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EXPERIMENTAL THERAPEUTICS

Efficacy of Azithromycin and Miltefosine in Experimental Systemic Pythiosis in Immunosuppressed Mice

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ABSTRACT We evaluated the efficacy of azithromycin (50 mg/kg, every 12 h [q12h] orally) and miltefosine (25 mg/kg, q24h orally) treatments in an experimental model of vascular/disseminated pythiosis in immunosuppressed mice. Azithromycin was the only treatment able to reduce mortality. The histopathological findings showed acute vascular inflammation, pathogen dissemination, necrotizing myositis, neuritis, and arteritis. The results suggest that azithromycin, but not miltefosine, may have clinical relevance in the treatment of vascular/disseminated pythiosis.

KEYWORDS *Pythium insidiosum*, azithromycin, miltefosine, treatment

Pythiosis is a life-threatening infectious disease in humans and other mammals for which there is no proven standard treatment (1, 2). Subcutaneous and gastrointestinal pythiosis are the clinical forms most commonly observed in animals, while in humans, ocular and vascular pythiosis account for approximately 90% of cases (1, 2) and have high rates of morbidity and mortality (2–6).

Although *Pythium insidiosum* has a similar morphology to filamentous fungi, monotherapy with antifungal drugs without aggressive surgical interventions has proved to be ineffective (1, 2, 6) in most of the cases evaluated, which can be explained by the incomplete ergosterol biosynthetic pathway (7) observed in this microorganism. In this context, the development of new therapeutic strategies is necessary to improve the treatment of pythiosis.

It is interesting to note that several classes of antibacterial drugs that are inhibitors of protein synthesis have *in vitro* (8, 9) and *in vivo* (10) antimicrobial activity against *P. insidiosum*. Indeed, two cases of presumptive *P. insidiosum* keratitis were successfully treated with a combination of azithromycin and linezolid (11) or voriconazole (12). However, favorable (9) and failure (13) responses to azithromycin-linezolid therapy in a series of cases of patients with *P. insidiosum* keratitis have been reported. Another drug we highlight is miltefosine. Although this drug is recognized for its antileishmanial activity, this compound has demonstrated antimicrobial activity *in vitro* and *in vivo* against pathogenic fungi (14) and *in vitro* against *P. insidiosum* (15).

Considering the difficulty of treating pythiosis (2–4), the cases of favorable responses to azithromycin treatment (9–12), and the potential cidal effect of miltefosine *in vitro* against *P. insidiosum* (15), the treatment of vascular pythiosis with these drugs should be explored. In this context, this study aimed to investigate the utility of a murine immunosuppressive model of systemic/vascular pythiosis in the evaluation of

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therapeutic approaches and to determine the efficacy of azithromycin and miltefosine against *P. insidiosum in vivo*.

Azithromycin (EMS Pharma, Brazil) in an oral suspension and miltefosine (Milteforan; Virbac, Brazil) in an oral liquid veterinary pharmaceutical composition were commercially acquired. *Pythium insidiosum* isolate LAPEMI 0290 (GenBank accession number KJ176713), with azithromycin and miltefosine MIC values of 2 μg/ml (10, 15), was used in this study. Thirty female Swiss mice (34.13 g $\pm$ 2.86 g, 6 to 8 weeks old) purchased from the bioterium of the Federal University of Santa Maria, Brazil, were utilized. The mice were housed at a temperature of 22°C with 12-h light/dark cycles and with food and sterile water *ad libitum*. All mice were rendered neutropenic by the intraperitoneal administration of cyclophosphamide (CYP, 100 mg/kg of body weight; Genuxal, Baxter Hospitalar Ltd., Brazil) on days -4 and -1 preinfection (16). On day 0, the mice were anesthetized by an intraperitoneal injection of ketamine-xylazine (Syntec Brasil Ltd., Brazil) administered at doses of 50 and 10 mg/kg, respectively, and were infected by subcutaneous injections of 2×10^4 zoospores/mouse (17).

