

Histopathology and antifungal treatment of experimental murine chromoblastomycosis caused by *Cladophialophora carrionii*

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Objectives: A murine model of chromoblastomycosis caused by *Cladophialophora carrionii* was used to compare the efficacy of posaconazole and voriconazole with that of terbinafine and itraconazole, the currently used drugs in the management of chromoblastomycosis.

Methods: Athymic nude mice were infected with 2×10^7 cfu of a clinical isolate of *C. carrionii*. When typical lesions were established, treatments with posaconazole at 20 mg/kg/day, voriconazole at 20 mg/kg/day, itraconazole at 50 mg/kg/day or terbinafine at 250 mg/kg/day were initiated. Treatment efficacy was evaluated for 4 months by measuring the size of the lesions, observing any histopathological changes and culturing the excised tissue.

Results: Posaconazole was the only drug that reduced the initial lesion size, while voriconazole and terbinafine reduced growth relative to controls.

Conclusions: This study suggests that the newer triazoles have potential in the treatment of chromoblastomycosis caused by *C. carrionii*.

Keywords: *C. carrionii*, mice, cutaneous infections

Introduction

Chromoblastomycosis is a chronic cutaneous and subcutaneous infection caused by several dematiaceous fungi that inhabit soil and decayed wood. The disease has a worldwide distribution, with *Fonsecaea pedrosoi* being the most common aetiological agent in tropical areas and *Cladophialophora carrionii* the predominant agent in dry countries and desert zones.¹ The infection is caused by traumatic inoculation of the aetiological agent to the skin, generally by contaminated thorns or splinters, which, in the case of chromoblastomycosis caused by *C. carrionii*, are strongly associated with plants of the *Cactaceae* family.²

The lesions usually start as small skin-coloured papules, which gradually enlarge and develop several morphologies.³ The disease is generally localized, but it can disseminate by extension of the lesions through lymphatic vessels or by self-inoculation through scratching.¹ The diagnosis relies on clinical manifestations, fungal isolation in culture and microscopic detection of thick-walled, brown-pigmented, rounded cells called sclerotic bodies.^{1–3} Management of chromoblastomycosis is complicated and requires long-term therapy, which in most cases does not prevent relapse of the infection. Treatments include surgery, chemotherapy, chemotherapy or combinations

of these.¹ There is no drug of choice for chromoblastomycosis; however, terbinafine and itraconazole, alone or in combination, are the currently recommended treatments, and new triazoles like posaconazole and voriconazole could become alternatives for this infection.^{3–6}

Based on an experimental murine model of chromoblastomycosis previously used for testing *F. pedrosoi*,^{7,8} we have compared the efficacy of newer azoles and traditionally used drugs in the treatment of infections caused by *C. carrionii*.

Methods

Establishment of the model

We used two clinical isolates of *C. carrionii* (FMR 10804 and FMR 10805) to develop a murine chromoblastomycosis. On the day of infection, 2 week cultures of each strain on potato dextrose agar (PDA) were suspended in sterile saline and filtered through sterile gauze to remove clumps of cells or hyphae. The resulting suspensions were adjusted to the desired inoculum based on haemocytometer counts, viability being confirmed by serial dilution plating on PDA. We used athymic CD-1 male mice with a mean weight of 30 g (Charles River, Criffa S.A., Barcelona, Spain). After being anaesthetized by isoflurane inhalation, mice were subcutaneously challenged over the left thigh.⁷ Inocula of

5×10^5 , 3×10^6 or 2×10^7 cfu in 0.1 mL of sterile saline were tested for each fungal isolate.

Groups of eight mice were randomly established and housed in standard boxes with corncob bedding and free access to a 3.1% fat diet and water. All animal care procedures were supervised and approved by the Rovira i Virgili University Animal Welfare and Ethics Committee.

