothergill AW, Rinaldi MG, Sutton DA. Antifungal susceptibility testing of *Exophiala* spp.: a head-to-head comparison of amphotericin B, itraconazole, posaconazole and voriconazole. *Med Mycol* 2009; **47**: 41–3.

9 Graybill JR, Najvar LK, Johnson E *et al*. Posaconazole therapy of disseminated phaeohyphomycosis in a murine model. *Antimicrob Agents Chemother* 2004; **48**: 2288–91.

10 Calvo E, Pastor FJ, Rodríguez MM *et al*. Murine model of a disseminated infection by the novel fungus *Fonsecaea monophora* and successful treatment with posaconazole. *Antimicrob Agents Chemother* 2010; **54**: 919–23.