The inoculated mice were randomly divided into three groups of 10 animals each and received one of the following treatments from day 0 (treatment was initiated 3 h after zoospores inoculation) to day 14 after infection: (i) sterile saline (PI group), (ii) azithromycin (50 mg/kg of body weight every 12 h; PI+AZI group), or (iii) miltefosine (25 mg/kg of body weight every 24 h; PI+MILT group). Throughout the study, the animals were monitored 3 to 4 times daily during the treatment and up to the 30th day after the end of treatment for clinical signs such as rapid or very slow shallow or labored breathing, weight changes, ruffled fur, hunched posture, impaired ambulation, or lethargy/drowsiness. Other signs taken into consideration to decide whether to euthanize the animals during the study included physical and mental alertness, chronic diarrhea, and bleeding.

Half of each organ (kidney, liver, lung, and spleen) was sliced and subjected to microbiological culture in corn meal agar at 37°C for 1 week or to direct detection of *P. insidiosum* DNA by a nested PCR (nPCR) (10, 18). All positive cultures were identified through the induction of zoosporogenesis and nPCR (17, 18). The other organ halves and representative tissues from adipose tissue, skeletal muscle, and spine were fixed in 10% buffered formalin for 24 h for the histopathological analysis (17).

The statistical evaluation of survival was performed by the Kaplan-Meier method and the log-rank test using SigmaPlot software version 12.5. Significant differences were considered at P values of <0.05. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee at the Federal University of Santa Maria, Brazil (protocol number 7882240317), according to the National Council for Animal Experimentation Control.

The survival curves for treated and untreated pythiosis groups are shown in Fig. 1. The survival probability for untreated mice (PI group) was 30%, and the mean of survival and standard error (MS $\pm$ SE) was 13.7 $\pm$ 5.89 days (95% confidence interval [CI], 2.15 to 25.24). We observed that azithromycin treatment (PI+AZI group) markedly reduced mortality compared with that of the PI group ($P = 0.028$). In the PI+AZI group, the survival probability was 80% with MS of 32.4 $\pm$ 6.79 days (95% CI, 19.07 to 45.72). Treatment with miltefosine (PI+MILT group) did not reduce the mortality significantly compared to that of the PI group ($P = 0.48$), showing 40% survival probability with MS of 18.1 $\pm$ 6.23 days (95% CI, 5.88 to 30.31).

All mice that died or were euthanized before the end of the experiment presented with unilateral or bilateral paralysis of the hind limbs (Fig. 2A and B and Table 1). One mouse in the PI+AZI group presented hind limb claudication, which did not progress to paralysis. The surviving mice developed ulcerated lesions at the pathogen inoculation site that progressed to complete resolution (Fig. 2C to F). Microbiological organ cultures demonstrated that *P. insidiosum* established infection in the liver, lung, spleen, and/or kidney tissues from all dead or euthanized mice. The nPCR analysis of organs was in line with the culture results. Histological examination showed marked neutrophilic periarteritis with thrombosis, perivascular

