Evaluation of the efficacy of amphotericin B, posaconazole, voriconazole and anidulafungin in a murine disseminated infection by the emerging opportunistic fungus *Sarocladium (Acremonium) kiliense*.

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Abstract

We evaluated and compared the efficacy of different antifungal drugs against Sarocladium kiliense (formerly Acremonium kiliense), a clinically relevant opportunistic fungus in a murine model of systemic infection. Three clinical strains of this fungus were tested and the therapies administered were as follows: posaconazole at 20 mg/kg (twice daily), voriconazole at 40 mg/kg, anidulafungin at 10 mg/kg, or amphotericin B at 0.8 mg/kg. The efficacy was evaluated by prolonged animal survival, tissue burden reduction and by (1→3)-β-D-glucan serum levels. In general, the four antifungal drugs showed high MICs and poor in vitro activity. The efficacy of the different treatments was only modest, since survival rates never were higher than 40% and no drug was able to reduce fungal load in all the organs for the three strains tested. Posaconazole, in spite of its high MICs (≥16 µg/ml), showed the highest efficacy. The (1→3)-β-D-glucan serum levels were equally reduced by all drugs evaluated.
Introduction

*Acremonium kiliense* is a saprobic fungus with a worldwide distribution, which has been reported in the last years as an emerging opportunistic pathogen able to cause a wide range of human infections (1-4). Localized infections, such as mycetoma, keratitis or onychomycosis are acquired mainly by immunocompetent patients through trauma (5-11). Invasive infections generally affect immunosuppressed hosts such as those undergoing transplantation or those with AIDS, resulting in a high degree of fatality (12-16). More rarely, though, invasive infections have also been reported in immunocompetent individuals (17, 18). Recently, based on molecular studies, this fungus has been transferred to the genus *Sarocladium* as *Sarocladium kiliense* (1). This species is the most clinically relevant of the genus and apparently also the most virulent (4, 19, 20). Considering that the morphological identification of these fungi is difficult it is likely that some clinical isolates of this species have been misidentified as *Acremonium strictum*, another clinically important species of *Acremonium* (19). Infections by *Acremonium* spp. are difficult to treat due to the intrinsic resistance to the current antifungal agents and an effective treatment has not yet been determined (19, 21-23). Amphotericin B (AMB) is the most commonly used drug to treat severe fungal infections caused by opportunistic moulds (4). Despite its poor *in vitro* activity and variable clinical results, this drug still remains the first therapeutic choice against invasive *Acremonium* infections (2, 4, 19). Clinical experience with other drugs, such as posaconazole (PSC), voriconazole (VRC) or the echinocandins, is very poor.
To evaluate possible therapeutic strategies, we tested the efficacy of PSC, VRC, anidulafungin (AFG) compared to AMB against *S. kiliense* in a recently developed murine model of disseminated infection (20).

**Material and Methods**

Three clinical strains of *Sarocladium kiliense*, UTHSC 01-2238, UTHSC 03-3197 and UTHSC 07-550, previously identified by sequencing of the internal transcribed spacer (ITS) region of the rRNA gene (19), were tested. The strains were stored in slant cultures covered with sterile paraffin oil and subcultured onto potato dextrose agar (PDA) plates at 25ºC for a good sporulation during 7-10 days. *In vitro* susceptibilities to AMB, PSC, VRC and AFG were tested by using a broth microdilution reference method (24).

For the *in vivo* study, the inocula were prepared flooding the surface of the agar plate with sterile saline solution, scraping the sporulating mycelium with a culture loop, and drawing up the resultant suspension with a sterile Pasteur pipette. The conidial suspensions were transferred to 100 ml of potato dextrose broth (PDB) and incubated at 25ºC for 7 days. Suspensions were filtered twice through several layers of sterile gauze to remove large clumps and hyphal fragments and then centrifuged at 3000 rpm for 20 minutes. The resulting pellets were suspended in sterile saline and the inocula adjusted to the desired concentration by haemocytometer counts and by serial plating onto PDA to confirm viability. Four-weeks-old OF-1 male mice (Charles River, Criffa SA, Barcelona, Spain) weighing 28-30 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
immunosuppressed 1 day prior to the infection by intraperitoneal (i.p.) administration of a single dose of 200 mg/kg of cyclophosphamide plus a single dose of 150 mg/kg of 5-fluorouracil given intravenously (i.v.) (25).

