Posaconazole Prophylaxis in Experimental Systemic Zygomycosis

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ABSTRACT

Three isolates of zygomycetes belonging to two different genera (*Rhizopus oryzae* and *Absidia corymbifera*) were used to produce a systemic infection in neutropenic mice. On days -2, -1 and 2 h prior to infection the mice received either posaconazole (POS) at doses ranging from 20 to 80 mg/kg/day or amphotericin B (AMB) at 1 mg/kg/day. Antifungal drug efficacy was assessed by: the prolongation of survival, the percentage of infected organs (brain, lung, spleen and kidney), and the histological examination in number of infection foci and their size in brain and kidney tissues.

AMB significantly prolonged the survival of mice infected with all isolates. POS significantly prolonged the survival of mice infected with zygomycetes.

Cultured organs from mice infected with *R. oryzae* were all positive, while treated mice challenged with *A. corymbifera* generally showed lower percentages of infected organs with respect to controls. Zygomycete isolates established an active infection (presence of hyphae) in the brain and the kidney of all controls. In mice challenged with *R. oryzae*, both antifungal drugs were effective at reducing the number and the size of infection foci in the kidney. Only AMB reduced the number, but not the size, of foci in the brain. Finally, both drugs significantly reduced the number and the size of foci in both tissues of mice infected with *A. corymbifera*. Our data suggest that prophylaxis with POS has some potential to prevent zygomycosis.
INTRODUCTION

Zygomycosis is a rare but highly aggressive filamentous fungal infection (4). The clinical manifestations of zygomycosis include primary rhinocerebral, pulmonary, gastrointestinal, cutaneous or subcutaneous, or allergic disease and disseminated disease (4, 6, 7, 13). The zygomycetes most commonly identified as etiologic agents of human diseases are *Rhizopus* species, *Rhizomucor* species, *Mucor* species, and *Absidia* species (13). The invasive zygomycosis cause angioinvasion followed by progressive, necrotic, and generally fatal infections in immunocompromised hosts, such as diabetics with ketoacidosis, neutropenic patients, patients taking corticosteroids, and subjects with burns or iron overload (4, 6, 7, 12, 13). The standard therapy for these life-threatening infections consists of removal of the predisposing factors, widespread surgical debridement, and high doses of intravenous amphotericin B (13). Nevertheless, the aggressive therapy, mortality is often above 50% (4, 6). It is clear that new strategies for the treatment of zygomycosis are urgently needed.

Posaconazole is a new broad-spectrum triazole with activity against many filamentous fungal pathogens, including zygomycetes (3, 8, 11, 13, 14). Experimental infection models and clinical data showed that POS might be useful for the treatment of zygomycosis (3, 11, 14). No data are available on the effects of POS prophylaxis against these infections. Therefore, in this study we evaluated the effect of POS prophylaxis against *Rhizopus oryzae* and *Absidia corymbifera* in an experimental model of neutropenic mice infection. (This work was presented in part at the 16th Congress of the International Society for Human and Animal Mycology, Paris, France, 25 to 29 June, 2006).
MATERIALS AND METHODS

Organisms. Three clinical isolates were used in this study: *Rhizopus oryzae* 4570, *R. oryzae* 4745 and *Absidia corymbifera* 4535. Both isolates of *R. oryzae* were recovered from the sputum of two oncology patients with systemic zygomycosis, while *A. corymbifera* was obtained from the sputum of a patient with pulmonary zygomycosis. Isolates were stored as conidial suspensions at -80°C in 10% glycerol until used.

Antifungal drugs. For both in vitro and in vivo studies, posaconazole (POS; Schering-Plough) was prepared in polyethylene glycol 200 (Sigma). For in vitro studies, amphotericin B (AMB, Sigma) was dissolved in dimethilsulphoxide (Sigma), while for in vivo studies (Fungizone, Bristol-Myers Squibb) it was dissolved in sterile saline.

In vitro studies. MICs were determined by the CLSI broth microdilution methodology (NCCLS M38-A document) (9). Each strain was grown on Sabouraud dextrose agar (SDA) plates at 35°C for a period of 5 days. The fungal colonies were then covered with 1 ml of sterile 0.85% saline and gently scraped with a sterile pipette. The resulting conidial suspensions were transferred to sterile tubes and heavy particles were allowed to settle. To obtain a final inoculum of approximately 10⁴ CFU/ml, the conidial suspension was further adjusted by hemocytometer. The microdilution trays, containing both drugs at concentrations ranging from 0.015 to 8.0 µg/ml, were incubated at 35°C for 21-26 h. The MIC endpoint for both drugs was the lowest drug concentration that prevents any discernible growth. Each strain was tested in quintuplicate.