Therapy study

Groups of eight CD-1 athymic mice were established randomly for each treatment, plus one group as control. Mice were infected subcutaneously with 2×10^7 cfu of the strain *C. carrionii* FMR 10804 in 0.1 mL of sterile saline solution over the left thigh. This was the only strain and inoculum tested that was able to produce chronic infections. The *in vitro* susceptibility of the strain to posaconazole, voriconazole, terbinafine and itraconazole was tested by using a broth microdilution method.⁹ Due to the slow growth of the isolate, plates were incubated for 72 or 96 h at 35°C before the first MIC determination. The drugs tested were: posaconazole at 20 mg/kg, itraconazole at 50 mg/kg, terbinafine at 250 mg/kg and voriconazole at 20 mg/kg. All drugs were given orally once daily. Treatments began 3 weeks after challenge, which allowed the infection to become established, and the therapy lasted for 4 months. Control groups received no treatment. Mice treated with voriconazole received an additional 0.25 mL of grapefruit juice by gavage twice daily, 30 min before and approximately 6 h after voriconazole administration.^{10,11} From days 1 to 7 after challenge, mice received 5 mg/day of ceftazidime subcutaneously to prevent bacterial infections.

To assess the efficacy of the different drugs, we measured the areas of the skin lesions at the beginning and at the end of therapy, with histopathological studies and qualitative skin cultures also performed.

For the histopathological study, 4 months after challenge and 24 h after the last antifungal dose, approximately half of the tissue of each lesion was excised and fixed with 10% buffered formalin. Samples were dehydrated, paraffin embedded and sliced into 2 µm sections, which were stained with haematoxylin-eosin or Giemsa and examined blind by light microscopy. Fine-needle aspiration cytologies of the nodules of control mice were also carried out to assess the presence of sclerotic bodies, thus confirming establishment of the infection. The remaining half of the excised tissue was aseptically separated into four or five pieces using a sterile scalpel and placed on the surface of PDA plates supplemented with chloramphenicol. Plates were then incubated at 30°C and examined daily for 7 days.

The sizes of the lesion areas were analysed using the Kruskal–Wallis test and, when this test was significant, we used the Mann–Whitney *U*-test to compare treatments with the control group. Statistical significance of the tissue culture data was estimated by Fisher’s exact test with a *P* value of ≤ 0.05 considered significant.

Results

The *in vitro* susceptibility test of the strain used showed MICs of 0.06 mg/L for posaconazole, 0.12 mg/L for terbinafine and voriconazole and 0.25 mg/L for itraconazole.

In the *in vivo* study, after 4 months of therapy the lesions were macroscopically characterized by the presence of dark, soft subcutaneous nodules, some of which were ulcerated. The changes in the sizes of the lesions at the end of the therapy are shown in Table 1 and Figure 1. The cultures of lesion biopsies were positive for *C. carrionii* in all cases. After 4 months of treatment, administering posaconazole was able to reduce the size of the initial lesions. In mice treated with terbinafine or voriconazole the lesions showed significantly lower growth than that of the

Table 1. Differences between lesion size areas in murine chromoblastomycosis caused by *C. carrionii*, at the beginning of the treatment and after 4 months of therapy

Drug and dose (mg/kg/day)	Differences in lesion size (mm ²)		
	median	IQR	<i>P</i> value ^a
Control	+5.27	3.43	—
ITC (50)	+0.82	5.44	0.065
POS (20)	-0.95	3.21	0.0002
TRB (250)	+0.90	2.95	0.0002
VRC (20)	+2.12	4.14	0.0019

ITC, itraconazole; POS, posaconazole; TRB, terbinafine; VRC, voriconazole. ^aIn comparison with the control group.

