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In vitro and *in vivo* efficacy of amphotericin B combined with posaconazole against experimental disseminated sporotrichosis

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ABSTRACT

We evaluated the combination of posaconazole with amphotericin B *in vitro* and in a murine model of systemic infections by *Sporothrix brasiliensis* and *S. schenkii sensu stricto*. *In vitro* data demonstrated a synergistic effect and although posaconazole alone was effective against sporotrichosis, efficacy in terms of survival and burden reduction was increased in the combination. This combination could be an option against disseminated sporotrichosis, especially when itraconazole or amphotericin B at optimal doses are contraindicated.

Antimicrobial Agents and Chemotherapy

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Antimicrobial Agents and Chemotherapy

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Disseminated infection is the most severe manifestation of sporotrichosis, occurring mainly in immunocompromised patients although has also been reported in immunocompetent people (16, 17). The *Sporothrix schenckii* complex encompasses four species able to cause sporotrichosis in humans (14, 19). Treatment of the disseminated infection is carried out with amphotericin B and maintenance with itraconazole showing variable outcomes (3, 9, 13). Posaconazole has shown low MICs against *Sporothrix* spp. (15) and although only one study has explored its clinical role, its safety and efficacy in animal models indicates that this compound could be a good alternative for the treatment of disseminated sporotrichosis (3, 6). Seeking to enhance the treatment against sporothrichosis, posaconazole was evaluated in combination with amphotericin B against murine systemic infections by *S. brasiliensis* and *S. schenckii sensu stricto*.

Two strains of *S. brasiliensis* (FMR 8319 and FMR 8326) and two of *S. schenckii sensu stricto* (FMR 8606 and FMR 8609) in the mould phase were included in the study. MICs of amphotericin B (Sigma–Aldrich Co., St. Louis, Missouri, USA) and posaconazole (Schering-Plough, Kenilworth, New Jersey, USA.) were determined from 7-day-old cultures by following the CLSI guidelines (4) and activity of the drug combination was tested using the checkerboard method. The fractional inhibitory concentration index (FICI) was calculated and combination was defined as synergistic at FICI $\leq$ 0.5, indifferent at 0.5 < FICI $\leq$ 4.0, and antagonist at FICI > 4.0 (5). Tests were carried out in duplicate

For the *in vivo* study, the inocula were prepared from the filamentous growth by flooding the surface of the cultures with saline solution and scraping the sporulating mycelium. The resulting conidial suspension was transferred to potato dextrose broth and incubated in an orbital shaker at 150 rpm at 30°C for 5 days. Cultures were then

Antimicrobial Agents and Chemotherapy
AAC
Antimicrobial Agents and Chemotherapy

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filtered through sterile gauze and centrifuged at 325 x g. The conidia suspension was adjusted to the desired concentrations by hemocytometer counting (6). Four-week-old OF-1 male mice (Charles River, Criffa S.A., Barcelona, Spain) with a mean weight of 30 g were infected intravenously (i.v.) via the lateral tail vein with 2 x 10^7 CFU in 0.2 ml of sterile saline. Six groups of 15 animals/group, 10 for survival and 5 for tissue burden studies, were established for each strain. Treatment groups received amphotericin B (Xalabarder Pharmacy, Barcelona, Spain) at 0.3 mg/kg given i.v. or posaconazole (Noxafil; Schering-Plough Ltd., Hertfordshire, United Kingdom) at 2.5 or 5 mg/kg twice a day (BID) by gavage. Combined treatments consisted on posaconazole at 2.5 or 5 mg/kg BID together with amphotericin B at 0.3 mg/kg. Additionally, mice infected with *S. brasiliensis* (FMR 8319) received posaconazole at 10 mg/kg alone or combined with amphotericin B 0.3 mg/kg. All treatments began 1 day after infection and lasted for 18 days with control groups receiving no treatment. When control mice started to die at 12 days post infection, five mice from each group were euthanatized and the liver and the spleen, which are the most affected organs in experimental systemic sporotrichosis (1), were mechanically homogenized and placed on PDA for CFU/g of tissue calculation. All animal care procedures were carried out in duplicate and supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Statistical analysis was done using Graph Pad Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA). The mean survival time was estimated by the Kaplan-Meier method and compared among groups by using the log rank test. The colony counts from tissue burden studies were analyzed using the Mann-WhitneyU test (*P* values of ≤ 0.05, statistically significant).

