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Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians

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Batrachochytrium dendrobatidis is one of the most pathogenic microorganisms affecting amphibians in both captivity and in nature. The establishment of *B. dendrobatidis* free, stable, amphibian captive breeding colonies is one of the emergency measures that is being taken to save threatened amphibian species from extinction. For this purpose, *in vitro* antifungal susceptibility testing and the development of efficient and safe treatment protocols are required. In this study, we evaluated the use of amphotericin B and voriconazole to treat chytridiomycosis in amphibians. The concentration at which the growth of five tested *B. dendrobatidis* strains was inhibited was 0.8 μg/ml for amphotericin B and 0.0125 μg/ml for voriconazole. To completely eliminate a mixture of sporangia and zoospores of strain IA042 required 48 h of exposure to 8 μg/ml of amphotericin B or 10 days to 1.25 μg/ml of voriconazole. Zoospores were killed within 0.5 h by 0.8 μg/ml of amphotericin B, but even after 24 h exposure to 1.25 μg/ml of voriconazole they remained viable. Amphotericin B was acutely toxic for *Alytes muletensis* tadpoles at 8 μg/ml, whereas toxic side effects were not noticed during a seven-day exposure to voriconazole at concentrations as high as 12.5 μg/ml. The voriconazole concentrations remained stable in water during this exposure period. On the basis of this data, experimentally inoculated postmetamorphic *Alytes cisternasii* were sprayed once daily for 7 days with a 1.25 μg/ml solution of voriconazole in water which eliminated the *B. dendrobatidis* infection from all treated animals. Finally, treatment of a naturally infected colony of poison dart frogs (Dendrobatidae) using this protocol, combined with environmental disinfection, cleared the infection from the colony.

Keywords *Batrachochytrium dendrobatidis*, amphibian, treatment, voriconazole

Introduction

Worldwide, amphibian populations are in severe decline with around one-third of the world's approximate 6,000 species now considered threatened, and up to 122 species may be extinct [1]. Several possible causes have been identified and the fungus *Batrachochytrium dendrobatidis* has been found to plays a key role in this process [1–3]. The decline of amphibian populations worldwide is so dramatic that it is thought by some that the only means to secure the survival of many species is the establishment of captive populations [4]. However, introducing new animals to establish captive populations carries with it the risk of introducing *B. dendrobatidis*. In fact, infections caused by this fungus are prevalent in captive collections [5–10]. Elimination of *B. dendrobatidis* is vital, not only for the health of captive populations but also to remove the fungus from the environment if reintroduction of amphibians is ever to be a possibility.

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Very little has been published concerning the adequate treatment of amphibian chytridiomycosis caused by this agent, but itraconazole has been proven efficacious to eliminate the infection [7,11,12]. However, the use of this antifungal causes skin depigmentation in tadpoles [12]. In addition, the proposed itraconazole treatment protocol requires daily soaking of animals for 5 min over several days, which is labor-intensive, especially when large numbers of animals have to be treated. Finally, the water-soluble formulation of itraconazole is not widely available. Treatment with formaldehyde and malachite green, as proposed by Parker *et al*. [13] might be of use, but its adoption is questionable since formaldehyde is toxic, especially to tadpoles [13,14]. Increasing the environmental temperature to 37°C [15], although possibly effective for clearing infection in some host species, is not tolerated by many amphibians. A recent study by Berger *et al*. [16] describes treatment failure using fluconazole and benzalkonium chloride. Fisher *et al*. [17] reported MIC_{50} of caspofungin in studies of 9 isolates of *B. dendrobatidis,* but the investigation did not include animal models [16,17]. Thus there is an urgent need for developing effective treatment protocols, both for pre- and postmetamorphic amphibians.

