In vitro and in vivo activities of posaconazole against zygomycetes with various degrees of susceptibility

Elisabetta Spreghini1, Fiorenza Orlando2, Daniele Giannini3 and Francesco Barchiesi1*

1Dipartimento di Scienze Biomediche, Clinica Malattie Infettive, Università Politecnica delle Marche, Azienda Ospedaliero-Universitaria, Ospedali Riuniti Umberto I-Lancisi-Salesi, Via Conca, Torrette, Ancona 60020, Italy; 2Experimental Animal Models for Aging Units, Scientific Technological Area N. Masera, INRCA IRRCS, Via Birarelli n. 8, Ancona 60100, Italy; 3Università Politecnica delle Marche, Centro di Gestione Presidenza Medicina e Chirurgia, Via delle Brecce Bianche, Loc. Monte Daga, Ancona, Italy

*Corresponding author. Tel: +39-0715963467; Fax: +39-0715963468; E-mail: f.barchiesi@univpm.it

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Objectives: We analysed the in vitro and in vivo effects of posaconazole and amphotericin B against three clinical isolates of zygomycetes: Lichtheimia corymbifera, F1; and Rhizopus oryzae, F5 and F6.

Methods: In vitro activities of both drugs were assessed by determining MICs, minimum fungicidal concentrations (MFCs) and fungal damage measured by the XTT assay against either the spores or the hyphal forms. Additionally, the survival curves of neutropenic mice systemically infected with the zygomycete isolates were used as the marker of antifungal response to amphotericin B (1 mg/kg/day) or posaconazole (2.5, 10 and 50 mg/kg/day).

Results: In terms of MICs, posaconazole proved to be active against the three isolates (MICs ranging from 0.125 to 1.0 mg/L). The median posaconazole MFCs were 0.25, 0.5 and 16 mg/L for F1, F5 and F6, respectively. The XTT assay showed that posaconazole was active against spores of all three isolates, but only partially effective against the hyphae. The survival studies showed that amphotericin B at 1 mg/kg/day and posaconazole at 10 mg/kg/day prolonged the survival of the animals infected with L. corymbifera F1. In mice infected with R. oryzae F5, only posaconazole at 50 mg/kg/day significantly prolonged survival, whereas amphotericin B at 1 mg/kg/day was the only regimen active against R. oryzae F6.

Conclusions: Our findings showed that posaconazole could be useful in the treatment of zygomycosis. Also, we report that an isolate of R. oryzae with low MFC responded to posaconazole, while another isolate with high MFC did not.

Introduction

Zygomycosis is a rare but highly aggressive filamentous fungal infection, occurring primarily in immunosuppressed patients and those with diabetes mellitus.1–3 The clinical manifestations include primary rhino-orbital-cerebral zygomycosis in patients with diabetes mellitus, and pulmonary infection in transplant recipients and patients with haematological malignancy.4 The zygomycetes most commonly identified as aetiological agents of human diseases are Rhizopus spp., Rhizomucor spp., Mucor spp. and Mycocladus spp.1–4 Invasive zygomycosis causes 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.1–3

Despite the aggressive standard therapy for these infections, consisting of widespread surgical debridement and high doses of intravenous liposomal amphotericin B, the mortality rate has remained >40%.2,3 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.5–13 Unlike other azoles, posaconazole has demonstrated both in vitro and in vivo activity against zygomycetes.5–20 Experimental infection models and clinical data have shown that posaconazole might be useful for the treatment of zygomycosis.14–20 In this study, we evaluated the in vitro and in vivo effects of posaconazole against zygomycetes with various susceptibility patterns.

Materials and methods

Organisms

One clinical isolate of Lichtheimia corymbifera (formerly Absidia corymbifera) (F1) and two of Rhizopus oryzae (F5 and F6) were utilized in the present study.
The F1, F5 and F6 strains were isolated from a necrotic lesion of the palate, from the sputum of a child with cystic fibrosis and from the sputum of a patient with a bone marrow transplant, respectively. Isolates were stored as spore suspensions at −80 °C in 10% glycerol, until used. The isolates were identified by using standard microbiological methods, and by molecular sequencing of genomic extracts using the primers ITS1 (5′-TCCGATAGTCAACTTCGCGG-3′) and ITS2 (5′-GCTCGCTTCTCATCAGTG C-3′) and the ABI PRISM sequencing apparatus (Applied Biosystems).