For the survival studies, five groups of 8 mice were established for each of the three strains tested, one for each treatment and one for control. Mice were challenged with $2 \times 10^8$ cfu in 0.2 ml of sterile saline into the lateral tail vein. Preliminary experiments, testing several strains, demonstrated that this concentration of fungal elements was the optimal dose for producing an acute infection, with all animals dying within 7 days (20). The different groups were treated as follows: AMB at 0.8 mg/kg/day i.v. (26) once daily, PSC at 20 mg/kg orally (p.o.) twice a day (BID) by gavage (27), VRC at 40 mg/kg p.o. once daily (28), and AFG at 10 mg/kg i.p. once daily (29). Drug level determinations were not performed. Mice that received VRC were given grapefruit juice instead of water 3 days before being infected (30). Animals were checked daily for 30 days. The same grouping and treatments were used for tissue burden but the animals were infected by administering $2 \times 10^6$ conidia i.v. in 0.2 ml of sterile saline. This inoculum was chosen in order to avoid the rapid killing obtained with the highest inoculum and to allow the fungal load of treated groups be compared with controls at same day(20). For $(1\rightarrow3)$-$\beta$-D-glucan serum level determinations the same animals tested in the tissue burden study were used. Fungal load and $(1\rightarrow3)$-$\beta$-D-glucan serum levels were determined at day 5, after 5 doses of antifungal in order to compare the results with the control group, since control mice start to die on that day. Additionally, a group of 3 uninfected mice was included as negative controls for the $(1\rightarrow3)$-$\beta$-D-glucan determination. Mice were anesthetized by sevoflurane inhalation (Sevorane; Abbott, Madrid, Spain).
Blood was collected by cardiac puncture and centrifuged at 3500 rpm and the serum obtained stored at -80°C until its use for determining (1→3)-β-D-glucan levels. Then, animals were euthanized and kidneys, liver, lung and spleen the most affected organ in *Acremonium* infection were aseptically removed (20). Approximately half of each organ was weighed, mechanically homogenized in 1 ml of sterile saline and serially 10-fold diluted. Dilutions were plated on PDA plates and incubated at 25°C for 7 days in order to determine the number of colony forming units (CFU) per gram. Serum levels of (1→3)-β-D-glucan were determined using the Fungitell kit (Associates of Cape Cod, East Falmouth, MA) following the manufacturer’s instructions. The other half of each organ was fixed with 10% buffered formalin, dehydrated, paraffin-embedded, sliced into 2 μm sections, stained with haematoxylin and eosin (H-E), periodic acid-Schiff and Grocott methamine silver. For the histopathological study, two distinct sections from every organ and treatment were examined qualitatively by evaluating the presence or absence of fungal cells and/or hyphae by light microscopy. In addition, the presence of tissue alterations, such as inflammation or necrosis was also considered. No quantitative studies were carried out.

Mean survival time (MST) was estimated using the Kaplan-Meier method and compared among groups with the Log Rank test. Differences in levels of (1→3)-β-D-glucan between treatments and strains were determined by Mann-Whitney U-test pairwise comparisons and the Dunnnett's method, considering control group as reference, was used to adjust their p-values. Organ burden data differences between treatments were detected by Mann-Whitney U-test pairwise comparisons and the Bonferroni method was used to correct p-values.
Data analysis was performed with GraphPad Prism 4 for Windows. P-values ≤ 0.05 were considered statistically significant.

Results

For the UTHSC 01-2238 strain the MICs of AMB, PSC, VRC and minimal effective concentration (MEC) of AFG were 8, 16, 2 and 8 µg/ml, respectively, for the UTHSC 03-3197 strain they were 4, 16, 0.5 and 4 µg/ml, respectively, and for UTHSC 07-550 strain were 2, >16, 2 and >16 µg/ml, respectively.