In vivo studies.

(i) Animals and immunosuppression. CD1 male mice of 25 grams (Charles River Laboratories, Calco, Italy) were utilized in all studies. Mice were rendered neutropenic by intraperitoneal (i.p.) administration of cyclophosphamide 200 mg/kg on days -4, +1, and +4 postinfection. Animal experiments were conducted with the approval of the University of Ancona ethics committee.
(ii) Prophylaxis and infection models. Prophylaxis was started two days before the infection with the latter dose given 2 h prior to challenge for a total of three daily doses (i.e.: on days -2, -1 and 0). AMB was given i.p. at 1 mg/kg/day (200 µl), while POS was administered by oral gavage at doses of 20, 30, 50 and 80 mg/kg/day (200 µl). Control mice received 200 µl of PEG 200 by oral gavage. Two hours after the last drug dose, the mice were infected intravenously (200 µl) with the spores of each isolate. Pilot studies were performed with *R. oryzae* 4570 and *A. corymbifera*, to determine the 90% lethal dose by testing three inoculum sizes. Final experiments were conducted by challenging the mice with $5 \times 10^6$ spores/mice of *R. oryzae* 4570, with $2 \times 10^6$ (high inoculum) and $1 \times 10^5$ (low inoculum) spores/mice of *R. oryzae* 4745 and with $5 \times 10^5$ spores/mice of *A. corymbifera*. To confirm the inoculum, dilutions were streaked on SDA plates, and the colonies were counted following 24 h of incubation at room temperature. Each experiment was performed once.

(iii) Survival studies. In survival studies, the mice were observed through day 10 postinfection. Deaths were recorded daily. Moribund mice were sacrificed, and their deaths were recorded as occurring on the next day. There were from 13 to 18 mice in each group.

(iv) Qualitative cultures. The mice were treated and infected as reported above. On day three postinfection, the animals were sacrificed. Brain, lung, spleen and kidney from each animal were aseptically removed and homogenized in 2 ml of sterile saline solution. Either diluted or undiluted homogenates (including the entire organ) were plated onto SDA plates. The limit of detection was 1 CFU/organ. The data are presented as percentage of infected organs (positive cultures) with respect to the total organs observed for each group (3). There were eight mice in each group.

(v) Histopathology. The mice were treated and infected as reported above. On day three postinfection, the animals were sacrificed. Brain and kidney from control and treated animals were aseptically removed, fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained with Grocott Gomori's methenamine-silver nitrate stains. For each stained section, the number and size of infection foci (clusters of hyphae) (15) were counted in 20 consecutive...
microscopic fields by at least two observers who were unaware of the treatment group using a Leitz Orthoplan light microscope equipped with a micrometric eyepiece (objective: NPL Fluotar 25/0.55; eyepiece: Periplan GW 10x/26). The total measured tissues area was equal to 15.7 mm$^2$ for each stained section. The data are presented as the mean number and the mean size of each infection foci from treated and untreated animals. There were five mice in each group.

**Statistical analysis.** Differences in survival were analyzed by log rank and plotted by Kaplan-Meier curves. Qualitative cultures were compared by Fisher’s exact test. Histopathology results were compared by ANOVA with Bonferroni’s correction for repeated measures. All $P$ values $<$ 0.05 were considered to be significant.
RESULTS

The overall susceptibilities of the two zygomycete isolates are reported in table 1. POS median MICs were 1.0, 1.0 and 0.25 µg/ml for *R. oryzae* 4570, *R. oryzae* 4745 and *A. corymbifera*, respectively. AMB median MICs were 2.0, 0.5 and 1.0 µg/ml for *R. oryzae* 4570, *R. oryzae* 4745 and *A. corymbifera*, respectively.

Figure 1 shows the results of survival studies. In experiments with *R. oryzae* 4570 and *A. corymbifera*, the mice were given POS at 20 and 80 mg/kg/day. In mice infected with *R. oryzae* 4570, AMB significantly prolonged the survival with respect to controls (P = 0.001). POS was not effective at any dose. In mice infected with *A. corymbifera*, both doses of POS significantly prolonged the survival against the controls (P = 0.0001, POS 20 mg/kg/day; P = 0.001, POS 80 mg/kg/day). Similarly, AMB was effective (P = 0.0001).

