Activity of the new antifungal triazole, posaconazole, against Cryptococcus neoformans

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The new antifungal derivative posaconazole was tested against three clinical isolates of Cryptococcus neoformans var. neoformans using a broth microdilution procedure performed according to the guidelines established by the NCCLS. Posaconazole MICs were 0.125, 0.25 and 1.0 mg/L for isolates 491, 2337 and 486, respectively. To investigate the in vivo activity of this new compound, we established an experimental model of systemic cryptococcosis in CD1 mice by iv injection of cells of each strain of C. neoformans. Low (3 mg/kg/day) and high (10 mg/kg/day) doses of posaconazole were compared with amphotericin B given at 0.3 mg/kg/day for 10 consecutive days. Survival studies showed that all treatment regimens were effective in prolonging the survival of mice infected with C. neoformans 486 (P < 0.001). Only posaconazole at 10 mg/kg and amphotericin B were effective in prolonging the survival in mice infected with C. neoformans 2337 (P from <0.01 to <0.001), while neither agent was effective in mice infected with C. neoformans 491. Tissue burden experiments performed 24 h after the end of therapy revealed that posaconazole at 10 mg/kg was effective at reducing the fungal burden in both lung and brain tissues of all three strains of C. neoformans. In particular, for C. neoformans 491 and 2337 posaconazole was superior to amphotericin B at reducing the fungal burden in the brain (P < 0.05). The efficacy of posaconazole was also confirmed by determining the capsular antigen serum levels of treated mice versus untreated mice. Our study underlines the excellent activity of posaconazole against this pathogenic yeast.

Introduction

Cryptococcus neoformans is the cause of the most common life-threatening opportunistic fungal infection in patients with AIDS. Although the occurrence of cryptococcosis among this group of patients has decreased in the last 3 years due to the introduction of triple HIV therapy, this incidence remains high, particularly in developing countries.

The ‘gold standard’ therapy for cryptococcosis remains amphotericin B with or without flucytosine. For suppression therapy a triazole, such as fluconazole or itraconazole, is the agent of choice.

Recently, the new investigational triazole posaconazole was shown to have potent activity against isolates of C. neoformans in vitro. Thus far, few studies have been conducted to correlate the in vitro data on posaconazole MICs for this pathogenic yeast with results in vivo. Therefore, in the present study we investigated the efficacy of this new triazole in an experimental model of systemic murine cryptococcosis caused by strains of C. neoformans with variable patterns of posaconazole susceptibility in vitro.

Materials and methods

Isolates

Three isolates of C. neoformans, each obtained from an AIDS patient, were used in this study (Table). Two strains
Table. *In vitro* and *in vivo* activities of posaconazole and amphotericin B against three isolates of *C. neoformans*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (mg/L)</th>
<th>Survival*</th>
<th>Median (range) fungal burden (log&lt;sub&gt;10&lt;/sub&gt; cfu/g)</th>
<th>Antigen serum titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCZ</td>
<td>AMB</td>
<td>time therapy&lt;sup&gt;b&lt;/sup&gt;</td>
<td>brain</td>
</tr>
<tr>
<td>486</td>
<td>1.0</td>
<td>0.5</td>
<td>none</td>
<td>18.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>PCZ 3</td>
<td>26.9 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PCZ 10</td>
<td>28.8 ± 0.5&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>7.5&lt;sup&gt;d&lt;/sup&gt; (6.8–8.1)</td>
<td>5.0&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt; (4.8–5.3)</td>
</tr>
<tr>
<td></td>
<td>AMB 31.0 ± 2.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.9&lt;sup&gt;d&lt;/sup&gt; (7.0–8.1)</td>
<td>6.9 (5.2–7.3)</td>
<td>4096&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>491</td>
<td>0.125</td>
<td>0.5</td>
<td>none</td>
<td>41.1 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>PCZ 38.6 ± 1.5</td>
<td>3.7 (5.1–7.6)</td>
<td>3.7 (5.4–6.3)</td>
<td>4096</td>
</tr>
<tr>
<td></td>
<td>AMB 50.6 ± 4.4</td>
<td>6.7&lt;sup&gt;d&lt;/sup&gt; (6.3–6.9)</td>
<td>5.9 (5.4–6.3)</td>
<td>8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>2337</td>
<td>0.25</td>
<td>0.5</td>
<td>none</td>
<td>18.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>PCZ 19.7 ± 1.1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PCZ 10 22.4 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.0&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt; (5.2–6.5)</td>
<td>4.6&lt;sup&gt;d&lt;/sup&gt; (3.4–5.1)</td>
<td>8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AMB 36.7 ± 2.0&lt;sup&gt;d&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7.2&lt;sup&gt;d&lt;/sup&gt; (6.3–7.4)</td>
<td>5.4&lt;sup&gt;d&lt;/sup&gt; (4.0–6.1)</td>
<td>8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

PCZ, posaconazole; AMB, amphotericin B; ND, not done.