The results of the survival studies are shown in figure 1. All the controls died within 7 days after fungal inoculation. PSC and AFG were the only drugs able to significantly prolong survival for the three strains assayed (P<0.0012), with the exception of the strain UTHSC 01-2238. Although at the end of the experiment the survival never was over 40%. VRC increased the survival with respect to the control group only for one strain (UTHSC 03-3197) (P=0.0015). AMB did not show efficacy against any strain (P>0.150).

The spleen was the most affected organ and its fungal load was at least two log units higher than liver, lung and kidneys (Fig 2). In general the efficacy of the different therapies depended on the strain and on the organ tested, and even in the cases that tissue burden was reduced, it was only modest. PSC, with a few exceptions, was able to reduce fungal load in all organs of mice infected with each of the three fungal strains, and in some cases was more effective than the other drugs. PSC reduced tissue burden in 9 of the 12 groups evaluated (3 fungal isolates and 4 organs tested for each isolate), VRC in 7 of them, AMB also in 7, and AFG only in 4.
The concentration of (1→3) β-D glucan in the serum of non-infected mice was 39.28 ± 0.42 pg/ml in contrast to that obtained from infected control mice which ranged from 210.2 to 499.8 pg/ml. All treatments were able to significantly reduce the (1→3)-β-D-glucan serum concentrations in comparison with that for the control group. However, differences between drugs were not observed (Fig 3).

The histopathological studies of untreated controls showed a low and focal presence of fungal cells in all organs studied with exception of kidney, which showed glomerular invasion by hyphae with no signs of inflammatory response, necrosis, edema or angioinvasion. A decrease of fungal cells in kidney section was observed only in those mice treated with PSC.

Discussion

Until now only a few studies on the antifungal susceptibility of Acremonium have been done, all of them being in vitro (3, 4, 19). Our azoles MICs are different from those reported by Khan et al., using E-test (4) which were significantly lower, which could be explained by the different methods used in both works. To our knowledge, this is the first study that has explored the efficacy of antifungal drugs in the treatment of an experimental infection by S. kiliense and unfortunately the results can not be compared with other studies. In our study PSC displayed the best results, prolonging the survival of the mice infected by each of the three strains tested and reduced fungal load of most of the organs tested. Until now the clinical experience on the use of PSC in disseminated infections by Acremonium is scarce. However, that drug resolved a pulmonary infection by A. strictum in a leukemic patient after failure of AMB (31).
The variable outcomes of the reported *Acremonium* infections, have not allowed a conclusion about the most suitable treatment. Perhaps this variability may be explained by the different virulence or susceptibility to antifungal of the strains or even by the misidentification of the isolates when they are complex of species. However, in our study although all strains tested were correctly identified and apparently *S. kiliense* is not a complex of species, they show similar virulence and susceptibility to antifungal, some variability on the effectiveness of treatments was also observed.

AMB has been the drug most commonly used to treat *Acremonium* infection on the basis of its *in vitro* activity, with very variable results (14, 19, 21, 23, 32-36). While a disseminated infection by *Acremonium* sp. in a patient with Addison’s disease was successfully treated with that drug (36), failure has been reported mainly in cases of fungemia or disseminated infections (21, 23, 32, 33). Although in our study this drug was able to reduce fungal load in some organs it was ineffective in prolonging survival for any of the strains tested, which suggest that the use of this drug in human infection should be taken with caution. Clinical experience with VRC is more limited; however, some cases of successful treatment with that drug have been reported although the MICs of the *Acremonium* strains were high (21, 23, 37, 38). Unfortunately in almost all the cases the *Acremonium* isolates were not identified at species level (38). Similarly to AMB, VRC showed discouraging efficacy in mice survival prolonging in our study.

Although only few isolates were tested, there was a clear lack of correlation between the *in vitro* and *in vivo* results observed in our study. AMB and VRC showed the lowest MICs, however their efficacy was poor, and PSC which
showed MICs ≥16µg/ml, indicative of resistance, demonstrated the highest efficacy.