In the experiments with *R. oryzae* 4745, POS was administered at 50 mg/kg/day and the mice were infected with high and low inoculum. While AMB was effective in mice infected with both inocula (P = 0.0001), POS significantly prolonged the survival only in mice infected with low inoculum (P = 0.001).

Then, we investigated the effect of prophylaxis on qualitative cultures of four different organs. These experiments were conducted with *R. oryzae* 4570 and *A. corymbifera*. In these studies, POS was given at 30 mg/kg/day. The results are presented in table 2. In mice infected with *R. oryzae* 4570, neither AMB nor POS show any potential for organ clearance. Actually, 100 % of treated animals showed to be infected in all organs tested. In the infection due to *A. corymbifera*, the percentage of positive organs in treated mice was generally lower than that observed in untreated controls, but the only significant difference was observed in the brain of mice treated with AMB (12.5% vs 100%, P = 0.0014).

Then, the effects of prophylaxis were further investigated in brain and kidney tissues by histological studies. In these experiments, we examined the number and size of infection foci (clusters of hyphae). As in the previous experiment, the mice were given POS at 30 mg/kg/day. The results are
reported in figures 2. In mice infected with \textit{R. oryzae} 4570, POS was effective at reducing the number of infection foci with respect to the control in the kidney \((P < 0.05)\), but not in the brain (figure 2A and figure 3). AMB was active in both organs \((P < 0.05)\). In mice infected with \textit{A. corymbifera}, either drug was effective at reducing the number of infection foci with respect to the control in both organs \((P < 0.05)\; \text{figure 2B})

Prophylaxes with both drugs were effective at reducing the size of foci in the kidney of mice infected with \textit{R. oryzae} 4570 \((P < 0.05)\), while neither drug was effective in the brain (figure 2C). In mice infected with \textit{A. corymbifera}, either drug was effective at reducing the size of foci with respect to the control in both organs \((P < 0.05)\; \text{figure 2D}).
DISCUSSION

In this study, we investigated the effects of POS in the prophylaxis of experimental systemic zygomycosis. The indices used to assess the outcome of infection were: (i) survival of animals; (ii) percentages of infected organs and (iii) histopathological examination measuring either the number or the size of infection foci. In terms of survival, POS was effective against *A. corymbifera* and one isolate of *R. oryzae*. However, the protective benefit of POS against *R. oryzae* was inoculum-dependent. These results are similar to those recently found by Ibrahim et al (5). These authors found that caspofungin improved the survival of mice with diabetic ketoacidosis infected with a small inoculum but not a large inoculum of *R. oryzae*.

AMB was effective against all isolates. These data are quite similar to those previously reported by Dannaoui *et al.* (3). They studied the effects of POS therapy in a nonimmunocompromised model of systemic infection sustained by three isolates of zygomycetes and found that the new triazole prolonged the survival of animals infected with *A. corymbifera, Rhizopus microsporus var. rhizopodiformis*, but not with *R. oryzae* (3).

Prophylaxis with POS was not effective in terms of organ sterilization in mice challenged with *R. oryzae*: 100% of all four organs yielded positive cultures. AMB prophylaxis was also ineffective. These results are in accordance with previous studies that showed that AMB prolonged survival of infected animals but did not clear the fungus from the organs (10). On the contrary, organ sterilization of mice given POS and infected with *A. corymbifera* was not a rare event. It must be noted, however, that the only significant difference between treated and control groups was found in the brain of mice treated with the polyene (12.5% vs 100% of positive cultures, respectively, \( P < 0.05 \)).

It has been previously reported that intravenous inoculation of mice with zygomycetes resulted in generalized distribution of spores, particularly in the liver and spleen, whereas the development of infection foci containing hyphae occurs in brain and kidney (1, 2). Therefore, we further investigated the effects of prophylaxis on the histopathological features of these two organs.
Interestingly, we found that both POS and AMB were effective at reducing the number and the expansion of infection foci in the kidney of mice challenged with *R. oryzae*, while only AMB was effective at reducing the number, but not the size, of fungal foci in the brain. These findings seem to correlate well with the survival results, in fact all moribund mice presented clinical evidence of neurological disorders which probably represented the ultimate cause of death. Furthermore, histopathological analysis of the brain and the kidney from mice infected with *A. corymbifera* and prophylactically treated with either POS or AMB showed a significant reduction in the number and the expansion of infection foci with respect to the control group.

To our knowledge, this is the first study analysing the effects of POS prophylaxis in experimental infections due to zygomycetes. We found that POS is effective in infections due to *A. corymbifera* in terms of prolongation of survival and reduction of active infections in organs. Although beneficial effects are also observed in infections due to *R. oryzae*, our data confirm that this opportunistic pathogen remains difficult to manage.
REFERENCES.


