Efficacy of posaconazole in murine experimental sporotrichosis.

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Abstract

We have developed a murine model of systemic sporotrichosis by using three strains of each of the two commonest species causing sporotrichosis, *Sporothrix schenckii sensu stricto* and *Sporothrix brasiliensis*, in order to evaluate the efficacy of posaconazole (PSC). The drug was administered at doses of 2.5 or 5 mg/kg twice a day by gavage and one group was treated with amphotericin B (AMB) as control of treatment. Posaconazole, especially at 5 mg/kg, showed good efficacy against all the strains tested regardless of their MICs, as measured by prolonging survival, tissue burden reduction and histopathology.
Introduction

Sporotrichosis is the most common and cosmopolitan subcutaneous mycosis (13). The most usual clinical manifestation of the disease is the subacute or chronic lymphocutaneous form followed by fixed cutaneous infections (5); however, disseminated infections have also been described in underlying alcoholism and immunosuppressed patients, especially those with AIDS or receiving chemotherapy or corticoids (10,13,17). Sporotrichosis is caused by a group of species belonging to the Sporothrix schenckii complex, which vary in individual in vitro response to antifungal agents. The differential antifungal activity among Sporothrix species could explain the variability in antifungal susceptibility reported for S. schenckii in studies previous to the recent recognition of the S. schenckii complex (5, 20, 21).

The recommended procedures for the management of sporotrichosis include local measures such as hyperthermia and systemic measures such as administration of a saturated solution of potassium iodide, or azoles (itraconazole and fluconazole), amphotericin B or terbinafine (15). Itraconazole has become the drug of choice for treatment of the lymphocutaneous and cutaneous forms. In those patients with intolerance to itraconazole, fluconazole is the recommended alternative. Amphotericin B is the first choice in the treatment of disseminated sporotrichosis and itraconazole is recommended as the step-down therapy after patients respond to the initial treatment with amphotericin B (18).

Posaconazole has shown activity in vitro against the species of the S. schenckii complex (21, 25). Since treatment options for disseminated sporotrichosis are limited and there is only scarce information on the effectiveness in vivo of
posaconazole, we have evaluated the response to this drug in a murine model of disseminated sporotrichosis.

Material and methods

The fungi were stored in slant cultures covered with sterile paraffin oil and subcultured on potato dextrose agar (PDA) plates at 30ºC for 7 days. Antifungal susceptibility to posaconazole in vitro of 5 isolates of *S. brasiliensis* and 10 of *S. schenckii* s.s. was determined using a broth microdilution method according to the CLSI guidelines for filamentous fungi (8).

For the in vivo studies, strains of *S. brasiliensis* (n=3) and *S. schenckii* s.s. (n=5) showing different MICs values were chosen. The inocula were prepared by flooding with saline solution onto the surface of the agar plate, scraping the sporulating mycelium. The resulting solutions were transferred to 100 ml of potato dextrose broth (PDB) and incubated in an orbital shaker at 150 rpm at 30º C for 5 days. Then, cultures were filtered twice through sterile gauze, and centrifuged at 325 g. The pellets were washed once with saline solution and the conidia concentrations were adjusted to the desired concentrations by haemocytometer counting. To verify the viability and size of inocula, 10-fold dilutions were placed in PDA to determine the CFUs.

Four–week-old OF-1 male mice (Charles River, Crippa S.A., Barcelona, Spain) with a mean weight of 30 g were used. Animals were housed in standard boxes with corncob bedding and free access to food and water. All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Mice were infected intravenously (i.v.) in the lateral tail vein with 2x10⁷ CFU in 0.2 ml of sterile saline. This inoculum level was chosen based upon previous studies with strain FMR 8314 that indicated
that this concentration was the minimum dose that killed all the infected animals within 18 days (data not shown).

Posaconazole, provided as Noxafil (Schering-Plough Ltd., Hertfordshire U.K.), was administered at 2.5 or 5 mg/kg twice a day (BID) by gavage. These doses were chose based upon preliminary studies using higher (10 and 20 mg/kg BID) and lower (1 and 2 mg/kg BID) doses of Posaconazole (Fig 1). Controls received no treatment. The efficacy of the posaconazole was evaluated as prolonging survival, reducing tissue burden and by studying the histopathology. Treatments began one day after infection, and lasted for 18 days. For survival studies, groups of 10 mice were randomly established for each strain and each treatment and checked daily for 30 days after challenge. For tissue burden studies, groups of 10 mice were also established and the animals were sacrificed on day 13 post-infection in order to compare the results with controls. Liver and spleen, since are the most affected organs in experimental systemic sporothrichosis (2), were removed aseptically and a half of them were homogenized in 1 ml sterile saline. Serial 10-fold dilutions of the homogenates were plated on PDA and incubated for 72 h at 30°C. The numbers of CFU/g of tissue were calculated. For the histopathology study, half of each organ was fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded and sliced into 2 μm sections, which were then stained with Haematoxylin-Eosin (H-E), Periodic acid Schiff (P.A.S) or Grocott methamine silver and examined in blinded fashion by light microscopy. Additionally, one group of 10 animals was infected with the strain of S. brasiliensis FMR 8326 and treated i.v. with amphotericin B (Fungizone™, Squibb Industria Farmacéutica S.A., Barcelona, Spain) at 0.8 mg/kg for 18 days.
The statistical analysis was made using Graph Pad Prism 5 for Windows. The mean survival time was estimated by the Kaplan-Meier method and compared among groups using the log rank test. The colony counts from tissue burden studies were analysed using the Mann-Whitney U test. The observed differences were considered statistically significant at p<0.05.

