Interactions between triazoles and amphotericin B in the treatment of disseminated murine infection by *Fusarium oxysporum*.

Running title: Azoles against *Fusarium oxysporum*

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ABSTRACT

We have evaluated and compared the efficacy of high doses of amphotericin B (3 mg/kg of body weight/day), voriconazole (60 mg/kg) and posaconazole (100 mg/kg) alone and combined in a murine model of disseminated infection by Fusarium oxysporum. The combination of AMB with PSC showed the best results prolonging the survival and reducing the organ fungal load in mice. This combination might constitute a therapeutic option for those infections where monotherapies fail.
Disseminated disease is the most frequent and challenging clinical form of fusariosis (13) showing a poor response to the antifungals (3, 8, 13, 14). For a long time, conventional therapy for invasive fusariosis has consisted of an amphotericin B-based regimen (2, 4, 12). However, clinical practice has demonstrated that this drug has poor activity and shows renal toxicity. Other treatment options include voriconazole (VRC), or posaconazole (PSC) which are indicated in patients with refractory disease or in those who are intolerant to certain antifungal agents (15, 16). However, the use of a drug alone to treat systemic fusariosis has not been completely satisfactory (13). The combined therapy could be another option, although its usefulness for the treatment of fusariosis has not yet been investigated. The combination of AMB with VRC has demonstrated some beneficial effects in a murine infection by *F. solani* (19). Clinical data confirmed the usefulness of such combination since it led to clinical improvement before neutropenia was reduced in two clinical cases (9, 18). We have evaluated the activity of triazoles (PSC and VRC) in combination with AMB in a murine model of systemic infection by *Fusarium oxysporum*. This species is the second most frequently reported cause of human fusariosis (1).

Two isolates of *F. oxysporum*, FMR 5205 and FMR 10281, were used in this study. They were subcultured on potato dextrose broth at 30ºC for 5 days. In vitro susceptibilities of both strains were tested using a microdilution reference method (5). Antifungal interactions were assessed by a checkerboard microdilution method (7). The fractional inhibitory concentration index (FICI) was used to classify drug interactions (10). For all the drugs tested and their combinations, we used a MIC-0 endpoint criterion.
Male OF1 mice were immunosuppressed by a single intraperitoneal injection of 200 mg of cyclophosphamide/kg of body weight, plus a single intravenous injection of 150 mg of 5-fluorouracil/kg one day before challenge (20). The procedure standards were approved by the Animal Welfare Committee of the Rovira i Virgili University.

Mice were challenged with $1 \times 10^7$ CFU in 0.2 ml of sterile saline, injected into the lateral tail vein. Preliminary experiments, demonstrated that this inoculum was appropriate for producing an acute infection, with 90% of the animals dying within 11 days (data not shown). Groups of 20 mice were established for each strain and each treatment. Ten mice were randomly chosen for survival and ten for tissue burden studies.

The different groups were treated once daily as follows: AMB at 3 mg/kg of body weight/dose given intraperitoneally, VRC at 60 mg/kg and PSC 100 mg/kg both given orally. In addition, AMB was combined with VRC or PSC at the same doses described above. All treatments began 1 day after challenge and the therapy lasted for 10 days. From three days prior to infection, the mice that received VRC were given 50% grapefruit juice in place of water (6). Mouse survival was evaluated daily for 20 days after challenge. For tissue burden studies mice were euthanized on day 6 of treatment and the spleens and kidneys were aseptically removed being the entire organs homogenized in 1 ml of sterile saline. Serial 10-fold dilutions of the homogenates were plated on potato dextrose agar and incubated for 48 h at 30°C.

The mean survival time (MST) was estimated by the Kaplan-Meier method, and compared among groups using the log-rank test. CFU counts were analyzed by the Mann-Whitney U-test. SPSS version 15.0.1 and Graph Pad
Prism version 4.0 for Windows were used. A $p$ value of $\leq 0.05$ was considered statistically significant.

The in vitro activities of the drugs tested were very poor and the combinations showed indifferent interactions (Table 1).

The monotherapies exerted very poor in vivo efficacy. Only PSC was able to prolong the survival for the strain FMR 10281. The combination AMB with VRC only improved the survival of mice infected with the strain FMR 10281. However, the combination AMB with PSC prolonged the survival for both strains (Fig. 1). AMB reduced significantly the fungal burden only in spleen tissue for both strains. VRC reduced the fungal burden only in kidney for the strain FMR 5205. PSC significantly reduced the fungal load in spleen for both strains and kidney load only in the strain FMR 5205. The combination AMB plus VRC was able to reduce the tissue burden in spleen and kidney only in the strain FMR 5205. Finally, the combination AMB plus PSC reduced significantly the fungal load in spleen for both strains and in kidneys for the strain FMR 5205 respect to the control group and the monotherapies (Fig. 2).

The lack of efficacy of AMB and VRC observed in our study agrees with clinical data, where approximately 55-70% of patients of fusariosis receiving such drugs died (13). However, in our study PSC was able to reduce the fungal load in spleen, the target organ in this experimental model. In a study of salvage treatment for invasive fusariosis, PSC showed an overall efficacy of 48% in patients with neutropenia, although the species of *Fusarium* involved in these cases were not determined (17). Although in general PSC shows high MICs against all the fusaria tested, this drug is apparently more active against *F. oxysporum* than *F. solani* (1, 21). Combined therapy using AMB and triazoles...
appear to be a promising alternative against fusariosis. However, up to now there was no data regarding the use of AMB plus PSC in experimental fusariosis. In our case, despite the high doses of both drugs used, the results were only modest though more effective than AMB plus VRC. By contrast, Lewis et al. (11) reported a lack of efficacy of AMB plus PSC, in a case of human fusariosis. However, according to the authors, the failure resulted from the subtherapeutic PSC levels achieved in serum due to the patient’s extremely poor diet.

In conclusion, the combination AMB with PSC could be an alternative therapy in *Fusarium oxysporum* infections. Further studies are needed to ascertain the real clinical relevance of this combination.

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    antifungal susceptibility patterns of 75 clinical isolates of Fusarium spp. from
Table 1. In vitro antifungal activities and interactions among antifungal drugs against two strains of *F. oxysporum*

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
<th>AMB + VRC</th>
<th>AMB + PSC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>AMB</td>
<td>VRC</td>
<td>PSC</td>
</tr>
<tr>
<td>FMR 5205</td>
<td>2</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>FMR 10281</td>
<td>4</td>
<td>16</td>
<td>&gt;16</td>
</tr>
</tbody>
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\(^a\) I, indifference
FIG. 1. Cumulative mortality of mice infected with *F. oxysporum* strains FMR 5205 (A) and FMR 10281 (B) and treatments with antifungal agents. AMB, amphotericin B at a dose of 3 mg/kg. VRC, voriconazole at 60 mg/kg. PSC, posaconazole at 100 mg/kg. Treatments started on day 1 post-infection and continued for 10 days. *P* < 0.05 versus control.
FIG. 2. Effects of the antifungal treatments on colony counts of *F. oxysporum* strains FMR 5205 (A) and FMR 10281 (B) in kidneys and spleen of mice. AMB, amphotericin B at a dose of 3 mg/kg. VRC, voriconazole at 60 mg/kg. PSC, posaconazole at 100 mg/kg. *a*, *P* < 0.05 versus control. *b*, *P* < 0.05 versus all treatments. The horizontal lines of scatter plots indicate mean values.