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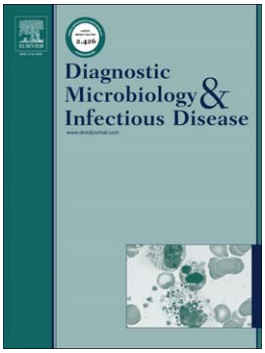

Experimental treatment of *Curvularia* infection

Katihuska Paredes, Javier Capilla, Deanna A. Sutton, Emilio Mayayo, Annette W. Fothergill, Josep Guarro

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Title: Experimental treatment of *Curvularia* infection.

Running title: Antifungal therapies against *Curvularia* infection.

Katihuska Paredes[1], Javier Capilla[1], Deanna A. Sutton[3], Emilio Mayayo[2], Annette W. Fothergill[3], and Josep Guarro[1#].

Unitat de Microbiologia[1] and Unitat d' Anatomia Patològica[2], Facultat de Medicina i Ciències de la Salut, IISPV, Universitat Rovira i Virgili, Reus, Tarragona, Spain.

Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas, USA[3].

[#] Corresponding author. Mailing address: Unitat de Microbiologia, Facultat de Medicina, Universitat Rovira i Virgili. Carrer Sant Llorenç, 21.43201 Reus, Spain. Phone 0034-977-759350. Fax: 0034-977-759322. E-mail: josep.guarro@urv.cat

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Abstract

We have evaluated the efficacy of amphotericin B, posaconazole, and voriconazole in immunosuppressed murine models of disseminated infection by *Curvularia spicifera* and *Curvularia hawaiiensis*. The three antifungals improved survival of mice in comparison to controls; however, only the two azoles were able to reduce significantly the fungal load.

Keywords: *Curvularia, Bipolaris hawaiiensis, Bipolaris spicifera*, animal model, fungal infection

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Introduction

Curvularia is one of the most relevant genera of dematiaceous fungi involved in phaeohyphomycosis. Despite allergic fungal sinusitis being the most common clinical manifestation attributed to this genus (Alvarez et al., 2011; Fryen et al., 1999), a wide variety of infections (Bashir et al., 2009; Bilu et al., 2004; Castelnuovo et al., 2004; Viola and Sutton, 2010), including disseminated disease in both immunocompromised and immunocompetent patients (Kobayashi et al., 2008; Revankar et al., 2002), have been linked to *Curvularia*. Based on molecular studies, the taxonomy of *Curvularia* recently underwent important changes and some species formerly included in the genus *Bipolaris* i.e., *B. spicifera* and *B. hawaiiensis*, have been re-accommodated into *Curvularia* (Manamgoda et al., 2012). Although infections by this genus are still rare, they have increased in recent years (da Cunha et al., 2012; El Khizzi et al., 2010).

Due to the scarcity of clinical cases, the treatment of choice for these infections has not yet been established. *In vitro* studies have shown variable activity of amphotericin B (AMB), although the newest azoles, posaconazole (PSC) and voriconazole (VRC) have shown good activity (da Cunha et al., 2012; Revankar and Sutton, 2010). Since there is no experimental *in vivo* data on the efficacy of the available antifungal drugs, the aim of this study was to evaluate the therapeutic efficacy of AMB, PSC and VRC using murine models of disseminated infection caused by the two species of this genus that are most commonly involved in human infection, *C. spicifera* and *C. hawaiiensis*.

Material and methods

Fungal isolates. One strain of *C. spicifera* (UTHSC 09-3102, isolated from maxillary sinus) and one of *C. hawaiiensis* (UTHSC 07-3226, from lung) were included in the study. Both strains were identified by comparing their internal transcribed spacer (ITS) sequences with those of reference strains. The *in vitro* antifungal susceptibility tests were carried out following the CLSI guidelines (CLSI, 2008). The MICs of AMB, PSC and VRC for *C. spicifera* were 0.25 µg/ml, 0.25 µg/ml and 1 µg/ml; and for *C. hawaiiensis* were 0.125 µg/ml, 0.125 µg/ml and 1 µg/ml, respectively.