Amphotericin B, a polyene drug, and voriconazole, a second generation triazole antimycotic for which water soluble formulations are available, have pronounced fungicidal effects on many diverse fungal species [18]. Therefore, we studied the usefulness of amphotericin B and voriconazole for the treatment of chytrid infections. For this purpose, the *in vitro* minimal inhibitory concentrations of both drugs against five *B. dendrobatidis* strains were determined, as well as the time needed to kill the fungus. However, since *B. dendrobatidis* infections are limited to the superficial keratinized tissues, the contact time of an antimycotic with the fungus needs to be long enough to eliminate this pathogen. We also assessed the killing activity of both antifungals on *B. dendrobatidis* zoospores and the toxicity of amphotericin B and voriconazole exposure to *Alytes muletensis* (Mallorcan midwife toad) tadpoles. Based on these data, voriconazole was evaluated for the treatment of postmetamorphic *Alytes cisternasii* (midwife toads) experimentally inoculated with *B. dendrobatidis*.

Materials and methods

Strains and culture conditions

Five *B. dendrobatidis* strains, four kindly provided by Dr J. Longcore and one (IA042) isolated from a dead *Alytes obstetricans* (common midwife toad) involved in a mass mortality event at Ibon Acherito (Spanish Pyrenees) reported by Garner *et al*. [19], were used in this study (Table 1). The strains were grown in TGhL broth (16 g tryptone, 2 g hydrolysed gelatin and 4 g lactose per liter distilled water) in 25 cm² cell culture flasks at 20°C for 5 days. For the collection of zoospores for the experimental inoculation, 2 ml of a 5-day-old broth culture was inoculated on TGhL agar (16 g tryptone, 2 g hydrolysed gelatin, 4 g lactose + 10 g agar per liter distilled water) and incubated for 5–7 days at 20°C. Zoospores were collected by flooding the agar with 2 ml of distilled water and collecting the fluid.

Table 1 Strains of *Batrachochytrium dendrobatidis* used in this study, with their minimal inhibitory concentration (MIC) of amphotericin B and voriconazole.

Strain	Amphibian species of origin	MIC (μg/ml)	
		Amphotericin B	Voriconazole
IA042 [19]	*Alytes obstetricans*	0.8	0.0125
JEL197 [27]	*Dendrobates azureus*	0.4	0.0125
JEL277 [28]	*Ambystoma tigrinum*	0.4	0.00625
JEL310	*Smilisca pheota*	0.4	0.0125
JEL423	*Rana catesbeiana*	0.8	0.0125

Determination of minimal inhibitory concentrations of amphotericin B and voriconazole for B. dendrobatidis

The minimal inhibitory concentrations (MIC) of amphotericin B and voriconazole for the *B. dendrobatidis* isolates were determined using a macrodilution method in 24 well plates. To each well, 200 μl of culture broth containing various concentrations of amphotericin B (SPRL Bristol-Myers Squibb, Brussels, Belgium) or voriconazole (VFend, Pfizer, Kent, UK) were added to 200 μl of a 5-day-old growing culture of each of the strains, containing a mixture of approximately 10^5 *B. dendrobatidis* sporangia and zoospores. The final assay concentrations of the drugs were 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625 and 0.003125 μg/ml. The MIC value was determined as the lowest concentration of amphotericin B or voriconazole at which no growth of the *B. dendrobatidis* strain was recorded. Growth was assessed after 5, 7 and 10 days incubation at 20°C using inverted microscopic examination of the wells. The experiment was carried out in triplicate.

Determination of the time to 100% killing of B. dendrobatidis by amphotericin B and voriconazole

The time needed for amphotericin B or voriconazole to kill 100% of a growing culture of *B. dendrobatidis* strain IA042 was assessed in 24-well plates as described above. In these experiments, the strain was exposed to a concentration of either 8 μg/ml or 0.8 μg/ml of amphotericin B or to 1.25, 0.125 or 0.0125 μg/ml of voriconazole. The exposure time

was 0, 1, 2, 4, 8, 12, 16, 24, 48, 72, 96, 120, 168, 192 or 240 h. After the exposure time, the medium containing amphotericin B or voriconazole was replaced by broth without antifungals. The time to 100% killing at a given antifungal concentration was determined as the earliest time point of medium replacement at which no growth of the strain was recorded after 10 days of incubation at 20°C. All experiments were carried out in triplicate.