**Antifungal drugs**

Posaconazole pure powder (Schering Plough Research Institute, Kenilworth, NJ, USA) was prepared in DMSO and polyethylene glycol 200 (PEG-200; Sigma) for *in vitro* and *in vivo* studies, respectively. Amphotericin B was used as a pure powder (Sigma; Bristol-Myers Squibb) for *in vivo* studies and as a commercial preparation (Fungizone; Bristol-Myers Squibb) for *in vitro* studies. It was dissolved in DMSO and in sterile water following the manufacturer’s instructions for *in vitro* and *in vivo* studies, respectively.

**Susceptibility tests**

MICs were determined by the CLSI broth microdilution methodology (CLSI M38-A2 document). Each strain was grown on Sabouraud dextrose agar (SDA) plates at 35 °C for 5 days. The fungal colonies were then covered with sterile saline solution containing 0.025% Tween 20 and gently scraped with a sterile pipette. The resulting sporangiospore suspensions were transferred to sterile tubes and heavy particles were allowed to settle. The supernatant was transfused in a new tube and the sporangiospore suspension was prepared following the CLSI recommendation to obtain a final inoculum of ~0.4×10⁸ to 5×10⁸ cfu/mL. The tests were performed in RPMI 1640 medium, with l-glutamine and phenol red but without sodium bicarbonate (Sigma), buffered to pH 7.0 with 0.165 M MOPS (Gibco Laboratories, Milan, Italy) buffer. Growth (drug-free) and sterility controls were included for each isolate tested. The microdilution trays, containing both drugs at concentrations ranging from 0.03 to 16 mg/L, were incubated at 35 °C for 24–48 h. Experiments were conducted in quintuplicate and repeated on two different days. The MIC endpoint for both drugs was the lowest drug concentration that prevented any discernible growth.

After the MICs were read, the minimum fungicidal concentrations (MFCs) were determined by plating onto SDA plates the whole volume from each well above the MIC.

Inoculated plates were incubated at 35 °C and MFCs were recorded after 48 h. The limit of detection was 1 cfu. The MFC was defined as the lowest concentration of drug that caused total inhibition of growth (100% of killing).

**Metabolic activity assay**

Metabolic activities of non-germinated spores and filamentous forms were assessed by a modified method of the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Briefly, testing was performed in RPMI 1640 medium (Sigma) with l-glutamine, but without phenol red and sodium bicarbonate, buffered to pH 7.0 with 0.165 M of MOPS using 96-well microtitration plates. All the incubations were performed at 35 °C.

The sporangiospore suspensions of the clinical isolates of zygomycetes were prepared at a final inoculum of 10⁴ cfu/mL and 100 μL/well was dispensed into 96-well microtitration plates. The activities of amphotericin B and posaconazole were determined by adding the drugs at 1/8x, 1/4x, 1/2x, 1x, 2x, 4x and 8x the MICs. To study the metabolic activities of the drugs on the two life-cycle stages, 100 μL of antifungal agent solutions at 2x the desired concentration was added to each well immediately (to study the sporangiospore metabolic activity) or after 24 h of incubation (to study the hyphal metabolic activity). Additional wells containing medium alone or the above-mentioned drug concentrations at a final volume of 200 μL were included in each plate to verify growth medium sterility and to correct the background absorbance. After 24 h of incubation, 20 μL of XTT-based in vitro toxicology assay kit (Tox-2, Sigma) was added to each well and plates were read at 450 nm with a reference wavelength of 690 nm after 4 h of incubation. The sporangiospore and the hyphal metabolic activities were calculated according to the following formula: % inhibition of metabolic activity = 100 − (X/C)×100, where 