Results

Two of 5 strains tested of *S. brasiliensis* and four of 10 strains of *S. schenckii* s.s., showed posaconazole MICs of 0.5 - 1 µg/ml, which are close to the suggested breakpoint indicative of the susceptibility of filamentous fungi i.e. ≤1 µg/ml for Posaconazole (8). The other strains of *S. brasiliensis* (3) showed MICs of 2 µg/ml (intermediate susceptibility). Three strains of *S. schenckii* s.s. showed intermediate susceptibility, and another 3 showed MICs > 2 µg/ml.

The posaconazole MIC values of those strains included in the *in vivo* study were as follows: *S. brasiliensis* 0.5 µg/ml (FMR 8319), 1 µg/ml (FMR 8314) and 2 µg/ml (FMR 8326). For *S. schenckii sensu stricto*, the MICs were 1 µg/ml (FMR 8606), 2 µg/ml (FMR 8609 and FMR 9018), 4 µg/ml (FMR 9010) and 8 µg/ml (FMR 8604).

Systemic infection caused 100% mortality in untreated control groups within 11 to 18 days after challenge regardless of the species or strain tested, with the exception of a strain of *S. schenckii* s.s. with a high MIC (FMR 9010 and FMR 8604), which did not cause death; so these strains were discarded from the treatment study.

Posaconazole at 2.5 or 5 mg/kg BID significantly prolonged survival with respect to the control (Fig 2) and all treated animals survived to the end of the experiment regardless of the species and strain tested.
Posaconazole at 2.5 or 5 BID was able to significantly reduce the fungal load in liver and spleen in comparison to the control group in animals infected with *S. schenckii* s.s. or *S. brasiliensis* (Fig 3). Moreover, the high dose of posaconazole significantly reduced the fungal load in both organs in comparison to the low dose (P<0.05). Amphotericin B was effective in reducing the fungal load in both organs in comparison to controls (p<0.0001) despite that amphotericin B MIC for this strain was 4 μg/ml. Histopathological studies of control mice showed an abundance of typical cigar-shaped cells in liver and spleen. In mice infected with *S. schenckii* sensu stricto, these fungal cells were usually surrounded by a granuloma, while in those infected with *S. brasiliensis* a massive infiltration of tissue with fungal cells was observed and an absence of granuloma (Fig 4 A-B). In general, there was a scarcity of fungal cells in mice treated with posaconazole at 2.5 BID (Fig 4C), with a recovery of tissue structure, and in those treated with both posaconazole at 5 mg/kg BID (Fig 4 D) or amphotericin B fungal elements were not observed and tissue structure was normal.

**Discussion**

Although cases of systemic sporotrichosis are fortunately not frequent, several cases have been described (1, 3,15,19,22, 26). Amphotericin B is the most common drug used in the treatment of disseminated sporotrichosis, but the dosage is limited by its toxicity and by the long-term therapy required. In this study, we have evaluated the activity *in vitro* and the efficacy *in vivo* of posaconazole against several strains of *S. schenckii* s.s. and *S. brasiliensis*. Only a few previous studies evaluated the *in vitro* activity of posaconazole against *Sporothrix* spp., and reported very variable results, with MICs values ranging from 0.03 to 16 μg/ml. The MICs obtained in our study are consistent
with those obtained by previous authors (12, 14, 21, 25), although they did not establish MICs for each individual species of the complex as was done later by Marimón et al. (21). Although some studies have evaluated the efficacy of some antifungals (16) and/or determined the virulence of strains of various origins (9) all of them were carried out prior to the reclassification of Sporothrix as a species complex. Only a comparative study on virulence in mice had been carried out for this complex of species (2). Our study shows that spleen and liver have high fungal load, which is in agreement with previous reports on animal models of sporotrichosis (2, 9, 11). To our knowledge, this is the first study to explore the efficacy of posaconazole in a murine model of disseminated sporotrichosis. Our results demonstrated good efficacy of posaconazole administered at 2.5 or 5 mg/kg BID against disseminated sporotrichosis by the two species tested. In addition, lower (1 or 2 mg/kg BID) and higher doses (10 or 20 mg/kg BID) showed in vivo efficacy for our model of infections. However, the best statistical significance was observed at 2.5 mg/kg and 5 mg/kg BID. These results should be considered excellent in prolonging survival since none of the infected mice died during the experiment, even considering that the doses were relatively low in comparison with those tested in other studies that had treated infections by other fungi under similar conditions (7, 23, 24). For instance, doses of 20 or 30 mg/kg BID against Cryptococcus gatti (7), Rhizopus oryzae (23) or Aspergillus terreus complex (24) only achieved survival rates of 20%, 40% and 60%, respectively.