The relatively poor drug efficacy shown here is, to some extent, similar to that shown with other morphologically similar fungi causing hyalohyphomycosis such as *Fusarium*, *Paecilomyces* or *Scedosporium*, which are refractory to most of antifungal agents (39). All these fungi share the common characteristic of sporulating in the infected tissue, which is a rare feature in pathogenic moulds (40). Perhaps, this ability to sporulate facilitates a quick dissemination to very distant organs limiting the effectiveness of the antifungal drugs. Nevertheless, we did not observe this feature in our histologic sections, most likely because the organs were removed too soon after infection.

Different biomarkers have been tested to help in the diagnosis of fungal infections (41). Detection of (1→3)-β-D-glucan has been useful for the diagnosis and therapeutic monitoring in experimental and clinical studies on invasive fungal infections (42-46). In our studies, the high serum levels of that marker in control mice were similar to those observed previously with *Acremonium* (4, 20), but also in experimental invasive fungal infections by *Fusarium* and *Aspergillus* (44-46). Although the animal models used in those studies were different than our models, since the infection was by conidial inhalation their results also showed a good correlation between the (1→3)-β-D-glucan levels, the progression of infection and the response to antifungal therapies (46). Our (1→3)-β-D-glucan levels from mice treated with different drugs showed that the treatments were able to significantly reduce these levels with respect to the control group; however no differences were noted between the different antifungal agents. Although it must be taken into account that (1→3)-β-D-glucan
is a panfungal marker (41) and the cutoff value is not determined for animal
models, our results suggest the potential use of this marker for the prompt
detection of infection and for monitoring treatment efficacy.

In conclusion, the results of this work suggest that further studies are needed to
explore the potential efficacy of PSC in the treatment of refractory human
infections incited by *S. kiliense*.

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Reference


compared with galactomannan and (1,3)-\(\beta\)-D-glucan detection. J Clin Microbiol. 50: 602-608.
Fig 1. Cumulative mortality of mice infected with $2 \times 10^8$ conidia/animal of three strains of *Sarocladium (Acremonium) kiliense*, UTHSC 01-2238 (A), UTHSC 03-3197 (B) or UTHSC 07-550 (C). $^a P < 0.05$ versus control; $^b P < 0.05$ versus amphotericin B (AMB); $^c P < 0.05$ versus voriconazole (VRC).

Fig 2. Effects of the antifungal treatments on colony counts in mice infected with $2 \times 10^6$ conidia/animal of three strains of *Sarocladium kiliense*, UTHSC 01-2238, UTHSC 03-3197 or UTHSC 07-550, in spleen, liver, lung and kidneys of mice. Amphotericin B (AMB) at 0.8 mg/kg; posaconazole (PSC) at 20 mg/kg twice a day; voriconazole (VRC) at 40 mg/kg; anidulafungin (AFG) at 10 mg/kg. $^a P$ value $< 0.05$ versus the control; $^b P$ value $< 0.05$ versus AFG; $^c P$ value $< 0.05$ versus AMB; $^d P$ value $< 0.05$ versus VRC. Horizontal lines indicate mean values.

Fig 3. (1→3)-β-D-glucan serum levels of mice infected with $2 \times 10^6$ conidia/animal of the strains of *Sarocladium kiliense*, UTHSC 01-2238, UTHSC 03-3197 or UTHSC 07-550, on day 5 after challenged. Amphotericin B (AMB) at 0.8 mg/kg; posaconazole (PSC) at 20 mg/kg twice a day; voriconazole (VRC) at 40 mg/kg; anidulafungin (AFG) at 10 mg/kg. $^* P$ value $< 0.05$ versus the control. Discontinuous lines indicates values cutoff $\geq 80$ pg/ml.
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Fig. 3. (1→3)-β-D-glucan serum levels of mice infected with 2x10⁶ conidia/animal of the strain of <i>Saccharomyces cerevisiae</i>, UTHSC 01-2238, UTHSC 03-3197 or UTHSC 07-550, on day 5 after challenged. Amphotericin B (AMB) at 0.8 mg/kg, posaconazole (PSC) at 20 mg/kg, voriconazole (VRC) at 40 mg/kg, and itraconazole (IRC) at 10 mg/kg. *P value < 0.05 versus the control. Discontinuous lines indicates values cutoff ≥ 80 pg/ml.