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Antimicrobial Agents and Chemotherapy

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The *in vitro* combination of posaconazole with amphotericin B was synergistic for all the isolates (FICI ≤ 0.5 for *S. brasiliensis* and ≤ 0.28 for *S. schenckii*) (Table 1). All the isolates caused systemic infection with 100% death in control animals with no significant differences between species or between strains of the same species ($p \geq 0.09$, in multiple comparisons). Treatments consisting of amphotericin B 0.3 mg/ml prolonged the survival of animals in comparison to their respective controls ($p \leq 0.043$). However, all animals receiving posaconazole alone at any concentration or in combination with amphotericin B 0.3 mg/kg survived through the experimental period (Figure 1).

Tissue burden studies correlated with survival studies i.e., amphotericin B reduced burden significantly but posaconazole alone or combined at any dose did so more efficiently (Figure 2). Posaconazole administered at 5 mg/kg was more effective in reducing burdens than at 2.5 mg/kg in all cases ($p \leq 0.0001$). The efficacy of posaconazole was better when combined with amphotericin B, with posaconazole at 5 mg/kg plus amphotericin B being the treatment that showed the highest burden reduction ($p \leq 0.03$ in comparison to the other treated and untreated groups). Interestingly, the data obtained demonstrated an equivalent efficacy in fungal reduction between posaconazole 5 mg/kg alone and posaconazole 2.5 mg/kg plus amphotericin B against *S. schenckii* and a trend to equivalence against *S. brasiliensis*.

The combination posaconazole 10 mg/kg plus amphotericin B against *S. brasiliensis*, strain FMR 8319, did not further improve the efficacy over the monotherapy with posaconazole 10 mg/kg or over 5 mg/kg even when combined with amphotericin B ($p \geq 0.061$).

FICI values were lower against *S. schenckii* than against *S. brasiliensis* and the combination was more effective against the strains of the former species than against *S.*

Antimicrobial Agents and Chemotherapy
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brasiliensis correlating the FICI with the animal outcome. There are several reported cases of systemic sporotrichosis having a fatal outcome despite having used the recommended treatments (8, 10, 18) which makes it desirable to explore new therapeutic options. Among them, voriconazole and terbinafine might be an option. The first has shown efficacy against *S. schenckii* but not against *S. brasiliensis* (7) and terbinafine has not been evaluated against systemic infections by *Sporothrix* although it has demonstrated activity *in vitro* (2, 11). Posaconazole has also demonstrated efficacy against systemic sporotrichosis, but in the present study, we show that such efficacy can be enhanced in combination with amphotericin B at low doses. Amphotericin B plus itraconazole at high doses has proven efficacy in the experimental disseminated infection by *S. brasiliensis* (12), although this combination failed in a clinical case due to toxic effects. Therapy was then changed to amphotericin B in combination with posaconazole resulting in a dramatic clinical improvement (3). The combination between posaconazole and suboptimal doses of amphotericin B deserves attention as alternative especially in those patients suffering disseminated sporotrichosis who do not respond to the treatment or when itraconazole or high doses of amphotericin B are contraindicated.

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Antimicrobial Agents and Chemotherapy
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Table 1. *In vitro* interaction of amphotericin B (AMB) and posaconazole (PSC) against *S. brasiliensis* and *S. schenckii*.

Species	Strain	MIC (mg/L) AMB / PSC		FICI	Effect
		Alone	In combination		
S. brasiliensis	8326	4 / 2	1 / 0.03	0.265	Synergism
	8319	4 / 0.5	1 / 0.125	0.50	Synergism
S. schenckii	8606	4 / 1	1 / 0.03	0.28	Synergism
	8609	4 / 2	1 / 0.03	0.265	Synergism

Figure 1. Effect of posaconazole and amphotericin B on survival of mice infected intravenously with $2x10^7$ CFU/animal of *S. brasiliensis* and *S. schenckii*. Posaconazole was administered at 2.5 and 5 mg/kg (PSC 2.5 and PSC 5) and amphotericin B at 0.3 mg/kg (AMB 0.3), both alone and in combination. Significant ($p < 0.05$) in comparison to [a] Control, [b]AMB 0.3.