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Inoculum. Strains were grown on oatmeal agar (OA; 30 g of filtered oat flakes, 20 g of agar, 1 liter of distilled water) for 12 days at room temperature. On the day of infection, cultures were flooded in sterile saline and filtered through sterile gauze to remove clumps of cells and hyphae. The suspensions were adjusted to $5x10^4$ colony forming units (CFU) per ml by haemocytometer count and the viability of the inoculum was determined by serial plating onto Dichloran Rose Bengala Chloramphenicol (DRBC) agar.

Infection. Male OF-1 mice (Charles River; Criffa SA, Barcelona, Spain) with a mean weight of 30 g were used in the experiment. Mice were housed in standard boxes with food and water *ad libitum*. All animal procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee. Animals were immunosuppressed 1 day prior to the infection by a single intraperitoneal (i.p.) injection of 200 mg/kg of body weight of cyclophosphamide (Genoxal; Laboratorios Funk SA, Barcelona, Spain) plus 5-fluorouracil (Fluorouracilo; Ferrer Farma SA, Barcelona, Spain) at 150 mg/kg given intravenously (i.v) (Ortoneda et al., 2004). Mice were challenged i.v., with $1x10^4$ CFU per animal in a volume of 0.2 ml into the lateral tail vein. Previous studies have shown that this inoculum is able to produce an acute infection, with all animals dying within 10 days (Paredes et al., 2013).

Treatments. Treatments were selected based on previous studies. AMB (amphotericin B deoxycholate, Xalabarder Pharmacy, Barcelona, Spain) was administered i.v., at doses of 0.8 mg/kg once a day (QD); PSC (Noxafil; Schering-Plough Ltd., Hertfordshire, United Kingdom) at 20 mg/kg given orally (p.o) by gavage, twice daily (BD) or VRC (Vfend; Pfizer S.A., Madrid, Spain) at 25 mg/kg p.o., by gavage QD. Mice treated with VRC received from 3 days before infection, grapefruit juice instead of water (Sugar and Liu, 2000). These doses allowed serum concentrations above the respective MICs (Calvo et al., 2010b; Warn et al., 2006; Wiederhold et al., 2006). All therapies began 1 day after challenge and lasted for 7 days. Control animals received no treatment. To prevent bacterial infections, all animals received ceftazidime at 5 mg/kg day subcutaneously. The efficacy of the treatments was evaluated by prolongation of survival and reduction of fungal burden and histopathology of lungs, due that it is the most affected organ in these type of infection (Paredes et al., 2013). For survival studies, groups of 8

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mice were randomly established for each strain and treatment. Mice were checked twice a day and mortality was recorded for 15 days. For tissue burden and histopathological studies, groups of 8 mice were established. In order to compare results, on day 5 post infection, which coincides with when untreated controls begin to die, mice of both treated and untreated groups were euthanized by CO_2 anoxia. Lungs were aseptically removed, and approximately half of each one was weighed and homogenized in 2 ml of sterile saline. Dilutions of the homogenates were then plated onto DRBC agar and incubated for 3 days at room temperature to determine CFU per gram of tissue. The remaining half of each organ was fixed with 10% buffered formalin and then dehydrated, paraffin embedded, and sliced into 2 µm sections, which were then stained with hematoxylin-eosin (H-E), periodic acid-Schiff (PAS) stain and Grocott methamine silver (GMS) for examination by light microscopy.

Statistical analysis. The mean survival times (MST) were estimated by the Kaplan-Meier method and compared among groups by using the log rank test. In tissue burden studies, colony counts were $\log_{10}$-transformed and compared by the two-tailed Mann–Whitney U-test, using Graph Pad Prism 5 for Windows. *P* values $\leq$ 0.05 were considered statistically significant.

