Evaluation of the Efficacies of Amphotericin B, Posaconazole, Voriconazole, and Anidulafungin in a Murine Disseminated Infection by the Emerging Opportunistic Fungus *Sarocladium (Acremonium) kiliense*

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We evaluated and compared the efficacies 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 therapy administered was as follows: posaconazole at 20 mg/kg of body weight (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 (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 were never 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.

Acremonium kiliense is a saprobic fungus with a worldwide distribution which has been reported in recent 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 drug most commonly used to treat severe fungal infections caused by opportunistic molds (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), and the echinocandins, is very poor.

To evaluate possible therapeutic strategies, we tested the efficacies of PSC, VRC, and anidulafungin (AFG) compared to that of AMB against *S. kiliense* in a recently developed murine model of disseminated infection (20).

MATERIALS 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 par-

affin oil and subcultured onto potato dextrose agar (PDA) plates at 25°C for a good sporulation during 7 to 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 by 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 25°C for 7 days. Suspensions were filtered twice through several layers of sterile gauze to remove large clumps and hyphal fragments and then centrifuged at 3,000 rpm for 20 min. The resulting pellets were suspended in sterile saline solution and the inocula adjusted to the desired concentration by hemocytometer counts and by serial plating onto PDA to confirm viability. Four-week-old OF-1 male mice (Charles River, Criffla SA, Barcelona, Spain) weighing 28 to 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 of cyclophosphamide/kg of body weight plus a single dose of 150 mg of 5-fluorouracil/kg given intravenously (i.v.) (25).

For the survival studies, five groups of 8 mice, three for each of the three strains tested, one for each treatment, and one for a control, were established. Mice were challenged with 2×10^8 CFU in 0.2 ml of sterile saline solution 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

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dying within 7 days (20). The different groups were treated as follows: AMB at 0.8 mg/kg/day administered intravenously (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 groupings and treatments were used for tissue burden assays, but the animals were infected by administering 2×10^6 conidia i.v. in 0.2 ml of sterile saline solution. 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 to be compared with that of the controls on the same day (20). For (1 $\rightarrow$ 3)- β -D-glucan serum level determinations, the same animals as those tested in the tissue burden study were used. Fungal loads and (1 $\rightarrow$ 3)- β -D-glucan serum levels were determined at day 5, after 5 doses of antifungal, in order to compare the results with the control group results, since control mice started to die on that day. Additionally, a group of 3 uninfected mice were included as negative controls for the (1 $\rightarrow$ 3)- β -D-glucan determination. Mice were anesthetized by inhalation of sevoflurane (Sevorane; Abbott, Madrid, Spain). Blood was collected by cardiac puncture and centrifuged at 3,500 rpm and the serum obtained stored at -80°C until its use for determining (1 $\rightarrow$ 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 solution, 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 CFU per gram. Serum levels of (1 $\rightarrow$ 3)- β -D-glucan were determined using a 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 -thick sections, and stained with hematoxylin 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 $\rightarrow$ 3)- β -D-glucan between treatments and strains were determined by Mann-Whitney U-test pairwise comparisons, and Dunnett's method, with the control group as the reference, was used to adjust their *P* values. Differences in organ burden data 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, and VRC and the minimal effective concentration (MEC) of AFG were 8, 16, 2, and 8 $\mu\text{g}/\text{ml}$, respectively; for the UTHSC 03-3197 strain, they were 4, 16, 0.5, and 4 $\mu\text{g}/\text{ml}$, respectively; and for the UTHSC 07-550 strain, they were 2, >16 , 2, and >16 $\mu\text{g}/\text{ml}$, respectively.

The results of the survival studies are shown in Fig. 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 strain UTHSC 01-2238, although at the end of the experiment the survival rate was never over 40%. VRC increased the survival with respect to the control group for only one strain (UTHSC 03-3197) ($P = 0.0015$). AMB did not show efficacy against any strain ($P > 0.150$).

FIG 1 Cumulative mortality of mice infected with 2×10^8 conidia/animal of *Sarocladium* (*Acremonium*) *kiliense* strain UTHSC 01-2238 (A), UTHSC 03-3197 (B), or UTHSC 07-550 (C). A superscript "a" indicates $P < 0.05$ versus control; a superscript "b" indicates $P < 0.05$ versus amphotericin B (AMB); a superscript "c" indicates $P < 0.05$ versus voriconazole (VRC).

The spleen was the most affected organ, and its fungal load was at least 2 log units higher than the liver, lung, and kidney fungal loads (Fig. 2). In general, the efficacy of the different therapies depended on the strain and on the organ tested, and even in the cases in which the tissue burden was reduced, it was only modest. PSC, with a few exceptions, was able to reduce the 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 the 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 in only 4.

The concentration of (1 $\rightarrow$ 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 $\rightarrow$ 3)-

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

FIG 3 (1→3)-β-D-Glucan serum levels of mice infected with 2×10^6 conidia/animal of *Sarocladium kiliense* strain UTHSC 01-2238, UTHSC 03-3197, or UTHSC 07-550 on day 5 after challenge. Amphotericin B (AMB) was administered 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. *, $P < 0.05$ versus the control. Discontinuous lines indicate cutoff values ≥ 80 pg/ml.

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 the exception of kidney, which showed glomerular invasion by hyphae with no signs of inflammatory response, necrosis, edema, or angioinvasion. A decrease of fungal cell levels in kidney sections 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, with all of them having been performed under *in vitro* conditions (3, 4, 19). The MICs of our azoles are different from those reported by Khan et al., using Etest (4), which were significantly lower, which could be explained by the different methods used in the two studies. 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 the results unfortunately cannot be compared with those of 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 the fungal load of most of the organs tested. Until now, the clinical experience on the use of PSC in disseminated infections by *Acremonium* has been scarce. However, that drug resolved a pulmonary infection by *A. strictum* in a leukemic patient after failure of AMB therapy (31).

The variable outcomes of the reported *Acremonium* infections have not allowed a conclusion to be reached about the most suitable treatment. Perhaps this variability may be explained by the different levels of virulence or susceptibility to antifungals of the strains or even by the misidentification of the isolates when they represent a complex of species. In our study, however, although all strains tested were correctly identified and apparently *S. kiliense* is not a complex of species, they showed similar levels of virulence and susceptibility to antifungals, and some variability with respect to the effectiveness of treatments was also observed.

AMB has been the drug most commonly used to treat *Acremo-*

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

Although only a 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 ≥ 16 $\mu\text{g/ml}$, 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 antifungal agents (39). All these fungi share the common characteristic of sporulating in the infected tissue, which is a rare feature in pathogenic molds (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 $\rightarrow$ 3)- β -D-glucan has been useful for 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 were also similar to those observed previously with experimental invasive fungal infections by *Fusarium* and *Aspergillus* (44–46). Although the animal models used in those studies were different from our models, since the infection was by conidial inhalation, their results also showed a good correlation between the (1 $\rightarrow$ 3)- β -D-glucan levels, the progression of infection, and the response to antifungal therapies (46). Our (1 $\rightarrow$ 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 $\rightarrow$ 3)- β -D-glucan is a panfungal marker (41) and that the cutoff value has not been 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 by *S. kiliense*.

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