Killing capacity of amphotericin B and voriconazole towards B. dendrobatidis zoospores

To determine the killing capacity of voriconazole and amphotericin B towards *B. dendrobatidis* zoospores, the uptake of propidium iodide (PI) after exposure to amphotericin B or voriconazole was assessed using flow cytometry. A suspension containing 10^6 zoospores/ml of distilled water was exposed to either 8 or 0.8 μg/ml of amphotericin B or to 1.25, 0.125 or 0.0125 μg/ml of voriconazole and incubated for 1, 2, 4, 8, 16 and 24 hours at 20°C. The suspensions were transferred to Falcon tubes and PI was added to achieve a final concentration of 1 μg/ml. After incubation for 5 min at room temperature in the dark, the samples were analyzed using a FACSCanto flow cytometry system (Becton Dickinson Biosciences, Belgium). Analyses were performed with FACSDiva Software v5.0.1 (Becton Dickinson Biosciences, Belgium). Live zoospores were used to set light scatter gates for zoospore characteristics and zoospores killed with heat were used to set gates for PI positivity.

Toxicity of amphotericin B and voriconazole for tadpoles of Alytes muletensis

Six tadpoles of *Alytes muletensis* per treatment were maintained individually and exposed for 7 days at 20°C either to 8 μg/ml amphotericin B, or to 1.25 or 12.5 μg/ml voriconazole B, or media with any antimycotic at all. After the exposure period, the tadpoles were humanely killed and hematoxylin and eosin stainings of paraffin embedded in formalin fixed sections were examined for the presence of histological lesions of toxicity.

Voriconazole stability in water

The intravenous formulation of voriconazole has proven to be quite stable when dissolved in water [20,21]. To determine whether voriconazole treatment should include daily replacement of the bathing solution, the concentration of voriconazole was measured from day 0 through day 7 in tank water of *A. muletensis* tadpoles, containing 1.25 mg voriconazole per liter water. The tadpoles were kept individually in 200 ml of the bathing solution. The concentration of voriconazole was determined using HPLC with UV detection [22].

Optimizing a treatment protocol of chytridiomycosis in experimentally infected midwife toads (Alytes cisternasii) with voriconazole

B.dendrobatidis *strain and growth conditions*. For the experimental infection, *B. dendrobatidis* strain IA 042 was used. The strain was grown on TGhL agar for 5 days at 20°C. Subsequently, zoospores were harvested by flushing the agar with *aq dest* and the number of zoospores was adjusted to approximately 10^7 zoospores per ml.

Experimental animals. All animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University). Experiments were performed following all necessary ethical and biosecurity standards. Twenty-five captive bred *A. cisternasii* at approximately 2 months post-metamorphosis were used. The animals were kept in filter-top cages at temperatures of approximately 22°C and were fed fruit flies or crickets *ad libitum*. The room temperature was monitored using an automatic data logger device. Before experimental inoculation, all animals tested negative for the presence of *B. dendrobatidis* with quantitative PCR (qPCR), as described by Boyle *et al*. [23].

Experimental inoculation and treatment. The toadlets were inoculated by topically application 0.1 ml of the zoospore suspension per animal three times at 1-week intervals. Treatment started one week after the last inoculation. From 1 week post-inoculation onwards, the toadlets were divided into three groups. The first group of six animals served as untreated positive control. The second group of seven animals was treated for 7 days with voriconazole (VFend, Pfizer, Kent, UK) at a concentration of 1.25 mg/l water. The third group was treated with voriconazole at a concentration of 0.125 mg/l water for 7 days. The treatment consisted of transferring the frogs daily to a disinfected container provided with tissue paper and subsequent spraying of the frogs and the container contents once a day with the respective solution. To assess whether spraying of the animals is necessary to clear the infection, in a second experiment, five experimentally inoculated toadlets were housed for 7 days on paper towels soaked in a solution containing voriconazole at a concentration of 1.26 mg/l.

Cotton tipped swabs were used to collect samples from the drinking patch of all animals weekly from one week after the last inoculation onwards. These swabs were examined for the presence of *B. dendrobatidis* using the qPCR described by Boyle *et al*. (2004) [17]. Toads were considered negative after five consecutive negative sampling results.