<table>
<thead>
<tr>
<th>Isolate/drugs</th>
<th>Median MICs (range), mg/L a</th>
<th>Median MFCs (range), mg/L b</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L. corymbifera F1</strong></td>
<td>0.0625 (0.0625)</td>
<td>0.125 (0.0625–0.125)</td>
</tr>
<tr>
<td>AMB</td>
<td>0.0625 (0.0625)</td>
<td>0.25 (0.125–0.25)</td>
</tr>
<tr>
<td>POS</td>
<td>&lt;0.03 (&lt;0.03)</td>
<td>0.125 (0.125)</td>
</tr>
<tr>
<td><strong>R. oryzae F5</strong></td>
<td>0.0625 (&lt;0.03–0.0625)</td>
<td>1.0 (0.5–1.0)</td>
</tr>
<tr>
<td>AMB</td>
<td>&lt;0.03 (&lt;0.03)</td>
<td>0.25 (0.25)</td>
</tr>
<tr>
<td>POS</td>
<td>&gt;16 (&gt;16)</td>
<td></td>
</tr>
<tr>
<td><strong>R. oryzae F6</strong></td>
<td>0.0625 (0.0625)</td>
<td>0.25 (0.25)</td>
</tr>
<tr>
<td>AMB</td>
<td>0.0625 (0.0625)</td>
<td>1.0 (0.5–1.0)</td>
</tr>
<tr>
<td>POS</td>
<td>0.125 (0.0625–0.125)</td>
<td>&gt;16 (&gt;16)</td>
</tr>
</tbody>
</table>

POS, posaconazole; AMB, amphotericin B.

aMIC, minimum inhibitory concentration was defined as the lowest drug concentration that prevented any discernible growth. Each testing was run in quintuplicate and repeated on two different days.

bMFC, minimum fungicidal concentration was defined as the lowest concentration of antifungal compound yielding no growth. Each testing was run in triplicate and repeated on two different days.
In vivo studies
CD1 male mice weighing 25 g (Charles River Laboratories, Calco, Italy) were utilized in all studies. Mice were rendered neutropenic by intraperitoneal (ip) administration of 200 mg/kg cyclophosphamide on days −4, +1 and +4 post-infection. Pilot studies were performed with *R. oryzae* and *L. corymbifera* isolates to determine the 90% lethal dose by testing three inoculum sizes. The viability of the spores at the time of infection was checked. Final experiments were conducted by challenging the mice with $\approx 2.5 \times 10^6$ *L. corymbifera* spores/mice and $2.5 \times 10^5$ *R. oryzae* spores/mice in a 0.2 mL volume. The drug regimens were started 2 h post-infection and were administered once daily for 5 consecutive days. Amphotericin B was given ip at 1 mg/kg/day, while posaconazole was administered by oral gavage at doses of 2.5, 10 and 50 mg/kg/day, both in a 200 μL volume. Control mice received 200 μL of PEG-200 by oral gavage. Deaths were recorded daily. Moribund mice were sacrificed and their deaths were recorded as occurring on the next day. There were 10–14 mice in each group. Animal experiments were conducted with the approval of the University of Ancona ethics committee.

Statistical analysis
Differences in survival were determined by log rank analysis and plotted by Kaplan–Meier curves. A *P* value of $<0.05$ was considered significant.

Results
MICs and MFCs of both drugs against the three clinical isolates of zygomycetes are shown in Table 1. Both drugs were active against the strains, with MICs of $\leq 1.0$ mg/L. At 48 h, amphotericin B and posaconazole MIC values ranged from 0.0625 to 0.25 mg/L and from 0.125 to 1.0 mg/L, respectively. Amphotericin B MFCs were...
ranged from 0.125 to 1.0 mg/L, while posaconazole MFCs ranged from 0.25 to 16 mg/L.

To further characterize the activity of these drugs in vitro, we determined the inhibition of metabolic activity either against spores or hyphae of the zygomycete isolates (Figure 1). Both drugs showed a dose-dependent reduction of metabolic activity against spores of all three isolates. Against the hyphal form, amphotericin B inhibited the metabolic activity of *L. corymbifera* F1 and *R. oryzae* F6 isolates in a dose-dependent manner, while the polyene showed a reduced activity against *R. oryzae* F5. Posaconazole partially inhibited the metabolic activity of the hyphal forms of all strains. In particular, the greatest activity of the triazole accounted for 59.7%, 55.3% and 19.3% growth inhibition at 8 × MIC against *L. corymbifera* F1, and *R. oryzae* F5 and F6 isolates, respectively.