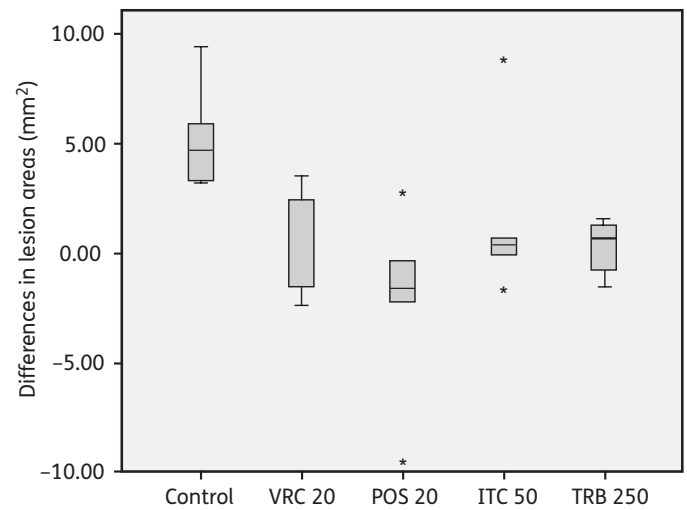


Figure 1. Box-plot of changes in the sizes of the lesions of murine chromoblastomycosis caused by *C. carrionii*. Horizontal lines indicate median values of eight mice. Asterisks represent the extreme outliers (values outside 3 IQRs measured from the 25th and 75th percentiles). VRC, voriconazole; POS, posaconazole; ITC, itraconazole; TRB, terbinafine; 20, 20 mg/kg/day; 50, 50 mg/kg/day; 250, 250 mg/kg/day.

control group. Itraconazole was the least active of the drugs tested. There were no significant differences among treatments.

The histological study of the control mice showed well-delimited subcutaneous nodules with central zones of necrosis, and a significant inflammatory response with the presence of polymorphonuclear neutrophils, histiocytes and a few giant multinucleated cells. The sizes of the nodules and the inflammatory responses were higher in control animals than in treated mice. Nodule sections showed pigmented hyphae and abundant brown-pigmented globose sclerotic bodies of different shapes and sizes, mainly around the necrosis areas (Figure 2). Fine-needle aspiration cytologies of control mice nodules 4 months after the inoculation showed a large number of hyphae and sclerotic bodies (Figure 3).

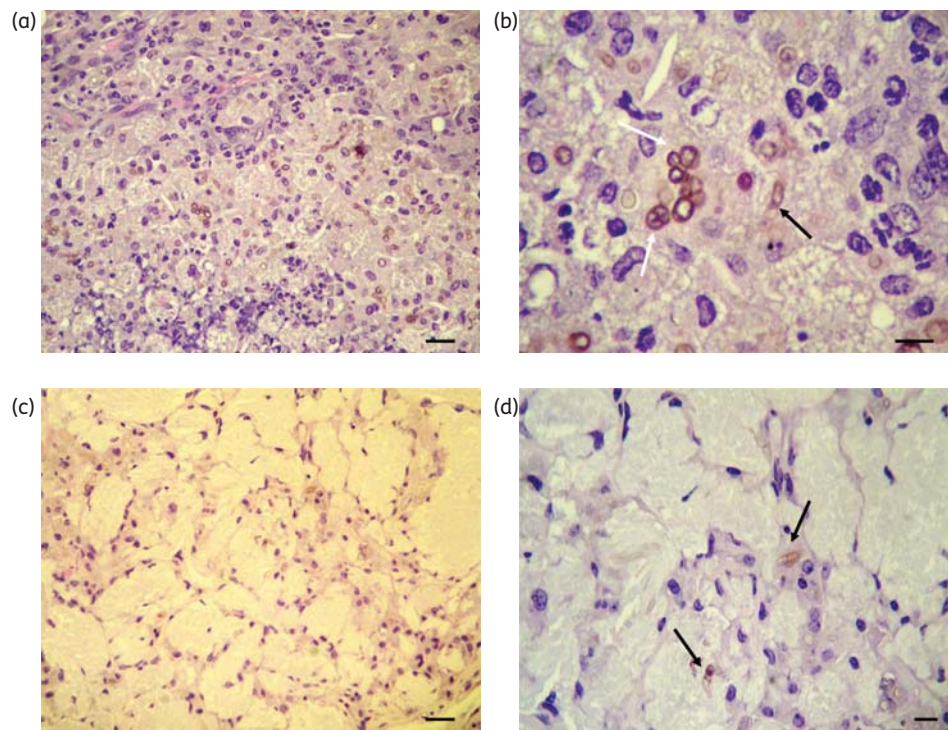


Figure 2. Histological sections of the nodular lesions in athymic mice 4 months after challenge with 2×10^7 cfu of *C. carrionii* FMR 10804. (a and b) Control mice showing an important inflammatory response with abundant melanized fungal hyphae (black arrows) and sclerotic bodies (white arrows). (c and d) Mice treated with 20 mg/kg/day posaconazole show few fungal elements. Haematoxylin-eosin stain. Bar=60 μ m (a and c) or 20 μ m (b and d). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Discussion