Treatment of a long-term experimentally infected colony of A. cisternasii (midwife toads) with voriconazole

To assess the efficacy of the treatment in a larger group of infected animals, 20 experimentally infected *A. cisternasii* (midwife toads) were treated by spraying the animals with

a voriconazole solution of 1.25 mg/l once a day for 7 days at 5 months after the experimental infection. After treatment, the animals were kept separately and weekly sampled to assess the presence of *B. dendrobatidis* as described above. If a positive sample was obtained, the animal was treated using the same treatment protocol.

Treatment of a naturally infected colony of poison dart frogs (Dendrobatidae) with voriconazole

On the basis of the results from the above-described treatment experiments, a captive colony of poison dart frogs (Dendrobatidae) with a history of chytridiomycosis was experimentally treated with voriconazole. Out of a colony of 52 animals, five animals were qPCR positive for chytrid infection. Positive animals belonged to the species *Dendrobates tinctorius* (3), *Phyllobates terribilis* (1) and *D. azureus* (1). The complete colony was treated by spraying the animals (once daily) with a 1.25 mg/l voriconazole solution for a period of 7 consecutive days. In addition, all contact materials, including terrariums, were treated by heating to at least 45°C for 3 days. During the treatment, all animals were transferred to tissue-lined plastic boxes and kept at approximately 25°C. After treatment, the colony was retested by qPCR.

Results

MIC values, time to 100% killing of B. dendrobatidis cultures, and B. dendrobatidis zoospore killing activity of amphotericin B and voriconazole

The results of the MIC values of *B. dendrobatidis* zoospores and sporangia in culture at a given concentration of amphotericin B or voriconazole are summarized in Table 1. Three strains were sensitive to a concentration of 0.4 μg/ml and two to 0.8 μg/ml of amphotericin B. Four strains were susceptible to a concentration of 0.0125 μg/ml and one strain to 0.00625 μg/ml of voriconazole. At 8 μg/ml of amphotericin B and at 1.28 μg/ml of voriconazole, growth was not observed after 48 h and 10 days of exposure, respectively. At 0.8 μg/ml of amphotericin B and at 0.0125 and 0.125 μg/ml of voriconazole, the *B. dendrobatidis* cultures were not killed within 10 days of exposure.

Zoospores exposed to voriconazole at 0.0125, 0.125 and 1.25 μg/ml were not killed within 24 h, as assessed using flow cytometry. In contrast, amphotericin B killed more than 95% of the exposed zoospores within 30 min at both 0.8 and 8 μg/ml.

Amphotericin B but not voriconazole is acutely toxic to Alytes muletensis tadpoles

No clinical signs were observed in tadpoles when exposed to one of both concentrations of voriconazole. No pathology was observed upon histological examination of the tadpoles that were either not exposed to an antifungal, or that were exposed to one of the two concentrations of voriconazole. Voriconazole concentrations also did not decrease in water containing an *A. muletensis* tadpole over a 7-day period (Fig. 1). By comparison, exposure to 8 mg/l amphotericin B resulted in the death of all exposed tadpoles within 4 h of exposure. Histological examination revealed hydropic epidermal degeneration, sometimes combined with multifocal intra-epidermal vesiculation (Fig. 2). Desquamation of epithelial cells was observed in the gills of the tadpoles treated with amphotericin B.

B. dendrobatidis persistently colonizes the skin of A. cisternasii

All inoculated and untreated toadlets were positive for *B. dendrobatidis* until the end of the experiment at 85 days post-inoculation (Fig. 3). At day 35 and especially at day 42 post-inoculation, a marked drop in the number of genomic equivalents of *B. dendrobatidis* in the skin swabs was observed. This drop coincided with an accidental rise of the temperature to ambient temperatures between 25 and 26°C at days 42 and 43. During the rest of the experiment, the average temperature ± standard deviation was 22.1 ± 1.8°C. Only a single toadlet died – at day 35 post-inoculation. qPCR on the skin of this animal revealed high numbers of *B. dendrobatidis* organisms (2.54×10^3 genomic equivalents). Histologically, epidermal hyperplasia and hyperkeratosis were observed and a large number of sporangia were noticed in the superficial skin layers (Fig. 4). None of the other untreated toads showed clinical signs of lethal chytridiomycosis, although we frequently found shed skin in the water basin and on the tissue, which was not the case in the uninfected toads.