* Mice were observed for 40 (*C. neoformans* 486 and 2337) or 60 (*C. neoformans* 491) days post-infection.
  
* Therapy was given for 10 consecutive days: none, no drugs; PCZ 3, PCZ at 3 mg/kg/day; PCZ 10, PCZ at 10 mg/kg/day; AMB 0.3, AMB at 0.3 mg/kg/day.

* Statistical significance for the following comparisons: *any treatment versus control; any treatment versus PCZ 3; AMB 0.3 versus PCZ 10; PCZ 10 versus AMB 0.3.
were obtained from blood (491 and 2337), while *C. neoformans* 486 was isolated from CSF. All isolates were identified as *C. neoformans* var. *neoformans* on the basis of no colour change on canavanine–glycine–bromothymol blue agar. All the strains were maintained on Sabouraud dextrose agar (SDA; Difco Laboratories, Detroit, MI, USA) slants at 4°C.

**Antifungal agents**

For *in vitro* studies, a stock solution of posaconazole (Schering Plough Research Institute, Kenilworth, NJ, USA) was prepared in polyethylene glycol (PEG 200; Janssen Chimica, Geel, Belgium), and a stock solution of amphotericin B (Sigma Chemical, Milano, Italy) in dimethyl sulphoxide (Sigma). Further dilutions of both drugs were made in the test medium. The final concentration of the solvent did not exceed 1% in any well.

For *in vivo* studies, posaconazole was prepared in PEG 200 while amphotericin B was purchased as Fungizone from Bristol-Myers Squibb (S.p.A., Latina, Italy).

**Susceptibility testing**

Antifungal susceptibility testing was performed using a broth microdilution method, adhering to the recommendations of the NCCLS. The test medium was RPMI 1640 (Sigma) buffered to pH 7.0 with 0.165 M MOPS (Gibco Laboratories, Milano, Italy). Final concentrations of both drugs ranged from 0.0078 to 4.0 mg/L. Yeast inocula ranged from 0.5 × 10^3 to 2.5 × 10^5 cfu/mL. The microdilution plates were incubated in air at 35°C and read at 72 h. The posaconazole MIC was defined as the lowest concentration of drug at which turbidity in the well was 80% less than that in the control well, while amphotericin B MIC was defined as the lowest concentration of drug at which no fungal growth was detectable. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were routinely tested in parallel with each MIC run.

**Animal studies**

A murine model of systemic cryptococcosis was established in CD1 male mice (weight 30 g; Charles River Laboratories, Calco, Italy) by injection via the lateral tail vein of viable yeast cells grown overnight in brain–heart infusion broth. Animal experiments were conducted with the approval of the University of Ancona ethics committee. Posaconazole was administered by oral gavage at concentrations of 3 and 10 mg/kg/day, while amphotericin B was given ip at 0.3 mg/kg/day. Therapy was started 24 h after the infection and continued for 10 consecutive days. In survival studies the mice were observed through days 40 or 60 and deaths were recorded daily. Moribund mice were killed, and their deaths were recorded as occurring on the next day. In tissue burden studies the mice were killed 24 h after the end of therapy, and the number of viable cfu per gram of brain and lungs of each animal was determined by quantitative plating of organ homogenates onto SDA plates. There were 10–13 mice per group in survival studies, and seven mice per group in tissue burden studies. Three additional mice per group were killed 24 h after the end of therapy and were used to study the effects of drugs on the clearance of cryptococcal capsular polysaccharidic antigen (PA) in serum. Testing was performed by a commercially available agglutination assay (Crypto-La Test, Bouty, S.p.A., Milano, Italy).

**Statistical analysis**

The log rank test was used to determine the difference between survival groups, and the Mann–Whitney *U*-test was used to determine the significance in tissue burden studies. The results of PA serum levels were determined by the analysis of variance followed by Bonferroni *t*-test. Differences were considered significant when *P* was < 0.05.

**Results**

Antifungal susceptibility testing of both drugs showed identical results in five independent experiments. Posaconazole MICs were 0.125, 0.25 and 1.0 mg/L for isolates 491, 2337 and 486, respectively, and the MIC of amphotericin B was 0.5 mg/L for all isolates (Table).