Results and Discussion

Irrespective of the infecting strain, all treatments significantly prolonged survival compared with the controls ($P \leq 0.02$) (Fig. 1). Animals receiving PSC or VRC succumbed to the infection within 10 to 12 days after infection; however, those treated with AMB at 0.8 mg/kg showed 62.5% and 30% of survival after 15 days of infection with *C. spicifera* or *C. hawaiiensis*, respectively. Although AMB increased slightly the survival over the other therapies, it was only significant in comparison to those VRC-treated animals infected with *C. spicifera*. For both species, AMB was unable to reduce the fungal load in lungs after infection by either species (mean Log_{10} CFU/g ± SD; 4.22 ± 0.13 and 4.18 ± 0.19 for *C. hawaiiensis* and *C. spicifera*, respectively) with respect to their controls (4.28 ± 0.26 and 4.15 ± 0.18, respectively). However, PSC and VRC did reduce the fungal load in that organ in *C. hawaiiensis* (3.176 ± 0.49 and 3.805 ± 0.18, respectively) and in C*. spicifera* (3.79 ± 0.2 and 3.808 ± 0.34, respectively). PSC was more effective than VRC against *C. hawaiiensis* ($P < 0.03$) (Fig. 2).

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The histological study confirmed invasion of lungs by abundant fungal elements. Sections of this organ from controls and treated mice showed similar histological alteration, with a presence of multinucleated giant cells and histiocytes surrounding septate dematiaceous hyphae, without an inflammatory response or signs of necrosis (data not shown).

Disseminated phaeohyphomicosis is infrequent in the clinical setting; however, these fungi can sometimes cause severe and fatal infections including important outbreaks like the recent USA multi-state outbreak by contaminated methylprednisolone injections with already 700 cases of meningitis or other central nervous system infections, mostly related to the dematiaceous fungus *Exserohillum rostratum*, morphologically similar to *Curvularia*. The initial recommendations by the Centers for Disease Control and Prevention for overcoming the crisis, include VRC and liposomal amphotericin B; however, 64 deaths have been reported so far (Stevens, 2013; CDC, 2014). Recently, an outbreak of fungal endophthalmitis due *C. hawaiiensis* was reported in association to triamcinolone administration (Mikosz et al., 2014).

Exserohillum and *Curvularia* produce infections that are difficult to treat principally because of the lack of clinical experience. Moreover, the *in vitro* data is scarce and often reported before to the current standardized methodology of susceptibility testing. In general, the drugs tested showed high MICs, although a recent study showed that azoles, with the exception of fluconazole, AMB and echinocandins have good activity against most *Curvularia* spp, (da Cunha et al., 2012), but clinical breakpoints have not been established for these species. In a retrospective review of 72 cases of disseminated phaeohyphomycosis, *Curvularia* and *Bipolaris* were the most common genera (15.2%) after *Scedosporium prolificans*, causing disseminated infections in immunocompetent and immunocompromised patients with a mortality rate of 72.7% despite aggressive antifungal therapy (Revankar et al., 2002). There are no standard therapies associated with favourable patient's outcome against disseminated *Curvularia* infections. In the absence of relevant clinical data on the management of these infections, animal models can play an important role in the assessment of the predictive value of the *in vitro* data (Guarro, 2011). No therapy has been experimentally tested previously against *Curvularia* infection. In the present study, despite the two strains tested showing low MICs, no remarkable antifungal efficacy was observed. Although the treatments were able to prolong mice survival for both species, mice treated with azoles died within 12 days. The better survival

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of AMB compared to azoles could be explained due the post antifungal effect of AMB versus the fungistatic effect described by azoles (Andes, 2006). By contrast, fungal burden was reduced modestly only by the two azoles. While some studies have used quantitative PCR techniques to determine fungal load in organs, we have preferred culture-based CFU determination, a simpler and cheaper method (Clemons & Stevens, 2009), that has demonstrated a similar efficacy than quantitative PCR for assessing antifungal drug efficacy (Singh et al., 2005).

It is likely that lower counts could be obtained with more days of treatment but due to the early death of the untreated animals and in order to compare fungal loads between them and the treated ones at same day post infection, the latest only received 5 doses of treatment. This could constitute a limitation of the study; however, previous pharmacokinetics studies demonstrated that the administration of PSC after 6 days of treatment (Calvo et al, 2010b) and VRC after a single dose (Andes et al, 2003), the mean of drug levels in serum were 9.72 $\pm$ 0.57 mg/L and 1.67 $\pm$ 0.69 mg/L, respectively.