The survival studies are reported in Figure 2. The mice infected with *L. corymbifera* F1 were treated with posaconazole at 2.5 and 10 mg/kg/day, and with amphotericin B at 1 mg/kg/day. Amphotericin B was effective at prolonging the survival with respect to the control group (*P* = 0.01). Posaconazole at 10 mg/kg/day, but not posaconazole at 2.5 mg/kg/day, significantly prolonged the survival compared with the controls (*P* = 0.048 and *P* = 0.0544, respectively). In the other two *in vivo* experiments, posaconazole was administered at 2.5, 10 and 50 mg/kg/day, and amphotericin B at 1 mg/kg/day. In mice infected with *R. oryzae* F5, only posaconazole at 50 mg/kg/day significantly prolonged the survival with respect to controls (*P* = 0.0215), while amphotericin B at 1 mg/kg/day was the only regimen active against *R. oryzae* F6 (*P* < 0.0001).

**Discussion**

In this study, we investigated the *in vitro* and *in vivo* effects of posaconazole and amphotericin B against zygomycetes with varying degrees of susceptibility. The MICs determined by us were within the ranges reported by others for *Rhizopus* spp. and *Absidia* spp.5,10–13

In our hands, posaconazole showed fungicidal activity against *L. corymbifera* and also against *R. oryzae* F5. In contrast, theazole yielded MFC values of >16 mg/L against *R. oryzae* F6. Published data on MFCs are limited. Recently, Rodriguez et al.,18 tested the *in vitro* activity of posaconazole against 50 strains of *R. oryzae* and found MFCs ranging from 0.5 to >16 mg/L.

The XTT assay results partially mirrored the susceptibility test findings. The inhibition of metabolic activity showed that posaconazole and amphotericin B were active in a dose-
dependent manner against spores of all three isolates. Against the hyphae, amphotericin B inhibited, in a dose-dependent manner, the metabolic activity of L. corymbifera F1 and R. oryzae F5, but not of R. oryzae F6, while posaconazole was only partially effective against the hyphal forms of all three isolates, even when tested at 8× MIC. Of note, hyphal forms of R. oryzae F5 were more susceptible to posaconazole than to amphotericin B, whereas the hyphae of F6 were more susceptible to the polyene.

In general, we observed a good correlation between in vitro and in vivo results with both drugs. Interestingly, MFCs rather than MICs as well the XTT assay on hyphae rather than sporangiospores were more predictive of outcome in our infection model. In fact, the mice infected with L. corymbifera F1 and R. oryzae F5, showing posaconazole MIC and MFC values <1.0 mg/L, responded to the triazole treatment. On the other hand, the triazole did not show any effect in mice infected with R. oryzae F6 (median posaconazole MICs and MFCs of 1.0 and >16 mg/L, respectively).

These results are only partially in agreement with those recently published by Rodriguez et al.19 They found that posaconazole was less, but still, effective against R. oryzae isolates with high MFCs (>16 mg/L), suggesting that high MFCs may be predictors of poorer outcomes.19 The higher number of spores utilized in our infection model (2×10^5 compared with 2×10^4 spores/mice) as well the different regimens of posaconazole administration (once compared with twice daily regimens) might explain the better outcomes reported by these authors.

Our in vitro data for amphotericin B correlate with the in vivo results. In fact, the efficacy of amphotericin B at 1 mg/kg/day was seen in vivo against F1 and F6 isolates (MFCs of 0.125 and 0.25 mg/L, respectively), but efficacy was not seen against R. oryzae F5 isolate (MFCs of 1.0 mg/L). Recently, Lewis et al.26 compared the kinetics of amphotericin B (liposomal formulation compared with lipid complex) lung accumulation in a neutropenic murine model of pulmonary mucormycosis caused by R. oryzae and found that the suppression of R. oryzae growth in the lung required amphotericin B tissue concentrations that approached the MFC for the infecting isolate. This result might explain why one isolate of R. oryzae responded to amphotericin B at 1 mg/kg/day, while the other did not.

We conclude that posaconazole could be useful in the treatment of zygomycosis caused by L. corymbifera and R. oryzae. Also, here we report for the first time that an isolate of R. oryzae with low MFC responded to posaconazole, while an isolate with high MFC did not. This preliminary research suggests that in vitro data could be of help in planning treatment against these difficult-to-treat infections. However, further studies with a larger number of strains are warranted before a firm conclusion can be drawn.

**References**


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**Transparency declarations**

None to declare.