Survival results are given in the Table. Mice were infected with 2.0 × 10^5, 4.5 × 10^5 and 4.8 × 10^5 cfu/mouse in experiments with *C. neoformans* 486, 491 and 2337, respectively. For *C. neoformans* 486, all treatment regimens were effective in prolonging survival against the controls (*P* < 0.001). The effectiveness of posaconazole was shown to be dose dependent, with posaconazole at 10 mg/kg/day being more effective than posaconazole at 3 mg/kg/day (*P* < 0.05). For *C. neoformans* 491, neither posaconazole nor amphotericin B was effective in prolonging survival. For *C. neoformans* 2337, only posaconazole at 10 mg/kg/day (*P* < 0.01) and amphotericin B (*P* < 0.001) were effective in prolonging survival. For this strain, amphotericin B was significantly more active than both triazole dosing regimens (*P* < 0.001).

The second set of experiments consisted of three tissue burden studies. Mice were infected with 1.8 × 10^5, 2.0 × 10^5 and 2.1 × 10^5 cfu/mouse in experiments with *C. neoformans* 486, 491 and 2337, respectively. Mice were treated for 10 days, and killed 1 day later. In these studies, posaconazole was only given at 10 mg/kg/day. The results are reported in the Table. For *C. neoformans* 486, both treatments were equally effective at reducing fungal burdens in the brain, whereas only the triazole was effective at reducing fungal burdens in the lung. Posaconazole was also more effective than amphotericin B (*P* < 0.05). Similar results were obtained for *C. neoformans* 491. In particular, for this
strain posaconazole was more effective than amphotericin B at reducing fungal burdens in both organs ($P < 0.05$). For C. neoformans 2337, both posaconazole and amphotericin B were effective at reducing fungal burdens in both organs, with the triazole more effective than the polyene in the brain tissue ($P < 0.05$).

Antigen titres are reported in the Table. Both drugs were significantly effective at reducing PA serum levels for all three strains ($P < 0.05$ to $< 0.001$). In addition, posaconazole was superior to amphotericin B against C. neoformans 486 ($P < 0.01$).

### Discussion

In this study, we correlated the in vitro activity of posaconazole with its efficacy in vivo against C. neoformans. Three clinical isolates of C. neoformans showing variable degrees of posaconazole susceptibility in vitro were used in the study. Our data on survival did not correlate with the in vitro data which showed that the most susceptible isolate (491) was resistant to treatment with either low or high doses of posaconazole, as it was with amphotericin B. On the other hand, the least susceptible strain (486) proved to be the most susceptible in vivo, with both posaconazole doses being effective at prolonging the survival compared with controls. These findings underline the lack of complete correlation between in vitro and in vivo results in testing this triazole against C. neoformans. It must be noted, however, that the posaconazole MICs only encompassed a three dilution difference between the most and the least susceptible isolate (0.125–1.0 mg/L), and that we did not use any highly resistant strain.

Unlike antibacterial agents, for which standardized susceptibility testing procedures and interpretive breakpoints are well established, reproducible methods and tentative breakpoints for antifungal agents have only recently been introduced.\(^1\) So far, most of the studies investigating the relationship between in vitro and in vivo results of antifungal efficacy have involved infections due to Candida spp.\(^1\) Only a few reports have tried to correlate the in vitro activity of a given antifungal agent (mainly fluconazole) with the clinical outcome of cryptococcosis.\(^2\)\(^–\)\(^4\)

In an early study, Casadevall et al.\(^5\) used a broth macrodilution method to analyse fluconazole and amphotericin B MICs for 13 strains of C. neoformans isolated from five AIDS patients, and showed a lack of correlation between in vitro data and clinical outcome. In contrast, Witt et al.\(^1\) used a modified broth microdilution method to test fluconazole MICs for clinical isolates of C. neoformans and found a statistically significant correlation between in vitro data and clinical success or failure.

The reason both posaconazole and amphotericin B were ineffective in prolonging the survival of mice infected with C. neoformans 491 is difficult to explain. It can only be hypothesized that failure to prolong survival of mice infected with this isolate might be due to the longer observation time (60 versus 40 days) than that applied in mice infected with the other two isolates of C. neoformans. This situation, owing to a progressive decrease of drug tissue levels over time, would facilitate the replication of the remaining fungi to a critical burden.

Unlike survival data, tissue burden experiments showed that posaconazole given at 10 mg/kg/day was effective at reducing the number of cfu per gram of brain and lung tissues in all isolates of C. neoformans. In particular, posaconazole was more effective than amphotericin B in the brain of mice infected with C. neoformans 491 and 2337 as in the lung of mice infected with C. neoformans 486 and 491. Determination of antigen serum levels of treated mice confirmed the potent in vivo efficacy of this new antifungal molecule.

Overall, our study underlines the excellent activity of posaconazole against this pathogenic yeast and indicates that this new antifungal molecule merits further investigation as a potentially useful agent for the treatment of human cryptococcosis.

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### References

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