Previous experimental studies of disseminated infections produced by other dematiaceous fungi such as *Fonsecaea*, *Exophiala* and *Wangiella* species (Calvo et al., 2010a, 2010b; Graybill et al., 2004; Rivard et al., 2007), have demonstrate the usefulness of PSC for the treatment of infections by pigmented fungi (Guarro, 2011). In clinical practice, infections by *Curvularia*/*Bipolaris* are rare and AMB has been the standard therapy. Unfortunately, the treatment often fails and its associated toxicity limits its use (Revankar and Sutton, 2010). Concerning the new triazoles, a few cases have been reported on the efficacy of itraconazole (Flanagan and Bryceson, 1997; Vásquez-del-Mercado et al., 2013) and VRC (Kobayashi et al., 2008; Rosow et al., 2011; Skovrlj et al., 2013) but not on the efficacy of PSC for treating *Curvularia* infections. In conclusion, all three antifungals tested prolonged mice survival, although AMB did it only over the two azoles. Only the azoles reduced fungal burden. Overall, no compound showed the desired effect in both survival and fungal burden. Further studies testing other therapeutic alternatives are needed aimed at reducing the mortality associated to systemic infections by *Curvularia*.

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Figure legends

Fig. 1 Cumulative mortality of immunosuppressed mice infected with $1x10^4$ CFU of *C. spicifera* UTHSC 09 -3102 (A) and *C. hawaiiensis* UTHSC 07-3226 (B). AMB 0.8, amphotericin B at 0.8 mg/kg QD; PSC 40, posaconazole at 20 mg/kg BD; VRC 25, voriconazole at 25 mg/kg QD. [a] $P < 0.05$ versus control, [b] $P < 0.05$ versus VRC 25.

Fig. 2 Effect of antifungal treatments on colony counts of *C. spicifera* UTHSC 09-3102 and *C. hawaiiensis* UTHSC 07-3226 in lungs of immunosuppressed mice. AMB 0.8, amphotericin B at 0.8 mg/kg QD; PSC 40, posaconazole at 20 mg/kg BD; VRC 25, voriconazole at 25 mg/kg QD. Horizontal lines indicate median values. [a] $P < 0.05$ versus control; [b] $P < 0.05$ versus AMB 0.8; [c] $P<0.05$ versus VRC.

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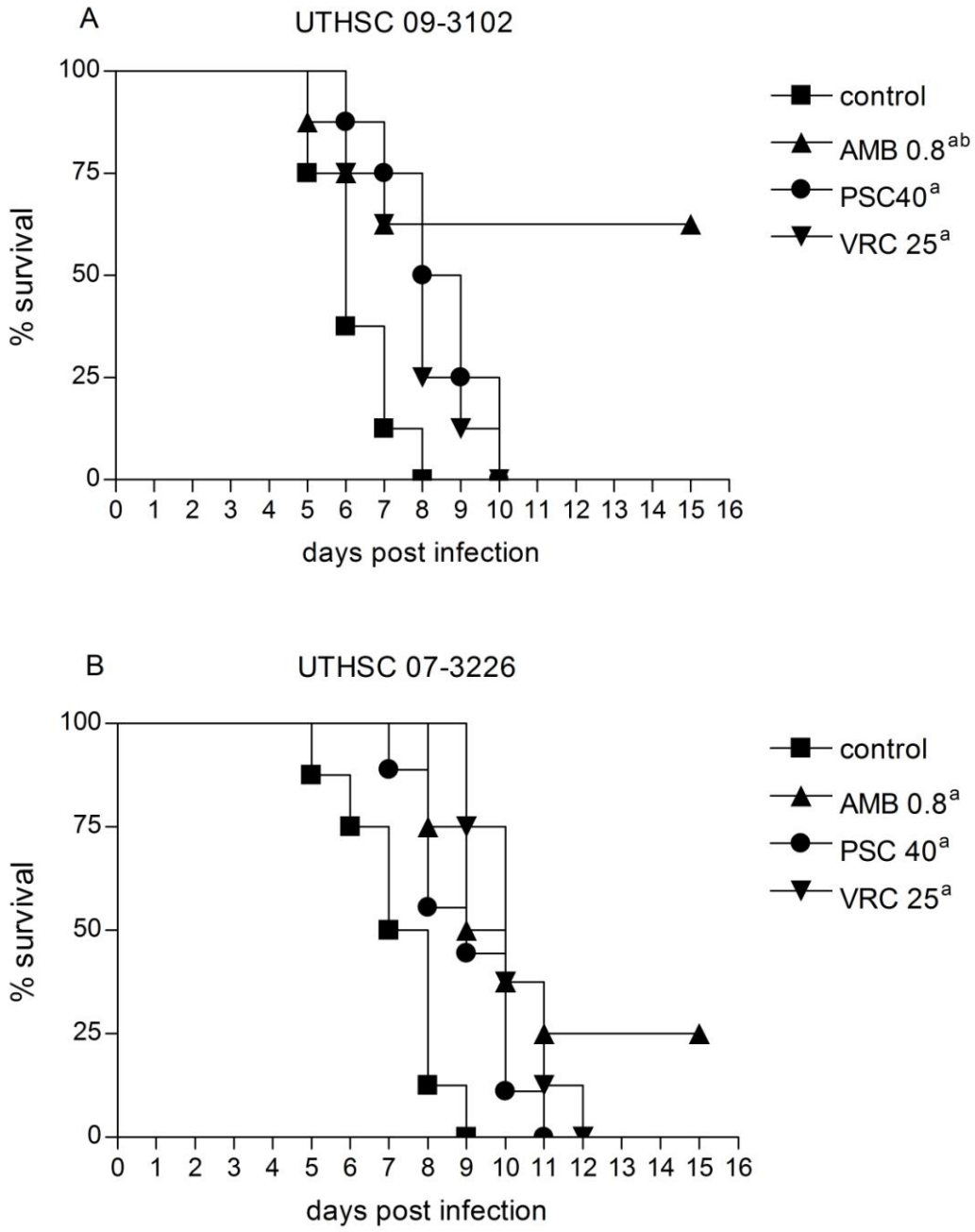

