Virulence and resistance to antifungal therapies of *Scopulariopsis* species.

Katihuska Paredes[a], Javier Capilla[a#], Emilio Mayayo[b] and Josep Guarro[a]

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

Runnig title: Virulence and antifungal resistance of *Scopulariopsis*

[#] Address correspondence to Javier Capilla, javier.capilla@urv.cat

Abstract:

Scopulariopsis is an emerging opportunistic fungus characterized by its high resistance to antifungal therapies. We have developed a murine model of disseminated infection in immunosuppressed animals, by intravenous inoculation of *S. brevicaulis* and *S. brumptii*, the most clinically relevant species, in order to evaluate their virulence and their response to conventional antifungal treatments. Survival and tissue burden studies showed that *S. brumptii* was more virulent than *S. brevicaulis*. The three drugs tested, liposomal amphotericin B, posaconazole and voriconazole, prolonged the survival of mice infected with *S. brumptii*, but none showed efficacy against *S. brevicaulis*. The different therapies were only able to modestly reduce the fungal burden of infected tissue, although in general, in spite of the high serum levels reached, they showed poor efficacy in the treatment of the infection. Unfortunately, the most effective therapy for *Scopulariopsis* infections remains unresolved.

Keywords: animal model, fungal infection, *Scopulariopsis,* antifungal therapy.

Introduction

The fungal genus *Scopulariopsis* of the ascomycetes includes hyaline and melanized species that are soil saprobic and show a wide geographical distribution. They are commonly isolated from air, wood, decaying organic matter, manure and animal remains (1) but are occasionally involved in human infections. They are mainly related to onychomycosis (2, 3), keratitis (4), otomycosis (5) and cutaneous infections (6) although disseminated infections have also been linked to high mortality rates in immunosuppressed (7, 8, 9) and more rarely in immunocompetent patients (10, 11). The most common species involved in human infections are *Scopulariopsis brevicaulis*, *Scopulariopsis gracilis*, *Scopulariopsis brumptii* (currently renamed as *Microascus paisii* (12)) *Scopulariopsis candida* and their relatives *Microascus cirrosus* and *Microascus cinereus* (7, 13). There is very little clinical and experimental data available on the management of infections by *Scopulariopsis*. In vitro susceptibility studies have shown high rates of resistance of these fungi to practically all current antifungal agents (14, 15, 16), which makes it difficult to treat such infections successfully. Surgery combined with antifungal therapy has been recommended for the treatment of infections by *Scopulariopsis* species, although no particular antifungal agent is mentioned (17). The aim of the present study was to develop murine models of invasive infections by two clinically relevant species in order to evaluate the efficacy of liposomal amphotericin B (LAMB), voriconazole (VRC) and posaconazole (PSC), since they are the most commonly used drugs against infections by filamentous fungi (17, 18).

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Materials and methods

Fungal isolates and inocula preparation

Two strains of *S. brevicaulis* (FMR 12216 from synovial fluid, FMR 12246 from aorta tissue) and two of *S. brumptii* (FMR 12240 from bronchoalveolar lavage and FMR 12229 from sputum), were included in the study. The isolates were identified by sequencing the D1/D2 domains of the 28S rRNA gene and a fragment of the elongation factor 1-α gene (*EF1-α*) (13) and comparing the sequences with those of the type strains. The in vitro antifungal susceptibility tests were carried out following the CLSI guidelines (19). Fungi were grown on potato-carrot agar (PCA; 20 g of filtered potatoes and carrots plus 20 g of agar in 1 L of distilled water) for 5 days at 25°C. On the day of infection, cultures were flooded with sterile saline and filtered through sterile gauze to remove clumps of conidia and hyphae. The resulting suspensions were adjusted by haemocytometer counts and to confirm viability 10-fold dilutions were cultured on PCA.

Animals

Male OF-1 mice (Charles River; Criffa SA, Barcelona), with a mean weight of 30 g were used in the assays. Mice were housed in standard conditions with access to food and water ad libitum. Mice were rendered neutropenic by an intraperitoneal (i.p.) injection of 200 mg/kg of cyclophosphamide (Genoxal; Laboratorios Funk SA, Barcelona, Spain) plus 150 mg/kg of 5-fluorouracil (Fluorouracilo; Ferrer Farma SA, Barcelona, Spain) given intravenously (i.v.) one day prior to the infection. This immunosuppression has demonstrated peripheral blood polymophonuclear

leukocytes (PMNs) counts < 100 PMNs/mL (20). All animal procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee.

Virulence study

The virulence of *Scopulariopsis* spp., was evaluated against both immunocompetent and immunosuppressed mice.