The management of chromoblastomycosis is generally complicated and usually shows only modest success rates.^{4,5} The outcome for patients depends on several factors, such as the stage and severity of the infection, as well as the causal agent of the disease—species like *C. carrionii* and *Phialophora verrucosa* seem to be more susceptible to antifungal drugs than other species causing this type of infection, such as *F. pedrosoi*.⁴ In the current study, we mimicked the route of human infection by inoculating the fungus subcutaneously, causing chronic and progressive verrucous dark lesions that, despite developing quickly in comparison with those in humans, in some cases disseminated and ulcerated.

All the tested drugs showed good activity *in vitro* against the isolate of *C. carrionii* used in our study. These *in vitro* results agree with previous studies on this fungus.^{12,13} For the *in vivo* study, the doses were selected on the basis of the observed plasma levels in mice in previous studies,^{14–17} which in all cases were far above the respective MICs of the drugs tested. These levels are similar to those achieved in humans receiving the recommended doses for the treatment of chromoblastomycosis.^{18–20} The results showed that administration of terbinafine and voriconazole was only able to limit the growth of the lesions, posaconazole being the only drug that reduced the size of the lesions at the end of the therapy. None of the drugs was able to achieve tissue fungal clearance after 4 months of treatment. To our knowledge, there is no experimental data on the efficacy *in vivo* of different antifungal drugs against chromoblastomycosis

caused by *C. carrionii*. Nevertheless, our results with *C. carrionii* agree with previous studies carried out with *F. pedrosoi*, which also demonstrated the good efficacy of posaconazole,^{8,21} even higher than that of itraconazole, terbinafine or voriconazole.⁸ To obtain a similar degree of infection in this study, we had to use a higher inoculum load than was used in a previous study that tested *F. pedrosoi* in similar experimental conditions, which suggests that our *C. carrionii* strain had lesser virulence. However, in the treatment study we also noted a generally poorer response to the therapies in mice infected with *C. carrionii* than those infected with *F. pedrosoi*, with the exception of voriconazole, which was ineffective against the latter.

In the clinical setting, monotherapies with itraconazole or terbinafine have proven to be effective against chromoblastomycosis,¹ and the combination of these drugs has even shown a synergistic interaction.²² On the other hand, the use of itraconazole may be limited by its drug interactions and by the induction of the resistance that its long-term administration can produce.^{4,23} In addition, itraconazole therapy has also been associated with adverse cardiac effects.²⁴ Regarding tolerance and efficacy, terbinafine is one of the recommended drugs for the treatment of chromoblastomycosis, showing a low side effect rate (2%–10%) and a relatively high cure rate (45%–70%).^{4,25} In contrast, the failure rate and reported cases of severe side effects like hepatic dysfunction,²⁶ make it necessary to find effective alternatives.

Although the use of posaconazole against chromoblastomycosis is very limited, it is considered a good option for the