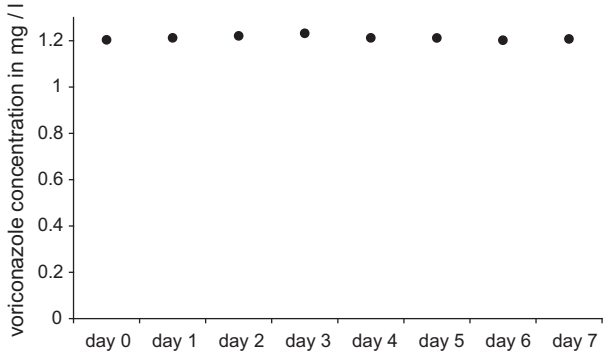

Fig. 1 Seven-day course of average voriconazole concentrations in a bathing solution containing one *Alytes muletensis* tadpole per 200 ml. At each time point, the concentration of voriconazole was determined in at least three containers.

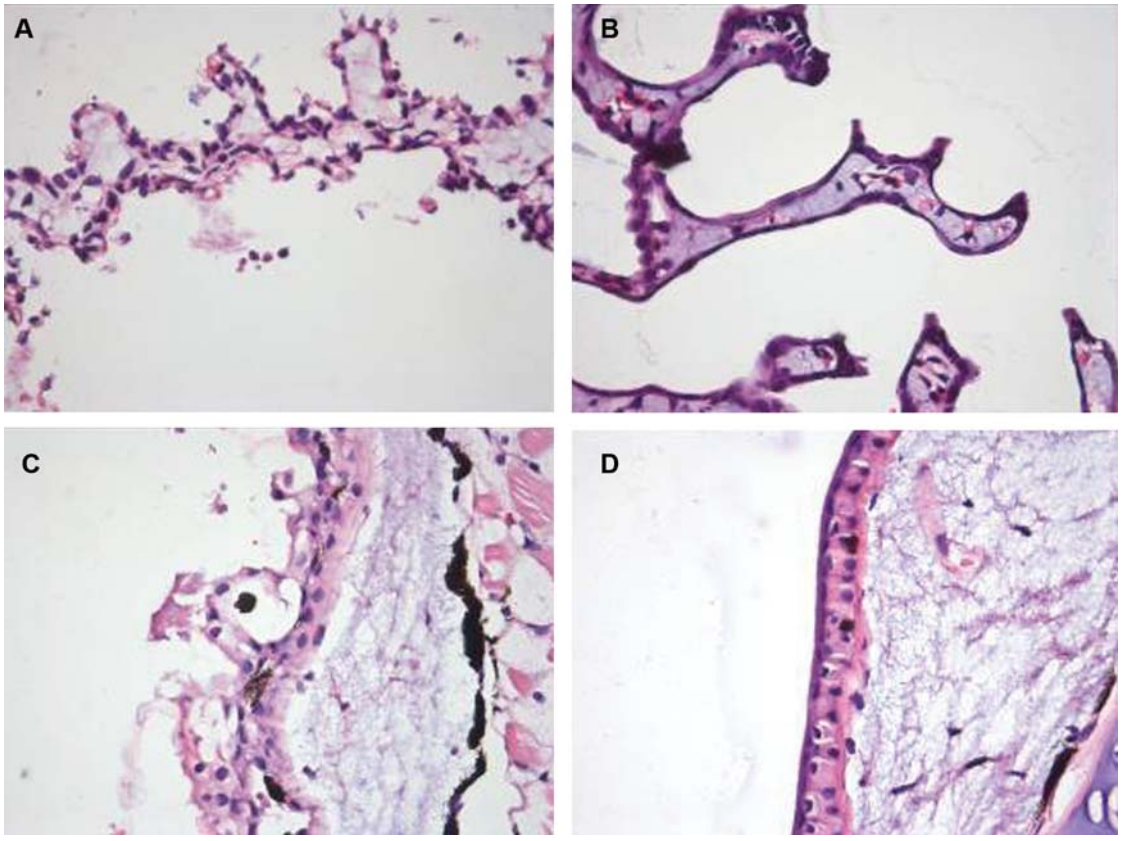

Fig. 2 Histological changes noticed in the gills (A) and skin (C) of an *Alytes muletensis* tadpole after 4 h of exposure to 8 mg/l amphotericin (haematoxylin and eosin staining). For comparison, the normal gill (B) and skin (D) structure of an untreated animal are shown. Notice hydropic epidermal degeneration, combined with multifocal intra-epidermal vesiculation in the skin and desquamation of epithelial cells in the gills of the amphotericin B-treated tadpoles.

Seven day voriconazole treatment eliminates B. dendrobatidis from experimentally infected postmetamorphic A. cisternasii

Immediately before the experimental treatment, all toadlets were positive, with an average load of *B. dendrobatidis* ± standard deviation in skin swabs of 17.8 ± 15.0 genomic equivalents (GE). *B. dendrobatidis* was absent in samples from all toadlets sprayed (once daily) with voriconazole at 1.25 mg/l for 7 days. However, three of five toadlets housed on tissue soaked in voriconazole at 1.25 mg/l were still positive immediately after the treatment, and 1 week later all five toadlets tested positive again. Three out of seven toadlets treated with voriconazole at 0.125 mg/l also remained positive.