The strain *S. brevicaulis* FMR 12246 was selected randomly to evaluate virulence in immunocompetent mice. Two groups of 8 mice were challenged with $1x10^5$ or $1x10^7$ CFU/animal, respectively. Inocula were administered i.v. in 200 µL of sterile saline via the lateral tail vein.

In the immunosuppressed mice model, one day prior to infection, animals were rendered neutropenic as explained above. We tested the two strains of *S. brevicaulis* and the two of *S. brumptii* mentioned previously, challenged with $1x10^5$, $1x10^6$ or $1x10^7$ CFU/animal. Additionally, the strain FMR 12246 of *S. brevicaulis* was challenged with $5x10^5$ CFU/animal. Experimental groups consisted of 16 mice per inoculum, 8 for survival studies and 8 for fungal load and histopathology. Animals were checked twice daily for 15 days post infection. In order to compare results those included in the tissue burden study were euthanased on day 6 post infection when controls began to die.

Tissue burden and histopathology studies

After euthanasia, kidneys, lungs, spleen, liver and brain of mice were aseptically removed and approximately half of each organ was weighed and mechanically homogenized in 2 mL of sterile saline. Serial 10-fold dilutions of the homogenates were placed onto PCA, and incubated for 3 days at 25ºC for CFU/g calculation. For

the histopathology study, the remaining portion of each organ was fixed with 10% buffered formalin, dehydrated, paraffin embedded, and sliced into 2 µm sections, which were stained with hematoxylin-eosin (H-E), periodic acid-Schiff (PAS) stain and Grocott methamine silver (GMS) for examination by light microscopy.

Treatments

Due to the low mortality rate observed in immunocompetent mice, the efficacy of the antifungal treatments was evaluated only in immunosuppressed animals inoculated with $5x10^5$ CFU of *S. brevicaulis* FMR 12246 and $1x10^6$ CFU of *S. brumptii* FMR 12240, which produced an acute infection with all mice dying within 15 days. The treatments evaluated were LAMB (Gilead Sciences S.A., Madrid, Spain) given at 10 mg/kg i.v., 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 60 mg/kg p.o, by gavage QD. These doses allowed serum drug concentrations to be higher than the respective minimal inhibitory concentrations (MICs) (21, 22). From 3 days before infection, mice receiving VRC were given grapefruit juice instead of water (23). All treatments began 1 day after challenge and lasted for 5 days. To prevent bacterial infections, mice received 5 mg/kg/day of ceftazidime (Ceftazidima; Normon, Madrid, Spain) subcutaneously. Animals from the survival study were checked twice a day for 15 days after challenge while animals from the fungal load study were euthanased at day 6 post infection, 4 hours after the last dosing.

Bioassay

Mice from the fungal burden group were anaesthetized by inhalation of sevoflurane (Sevorane; AbbVie, Madrid, Spain) and 1 mL of blood was obtained by cardiac

puncture. Blood was centrifuged and the obtained serum was used to determine concentrations of LAMB, PSC and VRC by diffusion assay following previous studies (24, 25). In brief, 22 mL of Yeast Nitrogen Base (YNB; 6.9 g/L nitrogen yeast base, 10 g/L peptone dextrose, 5 g dextrose and 15 g/L of agar) and an inoculum of *Candida parapsilosis* ATCC 22019 at $2x10^6$ CFU/mL were mixed at 45-50° C and poured into a sterile Petri dish. The plates were allowed to solidify at room temperature. Then, wells of 4 mm of diameter were perforated with a sterile borer. Thirty microliters of each standard of LAMB, PSC, VRC and serum samples were dispensed in the wells. Plates were incubated at 35 ºC for 24 h. The diameter of growth inhibition was measured and used to determine drug concentration in samples by linear regression analysis. Tests were carried out in duplicate.

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 6 for Windows. *P* values $\leq$ 0.05 were considered statistically significant.

Results

Virulence study

S. brevicaulis FMR 12246 showed a reduced virulence in immunocompetent animals with 100% and 80% of survival after infection with $1x10^5$ and $1x10^7$ CFU/animal, respectively (data not shown). Due to this low mortality rate and as

mentioned above, only immunosuppressed animals were used in subsequent experiments.

In immunosuppressed mice, the lowest inoculum tested, i.e., $1x10^5$ CFU/animal, caused the death of 62.5% of animals infected with any the two strains of *S. brevicaulis*, and the 87.5% and 100% of mortality in mice infected with the strains FMR 12240 and FMR 12229 of *S. brumptii*, respectively, while the highest inoculum caused the death of all the animals within 15 days after challenge (Fig. 1).