**TABLE 1.** In vitro susceptibility of zygomycetes isolates to amphotericin B and posaconazole

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Drugs</th>
<th>MIC₅₀ (µg/ml) reported as:</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Median</td>
<td>Range</td>
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<tr>
<td><em>R. oryzae</em> 4570</td>
<td>POS</td>
<td>1.0</td>
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</tr>
<tr>
<td></td>
<td>AMB</td>
<td>2.0</td>
<td>2.0-4.0</td>
</tr>
<tr>
<td><em>R. oryzae</em> 4745</td>
<td>POS</td>
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<td>1.0</td>
</tr>
<tr>
<td></td>
<td>AMB</td>
<td>0.5</td>
<td>0.5-1.0</td>
</tr>
<tr>
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<td>POS</td>
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<td>0.25-2</td>
</tr>
<tr>
<td></td>
<td>AMB</td>
<td>1.0</td>
<td>1.0-2.0</td>
</tr>
</tbody>
</table>

* Each testing was run in quintuplicate and repeated on two different days.

* POS, posaconazole; AMB, amphotericin B.
**TABLE 2.** Culture results for control and treated mice infected with *R. oryzae* 4570, and *A. corymbifera*.  

<table>
<thead>
<tr>
<th>Isolates and group</th>
<th>Brain</th>
<th>Lung</th>
<th>Spleen</th>
<th>Kidney</th>
</tr>
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<td><em>R. oryzae</em> 4570</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Control</td>
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<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>POS</td>
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<td>100.0</td>
<td>100.0</td>
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</tr>
<tr>
<td>AMB</td>
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<tr>
<td><em>A. corymbifera</em> 4535</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>62.5</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>POS</td>
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<td>12.5</td>
<td>100.0</td>
<td>75.0</td>
</tr>
<tr>
<td>AMB</td>
<td>12.5*</td>
<td>25.0</td>
<td>100.0</td>
<td>62.5</td>
</tr>
</tbody>
</table>

*a* Eight animals were present in each group.

*b* POS, Posaconazole was given at 30 mg/kg/day by oral gavage; AMB, Amphotericin B was given at 1 mg/kg/day (i.p.).

* Significantly different from the values obtained for the control groups (*P* < 0.05).
**FIGURE 1.** Survival of neutropenic mice infected intravenously with $5 \times 10^6$ and $5 \times 10^5$ spores/mice of *R. oryzae* 4570 and *A. corymbifera* 4535, respectively, and with $2 \times 10^6$ (high inoculum) and $1 \times 10^5$ (low inoculum) spores/mice of *R. oryzae* 4745. Mice were treated orally with POS at 20, 50 or 80 mg/kg/day daily, and AMB (i.p.) at 1 mg/kg/day on days -2, -1 and 2 h prior to infection (3 total daily doses). The animals were observed through day 10 postinfection and deaths were recorded daily. Moribund mice were sacrificed, and their deaths were recorded as occurring on the next day. There were 13 to 18 mice in each group. Asterisks indicate groups with prolonged survival over the controls ($P < 0.05$).
FIGURE 2. Histopathological analysis of antifungal prophylaxes in systemic zygomycoses. Number of infection foci (clusters of hyphae) in mice infected with *R. oryzae* 4570 (A) and *A. corymbifera* 4535 (B). Sizes of infection foci in mice infected with *R. oryzae* 4570 (C) and *A. corymbifera* 4535 (D). Brain and kidney were retrieved three days postinfection from control mice (*n* = 5, black histograms), posaconazole treated mice (*n* = 5, white histograms) and amphotericin B treated mice (*n* = 5, grey histograms). Tissues sections were prepared and stained with Grocott Gomori’s methenamine-silver nitrate. At least 20 microscopic fields were examined for each mouse, and the number of infection foci and their sizes were determined. Data are reported as means ± standard errors of the means. The ANOVA test with Bonferroni’s correction was used for statistical analysis. Asterisks indicate a significant difference between the treated and untreated group (*P* < 0.05).
FIGURE 3. Representative results of histopathological sections of brain (A, B) and kidney (C, D) tissues stained with Grocott's methamine silver nitrate (original magnification x 160) from mice infected with *R. oryzae* 4570. Control mice (A, C) show typical infection foci in both tissues. Posaconazole, given for three consecutive days at 30 mg/kg/day, was not effective in the brain (B), while it reduced the hyphae growth in the kidney (D).