Surprisingly, our results indicate that outcome does not seem to correlate with MICs in the range observed among our strains, since in those strains with MICs \( \leq 0.5 \) or with MICs \( = 2 \), tissue burden reduction was similar (approx. 4-5 log with
respect to the control group). Furthermore, we also observed that the efficacy of posaconazole was similar against the two species tested.

Having previously been demonstrated experimentally (16) and corroborated in clinical reports (19, 26) amphotericin B is effective in the treatment of disseminated sporotrichosis. It should be noted that *Sporothrix* infections are frequently chronic and consequently require long-term therapy. Our results suggest that treatment with posaconazole could be an alternative to amphotericin B. Despite of the reduction of fungal load in posaconazole-treated animal was lower than those achieved by using amphotericin B the reduction was notably significant with respect to untreated animals, also the reduced toxicity reported for posaconazole allow proposing as an alternative therapeutic to sporotrichosis. However, further studies including more strains and treatment durations are required in order to establish the usefulness of posaconazole against sporotrichosis. We can expect that in the future posaconazole could be effective at lower doses if intravenous formulations are available.
References


FIG 1. Effects on fungal load of mice infected with *S. brasiliensis* FMR 8314 (MIC = 1 μg/ml) following antifungal treatment with varying doses of posaconazole (PSC). *P* < 0.005 versus control, *b*P* < 0.05 versus PSC 1, *c*P* < 0.05 versus PSC 2, *d*P* < 0.05 versus PSC 2.5. Posaconazole was administered twice daily (BID) by gavage at 1 mg/kg, 2 mg/kg, 2.5 mg/kg, 5 mg/kg, 10 mg/kg or 20 mg/kg. Horizontal lines indicate median values.

FIG 2. Cumulative mortality of mice infected with the following strains and treated with posaconazole (PSC) (A-F) or amphotericin B (AMB) (G). *S. brasiliensis* (A) FMR 8314 (MIC = 1 μg/ml), (B) FMR 8319 (MIC = 0.5 μg/ml), (C) FMR 8326 (MIC = 2 μg/ml); *S. schenckii s.s.* (D) FMR 9018 (MIC = 2 μg/ml), (E) FMR 8609 (MIC = 2 μg/ml), (F) FMR 8606 (MIC = 1 μg/ml). (G) FMR 8326 (MIC = 4 μg/ml). *P* < 0.005 versus control. Posaconazole was administered twice daily (BID) by gavage at 2.5 mg/kg or 5 mg/kg. Amphotericin B was administered at 0.8 mg/kg i.v. once a day.

FIG 3. Effects of the antifungal treatment with posaconazole (PSC) (A-F) or Amphotericin B (AMB) (G) on fungal load of mice infected with following strains: *S. brasiliensis* (A) FMR 8314 (MIC = 1 μg/ml), (B) FMR 8319 (MIC = 0.5 μg/ml), (C) FMR 8326 (MIC = 2 μg/ml); *S. schenckii s.s.* (D) FMR 9018 (MIC = 2 μg/ml), (E) FMR 8609 (MIC = 2 μg/ml), (F) FMR 8606 (MIC = 1 μg/ml). (G) FMR 8326 (MIC = 4 μg/ml). *P* < 0.005 versus control, *b*P* < 0.05 versus PSC 2.5. Posaconazole was administered twice daily (BID) by gavage at 2.5 mg/kg or 5 mg/kg. Amphotericin B was administered at 0.8 mg/kg i.v. once a day. Horizontal lines indicate median values.
FIG 4. Histopathology of liver of mice infected with *Sporothrix brasiliensis* and treated with posaconazole. (A-B) Liver sections showing abundance of fungal cells with massive infiltration of the hepatic tissue (A) (Grocott X100); (B) (Grocott X 600). (C) Scarcity of fungal cells in mice treated with posaconazole 2.5 mg/kg BID (P.A.S X 600) (arrows indicate fungal cells into Kupffer cells) and (D) liver of mice treated with 5 mg/kg BID showing the absence of fungal elements and the absence of inflammatory response (P.A.S X 600).