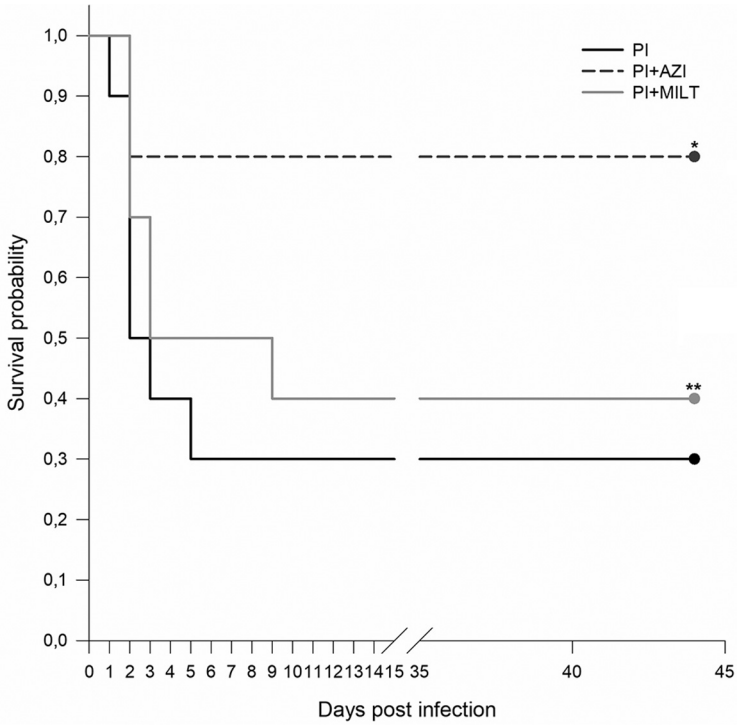

FIG 1 Survival of immunosuppressed mice experimentally infected via the subcutaneous route with 2×10^4 *Pythium insidiosum* zoospores. Mice were rendered susceptible with two doses of cyclophosphamide (100 mg/kg at 96 h and 24 h before infection). Mice were treated with azithromycin (PI+AZI; 50 mg/kg every 12 h [q12h] orally), miltefosine (PI+MILT; 25 mg/kg q24h orally), or sterile saline (PI, control group); $n = 10$ mice per group. Therapy was continued for 2 weeks, and survival was monitored up to 30 days after the end of treatment. *, $P = 0.028$; **, $P = 0.48$ by log-rank test compared with untreated mice (PI).

inflammatory infiltrate, neural and perineural neutrophilic inflammation, and vascular thrombus. Immunolabeled hyphae of *P. insidiosum* were observed in the walls of the vessels, in the perivascular inflammatory infiltrates, among peripheral nerve fibers and myocytes, in the red pulp of the spleen, and entering the renal interstitium.

In this study, we established a new protocol to model systemic pythiosis in Swiss mice immunosuppressed with CYP, and we provided the first evidence of the applicability of this model to test therapeutic approaches. Azithromycin treatment markedly reduced the mortality (from 70% to 20%), while miltefosine did not alter the outcome of the disease significantly compared to that of the control group. Recently, BALB/c mice were postulated as a feasible animal model to study pythiosis, and the authors highlighted the need for immunosuppression for the development of the disease (17). They used a combination of CYP and hydrocortisone acetate (HCA) and reported a mortality rate of 60% (17). Our study demonstrated that two intraperitoneal injections of CYP before infection rendered the mice susceptible to pythiosis with mortality rate of 70% if untreated.

Loreto et al. (19) were the first to describe the *in vitro* antimicrobial activity of azithromycin against *P. insidiosum*, and then Jesus et al. (10) confirmed its potential use in a rabbit model of pythiosis. After that, azithromycin was used in human cases of ocular pythiosis with favorable (9, 11, 12) and unfavorable (9, 13) clinical outcomes. The likely mechanism of action of azithromycin against *P. insidiosum* is the inhibition of protein synthesis, similar to that described for erythromycin against *Pythium ultimum* (20). Moreover, azithromycin has a rapid and extensive distribution from serum into intracellular compartments and to the tissues (21), a notable intraphagocytic uptake and retention in both polymorphonuclear leucocytes and macrophages (22) associated