Table 1 shows the results of the tissue burden studies. Mice infected with any strain showed detectable fungal load on day 6 post-infection in the five organs tested. Quantitative cultures correlated with the size of the inoculum tested, the animals infected with the highest inoculum showed the highest CFU/g. The most affected organs after infection by *S. brevicaulis* were lung and spleen while *S. brumptii* affected majorly spleen and liver. Histological findings, at day 6 post-infection, showed a clear fungal invasion with presence of hyphae in all the organs. Lungs were particularly affected, which displayed interstitial disease, with vascular congestion, focal atelectasis and alveolar hemorrhage. Spleen showed congestion in sinuses. Dilated and congested vessels were observed in liver and kidney tissue. Neither inflammatory response nor necrosis was observed. No differences were found between species.

Drug efficacy study

The MICs of AMB, VRC and PSC against *S. brevicaulis* FMR 12216 and *S. brumptii* FMR 12229 were > 16 µg/ml for all three compounds, while against *S.*

brevicaulis FMR 12246 were > 16 µg/mL, 4 µg/mL and 2 µg/mL and against *S. brumptii* FMR 12240 were > 16 µg/mL, 4 µg/mL and 1 µg/mL, respectively.

The three drugs prolonged significantly the survival of mice infected with *S. brumptii* in comparison with the control group ($P \leq 0.0037$), while none of them showed efficacy against *S. brevicaulis* ($P \geq 0.13$) (Fig. 2).

Although the three drugs tested reduced moderately the fungal load of any of the organs studied, none of them was able to reduce the tissue burden in all the organs tested in any of the two strains evaluated. (Fig. 3).

After the completion of the treatment, PSC and VRC levels in serum (mean ± standard deviation, 5.02 ± 0.77 and 12.42 ± 0.97 µg/mL, respectively) were above the corresponding MICs (4 µg/mL and ≤ 2 µg/mL, respectively). Despite the high dose of LAMB given, serum concentration of this drug (15.41 ± 1.56 µg/mL) was lower than the AMB MIC value (>16 µg/mL).

Discussion

Although aspergillosis is the most frequent invasive mould infection, severe infections by other opportunistic filamentous fungi are emerging, especially in the immunocompromised population (26, 27). *Scopulariopsis*, although a rare fungus, infects humans too and, contrary to aspergillosis, there are no recommended therapies. *Scopulariopsis* is associated mainly with localized infections in immunocompetent patients (2, 3). More rarely, invasive infections by this fungus have also been described especially in neutropenic patients. The delayed diagnosis and the high level of resistance of *Scopulariopsis* to the conventional

197 antifungals (13, 14, 16) are responsible for the high mortality associated to the
198 disseminated infections (5).
199 To our knowledge, this is the first animal study that has explored the virulence of
200 *Scopulariopsis*. In general, immunocompetent mice showed a high survival rate,
201 despite the high inocula employed, probably due to the efficacy of the immune
202 system in controlling the infection, which explains the low number of cases of
203 invasive infections in immunocompetent patients reported (11). By contrast, our
204 results show high virulence of the four strains tested in immunosuppressed animals
205 because all of the animals died after being challenged with 5 x 10^5, 1 x 10^6 and 1 x
206 10^7 CFUs and high fungal loads were recovered from all studied organs. Animals
207 infected with *S. brumptii* at 1 x 10^5 CFU also showed a lower survival rate than
208 those challenged with *S. brevicaulis*, which suggests it is more virulent. In terms of
209 fungal load, both species invaded similarly all the studied organs, lung and liver
210 being the most affected after infection by *S. brevicaulis* and liver and spleen in the
211 case of *S. brumptii* (Table 1).
212
213 Histopathological and tissue burden findings were similar among species, all the
214 strains tested being able to disseminate to all studied organs, including brain.
215 These findings agree with clinical reports where disseminated infection by
216 *Scopulariopsis* has been reported to involve multiple organs, including liver,
217 spleen, kidney and brain (5). However, conidia, swollen structures or ascospores
218 were not found, as described in some reports (7) possibly due to the short period of
219 infection.

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Guidelines for hyalohyphomycosis do not recommend any particular antifungal treatment for invasive infections (17). However, invasive *Scopulariopsis* infections are challenging to treat and are often fatal despite aggressive medical and surgical management. Some response to LAMB (28) and VRC (29) has been occasionally documented, while PSC has only shown good in vitro activity. Nevertheless, no therapy has been experimentally tested previously against this infection. In the absence of relevant clinical data and an absence of standard therapies linked to a favourable outcome for patients, animal models can play an important role in guiding the use of empirical treatments (18). In the present study, *S. brevicaulis* and *S. brumptii* showed high MIC values against AMB although LAMB was effective in prolonging the survival of mice infected with *S. brumptii*.