Figure 2. Effect of posaconazole and amphotericin B on fungal loads in spleen and liver of mice 12 days after intravenous infection with $2x10^7$ CFU/animal of *S. brasiliensis* and *S. schenckii*. Posaconazole was administered at 2.5 and 5 mg/kg (PSC 2.5 and PSC 5) and amphotericin B at 0.3 mg/kg (AMB 0.3), both alone and in combination. In one strain (FMR 8319), posaconazole was also administered at 10 mg/kg (PSC 10) alone and combined with AMB 0.3. Horizontal bars represent the median. Significant ($p < 0.05$) in comparison to [a]Control, [b]AMB 0.3, [c]PSC 2.5, [d]PSC 2.5+AMB 0.3, [e]PSC 5.

Antimicrobial Agents and Chemotherapy
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223

224

225

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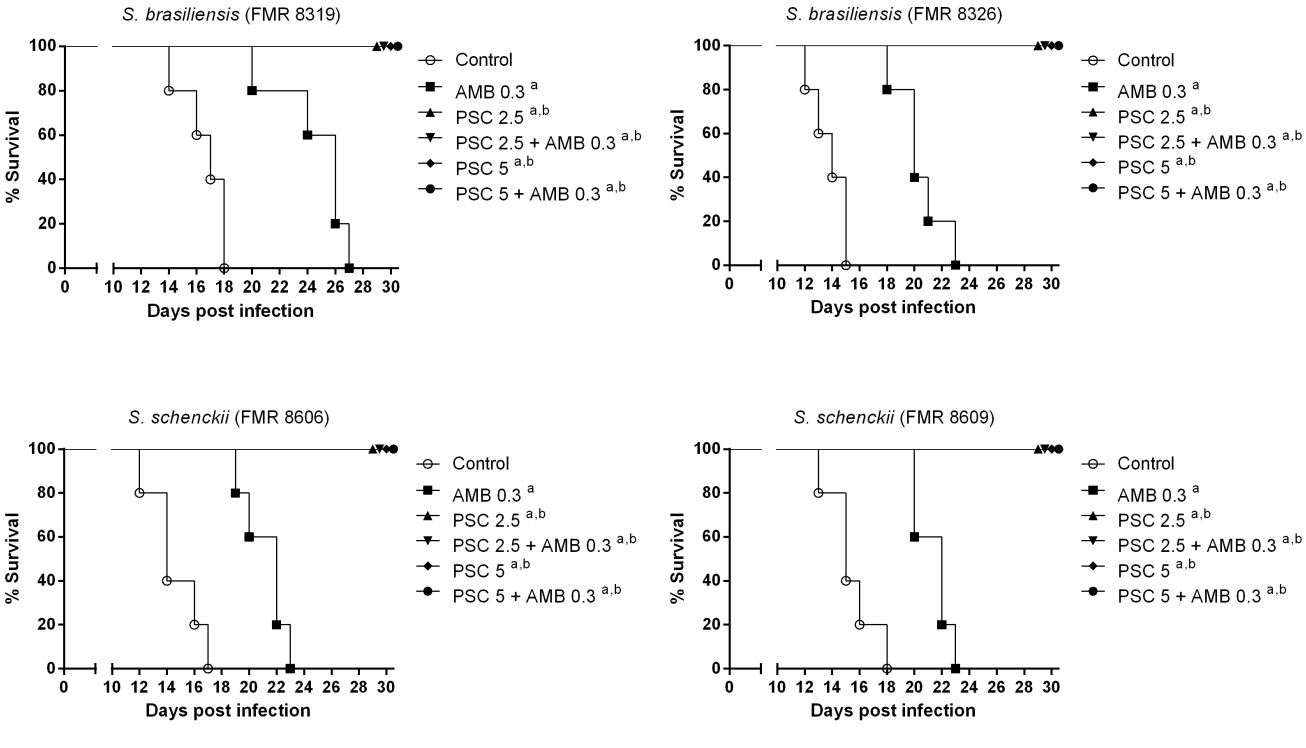

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