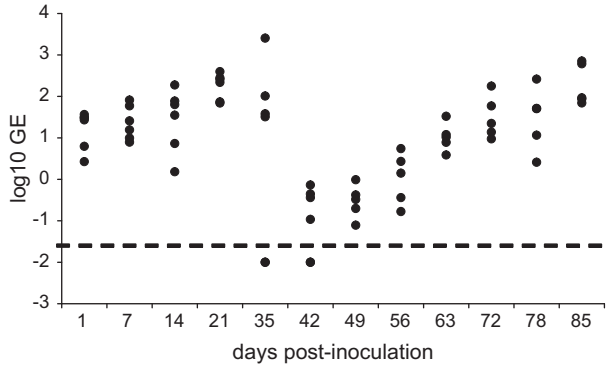

Fig. 3 Load of genomic equivalents (GU) of *Batrachochytrium dendrobatidis* in skin swabs collected from *Alytes cisternasii* and determined using qPCR, after experimental inoculation with *B. dendrobatidis* strain IA042. The dashed line represents the detection limit. A log10 GU of −2 is considered a negative sample.

From the group of 20 experimentally inoculated animals treated at 5 months post-inoculation by spraying the animals (once daily) with voriconazole at 1.25 mg/l water for 7 consecutive days, one animal remained positive after treatment for three consecutive samplings. This animal was retreated using the same protocol. This second treatment cleared the *B. dendrobatidis* infection from this animal.

Voriconazole treatment eliminates B. dendrobatidis from a colony of naturally infected poison arrow frogs

Five of the 52 animals sampled before treatment were positive for chytrid infection. These five animals belonged

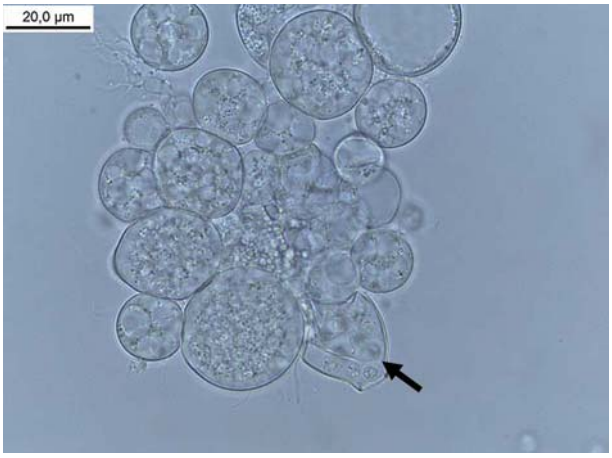

Fig. 4 Microscopic picture of a growing culture of *Batrachochytrium dendrobatidis* (scale bar = 20 µm). Sporangia with rhizoids can be seen, as well as zoospores in a discharge tube (arrow).

to the species *Dendrobates tinctorius* (3 animals), *Phyllobates terribilis* (1 frog) and *D. azureus* (1 frog). After treatment, all samples were negative for the presence of *B. dendrobatidis*. No adverse effects were noticed in any of the animals treated.