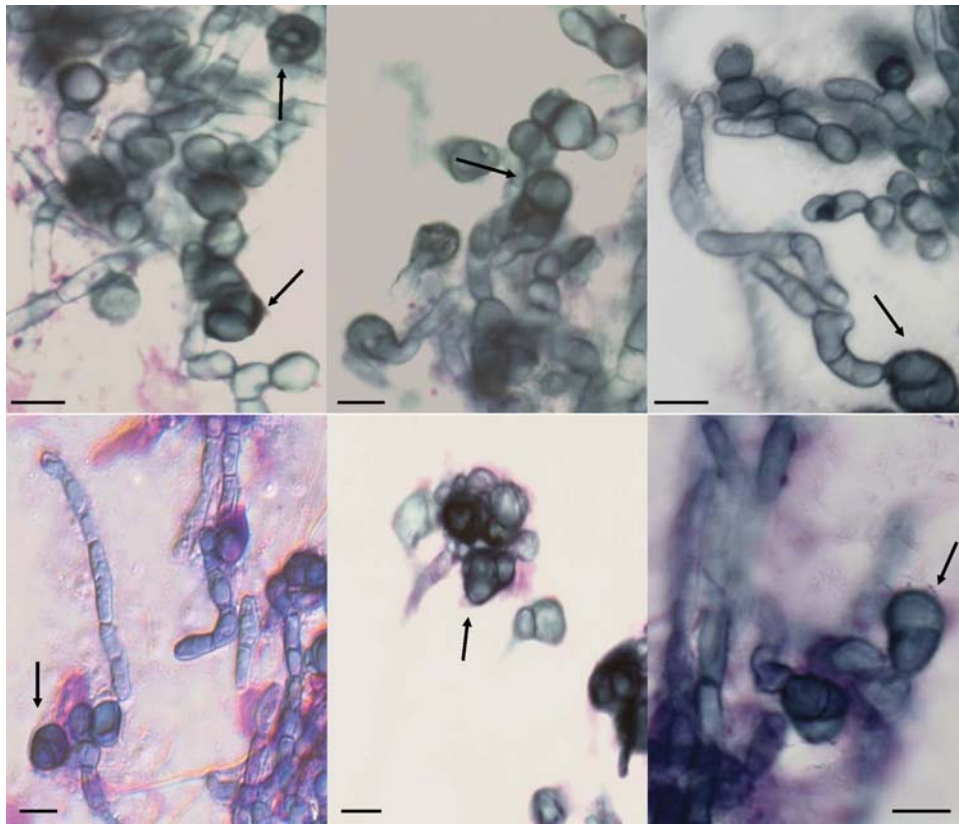


Figure 3. Fine-needle aspiration cytology of a nodule of athymic mice 4 months after challenge with 2×10^7 cfu of *C. carrionii* FMR 10804 showing septate hyphae and sclerotic bodies of different shapes and sizes (arrows). Giemsa stain. Bar = 5 μ m. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

treatment of infections caused by dematiaceous fungi,⁵ and is the drug that shows high efficacy against the widest spectrum of opportunistic fungi.²⁷ It was reported to be effective in a case of chromoblastomycosis caused by *C. carrionii* in a kidney and pancreas recipient,²⁸ and in chromoblastomycoses caused by *F. pedrosoi* refractory to conventional therapies such as itraconazole and terbinafine.^{29,30} Because of its good antifungal activity against this kind of fungus and its deeper skin penetration and tolerance in long-term therapies,^{18,31} posaconazole has become a promising drug in the management of chromoblastomycosis. Concerning voriconazole, despite having been reported to show slightly higher MICs than other azoles for dematiaceous fungi, its use against infections caused by these fungi is common.³² The drug has been successfully used in the treatment of cutaneous phaeohiphomycosis,⁶ and, due to its excellent cerebral penetration,³³ in cerebral phaeohiphomycosis, although with contradictory results in the latter.^{34–36} There are no data on the use of voriconazole in the management of chromoblastomycosis caused by *C. carrionii*. Nevertheless, four patients with infections by *F. pedrosoi*, with poor response to itraconazole and terbinafine, showed some improvement after voriconazole therapy.^{30,37}

In summary, in our experimental model of chromoblastomycosis, posaconazole showed good efficacy, while that of voriconazole was moderate. These results confirm the potential use of posaconazole against chromoblastomycosis caused by

C. carrionii, although, despite these encouraging results, the high cost of the newer azoles (voriconazole and posaconazole) strongly limits their use, particularly in those areas where this infection is endemic.¹

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Transparency declarations

None to declare.

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