Figure 1

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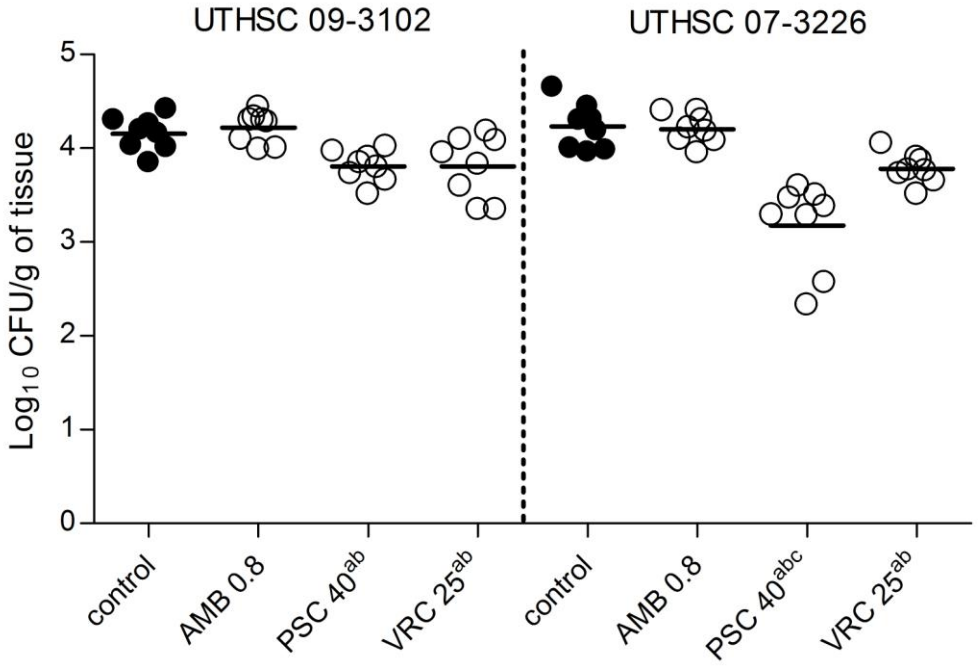

Figure 2

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Highlights

- We examined the efficacy of amphotericin B, posaconazole, and voriconazole in immunosuppressed murine models of disseminated infection by *Curvularia spicifera* and *Curvularia hawaiiensis*.
- Amphotericin B showed better survival in both species studied.
- Voriconazole and posaconazole were more effective reducing fungal load.

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