Discussion

The ultimate goal of *in vitro* testing is the prediction of the clinical outcome of therapy. However, our *in vitro* results were poorly correlated with *in vivo* findings. Amphotericin B killed all *B. dendrobatidis* organisms *in vitro* within 48 h at 8 µg/ml and was highly toxic to the fungal zoospores. These findings would suggest that a brief 2–3-day treatment using amphotericin B at 8 µg/ml should be sufficient to eliminate chytrid infections from affected animals. However, treatment trials using amphotericin B in postmetamorphic *A. cisternasii* showed that after 1, 3 and 5 days of treatment some infections were maintained, and comprehensive clearance of infection only occurred after 7 days of treatment (data not shown). Since acute adverse effects were observed in the tadpoles and 7 days of treatment is necessary, we do not recommend the use of amphotericin B in amphibians unless extensive toxicity studies for the target species have been carried out.

In contrast, voriconazole performed poorly during *in vitro* assays: the time required to kill all *B. dendrobatidis* organisms *in vitro* exceeded 7 days at 1.25 µg/ml, and voriconazole did not comprehensively kill zoospores over shorter time spans. However, all voriconazole-treated toads tested negative for *B. dendrobatidis* after 7 days of treatment.

On the basis of the results of this study, voriconazole at 1.25 mg/l water (once daily spraying) applied over a period of 7 days appears to be suitable for treating *Alytes cisternasii* and poison arrow frogs against *B. dendrobatidis* infections. Although amphotericin B is also capable of eliminating the fungus from infected postmetamorphic amphibians after 7 days of treatment at 8 mg/l (data not shown), the severe acute toxicity we detected in *A. muletensis* tadpoles precludes its general use for amphibians. Voriconazole was able to eliminate *B. dendrobatidis* from all infected tadpoles without inducing any obvious adverse effects, even at a concentration 10 times higher than the one used for treatment. Voriconazole also remains very stable in water, thus a simple treatment protocol for aquatic amphibians could consist of a permanent voriconazole bath over a 7-day treatment period. When treating terrestrial amphibians, it is vital that the animals be sprayed daily with the treatment solution, as only 2 of 5 infected *A. cisternasii* toadlets tested negative for *B. dendrobatidis* after 7 days of treatment with a soaked tissue substrate.

The proposed voriconazole treatment eliminated the chytrid infections within 7 days in all experimentally treated toadlets and in the group of naturally infected poison dart frogs.

This treatment protocol was thus applied to a group of 20 long-term infected *A. cisternasii*. Although the infection was cleared from 19 out of 20 of these animals after the 7 days of voriconazole treatment, one animal remained positive after sampling and was treated again using the same treatment protocol, after which no further chytrid DNA was detected. This finding highlights the fact that testing the animals after treatment is vital to confirm their chytrid-free status.

Despite being heavily and persistently colonized 3 months after experimental inoculation, and despite being a member of a supposedly *B. dendrobatidis*-sensitive genus (*A. obstetricans*, [24]; *A. muletensis*, Garner, unpubl. data), only one *A. cisternasii* toadlet died after exposure to *B. dendrobatidis*. It is notable that the animal that died was heavily infected with *B. dendrobatidis* (high genomic equivalent estimates of *B. dendrobatidis* using molecular diagnostics and histological signs of chytridiomycosis). This finding challenges the hypothesis that decline due to chytridiomycosis may be taxonomically selective at higher taxonomic levels [25]. It may also support the hypothesis that chytridiomycosis could be considered an opportunistic pathogen in at least some amphibian species and that clinical signs may be evoked through environmental or developmental stress [26], but further experimentation is required to confirm this in *A. cisternasii*.

In conclusion, we are proposing a safe and effective treatment against chytrid infections in amphibians, consisting of spraying once daily with voriconazole for 7 days at 1.25 mg/l.

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