Only PSC and VRC concentrations in serum were above the respective MICs, wich agrees with previous studies (30, 31, 32). Despite that, they were only effective against *S. brumptii*. Fungal burden of two strains tested was reduced only slightly by the three antifungals.

In summary, the lack of correlation between in vitro and in vivo studies and the poor efficacy of antifungals, makes difficult to manage this infection, which underlines the need for further studies to explore therapeutic alternatives. Synergistic in vitro interactions of antifungals against *S. brevicaulis* have been reported (15) and might be a new line of research for the treatment of *Scopulariopsis* infections.

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372 **Table 1.** Fungal load of different organs on day 6 post infection from
373 immunosuppressed mice infected with the indicated inoculum of *Scopulariopsis*
374 spp. Data correspond to 8 animals per experimental group.

Strain	Inoculum	Mean log10 CFU/g of tissue ± Standard deviation				
	CFU/animal	liver	kidney	brain	spleen	lung
S. brevicaulis	$1x10^5$	3.34 ± 0.05	1.92 ± 0.09	0.71 ± 0.53	2.54 ± 0.25	3.41 ± 0.06
FMR 12246	$5x10^5$	3.29 ± 0.08	3.12 ± 0.11	2.56 ± 0.3	3.43 ± 0.25	4.66 ± 0.2
	$1x10^6$	3.85 ± 0.08	3.78 ± 0.15	2.88 ± 0.08	4.19 ± 0.16	5.19 ± 0.19
	$1x10^7$	5.73 ± 0.04	3.64 ± 0.18	3.32 ± 0.14	5.75 ± 0.08	5.42 ± 0.25
S. brevicaulis	$1x10^5$	3.8 ± 0.24	1.72 ± 0.11	0.63 ± 0.5	4.68 ± 0.2	1.29 ± 0.63
FMR 12216	$1x10^6$	4.46 ± 0.24	2.61 ± 0.27	1.41 ± 0.24	4.52 ± 0.25	3.32 ± 0.54
	$1x10^7$	5.64 ± 0.16	3.55 ± 0.11	2.3 ± 0.16	5.8 ± 0.09	3.89 ± 0.05
S. brumptii	$1x10^5$	3.9 ± 0.03	3.02 ± 0.18	0.89 ± 0.58	4.15 ± 0.11	2.14 ± 0.05
FMR 12240	$1x10^6$	5.04 ± 0.19	3.98 ± 0.06	2.11 ± 0.07	5.31 ± 0.18	3.77 ± 0.48
	$1x10^7$	5.86 ± 0.33	5.05 ± 0.3	2.83 ± 0.03	6.05 ± 0.42	4.87 ± 0.27
S. brumptii	$1x10^5$	3.9 ± 0.03	0.73 ± 0.81	0.28 ± 0.44	3.73 ± 0.04	1.85 ± 0.11
FMR 12229	$1x10^6$	5.12 ± 0.12	3.24 ± 0.29	1.43 ± 0.18	5.2 ± 0.19	3.18 ± 0.24
	$1x10^7$	6.11 ± 0.11	4.69 ± 0.23	2.57 ± 0.09	6.32 ± 0.1	4.36 ± 0.14

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Figure. 1. Cumulative mortality of immunosuppressed mice (8 mice per experimental group) infected with *S. brevicaulis* FMR 12246 (A), FMR 12216 (B) and *S. brumptii* FMR 12240 (C), FMR 12229 (D). [a] $P < 0.05$ versus $1x10^5$ CFU/animal; [b] $P < 0.05$ versus $5x10^5$ CFU/animal; [c] $P < 0.05$ versus $1x10^6$ CFU/animal.

Figure. 2. Cumulative mortality of immunosuppressed mice (8 mice per experimental group) infected with *S. brevicaulis* FMR 12246 (A) or *S. brumptii* FMR 12240 (B), treated with LAMB 10, liposomal amphotericin B at 10 mg/kg QD; PSC 20 BID, posaconazole at 20 mg/kg BID; or VRC 60, voriconazole at 60 mg/kg QD. [a] $P \leq 0.05$ versus control.

Figure 3. Effects of the antifungal treatments on colony counts of organs in immunosuppressed mice (8 animals per experimental group) infected with *S. brevicaulis* FMR 12246 (A) or *S. brumptii* 12240 (B) 6 days after infection. LAMB 10, liposomal amphotericin B at 10 mg/kg QD; PSC 20 BID, posaconazole at 20 mg/kg BID; or VRC 60, voriconazole at 60 mg/kg QD. [a] $P \leq 0.05$ versus control, [b] $P \leq 0.05$ versus LAMB 10, [c] $P \leq 0.05$ versus PSC 40, [d] $P \leq 0.05$ versus VRC 60.