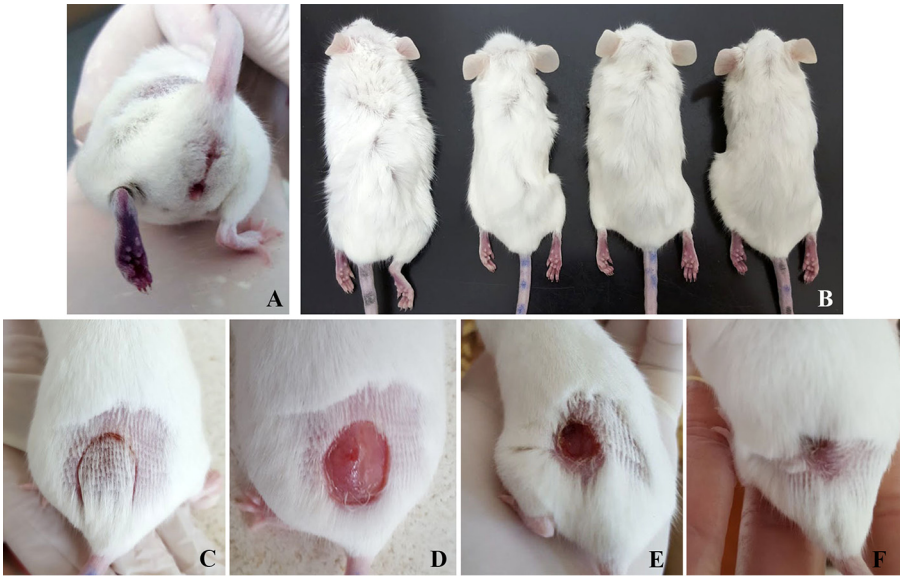

FIG 2 Signs of *Pythium insidiosum* infection in mice immunosuppressed with cyclophosphamide. (A) Ischemic aspect of a hind limb observed 2 days after the subcutaneous inoculation of the pathogen. (B) Hind limb paralysis. (C to F) Resolution of the ulcerative process with the healing of the subcutaneous lesions in the surviving mice. The inoculation area was trichotomized, and the figure sequences were taken on days 6, 8, 15, and 22 after the subcutaneous inoculation of the pathogen.

with delivery and release at the sites of infections (23), and immunomodulatory effects that are associated with macrophage phenotype alteration and a profile of anti-inflammatory cytokines (24, 25). Interestingly, azithromycin-induced M2 macrophage polarization was associated with reparative properties in spinal cord injury (26) and with protection against ischemic stroke (27).

Miltefosine previously demonstrated both efficacy (28) and limited activity (29) in murine models of cryptococcosis. However, it has been described as salvage therapy in *Lomentospora* (*Scedosporium*) *prolificans* bone and joint infections (30, 31). It is possible that the inefficacy of miltefosine in the treatment of experimental pythiosis is related to the rapid and aggressive progression of the disease, since although this drug shows good bioavailability after oral administration (82% to 94% in rats and dogs, respectively), it is absorbed slowly with a delay of up to 48 h to reach the maximum plasma concentration (32).

The histological findings were similar to those reported by Tondolo et al. (17). The systemic/vascular dissemination of the pathogen was evidenced by acute vascular inflammation associated with the observation of hyphae and hyphal fragments in the liver, kidney, lung, and spleen tissues. The data from the spine and surrounding skeletal muscles showed signs of necrotizing myositis, neuritis, and arteritis that explain the hind limb paralysis of mice.

The data from the present study provide support for future investigations on the treatment of pythiosis. However, it is important to note that (i) mice are not naturally occurring hosts of the disease, and only one treatment regimen was used for each drug analyzed; (ii) the treatments were started on the same day of *P. insidiosum* inoculation, and azithromycin is likely to have a favorable potential in preventing the spread of the disease. Therefore, further research is needed to determine the effectiveness of this treatment in cases where the pythiosis is advanced. (iii) Previous data on the *in vitro* susceptibility of *P. insidiosum* to azithromycin have shown that clinical isolates from animal and human origin have MICs ranging from 0.03 to 16 μg/ml (8) and 0.02 to 32 μg/ml (9), respectively. Considering there are no defined breakpoints for *P. insidiosum*, future studies should correlate the therapeutic response of pythiosis cases with the susceptibility profile of the recovered clinical isolates.

TABLE 1 Morbidity and mortality in experimental pythiosis in immunosuppressed mice

	PI[b]			PI+AZI[c]			PI+MILT[d]		
Cause of D/E[a]	*n*	Mean no. of days to D/E	Hd[e] (*n*)	*n*	Mean no. of days to D/E	Hd	*n*	Mean no. of days to D/E	Hd (*n*)
Spontaneous death, without obvious signs of morbidity	1	1	Liver, kidney, spleen	1	2	Kidney, liver			
Spontaneous death, appearance of animals indicated hind limb paralysis	1	2	Liver, kidney, spine, spleen				5	3.8 (range, 2–9)	Kidney (3), liver (3), spine (2), spleen (2)
Euthanized due to bilateral hind limb paralysis	1	2	Kidney, liver, spine	1	2	Kidney, liver, spine	1	2	Kidney, spine, spleen
Euthanized due to unilateral hind limb paralysis	4	3 (range, 2–5)	Kidney (3), liver (1), spine (3), spleen (1)						
Euthanized at the end of the experimental period without obvious signs of morbidity	3	44	ND[f]	8[g]	44	ND	4	44	ND

[a]D/E, death/euthanasia.
[b]PI group, disease control, sterile saline.
[c]PI+AZI group, azithromycin, 50 mg/kg of body weight every 12 h.
[d]PI+MILT group, miltefosine, 25 mg/kg of body weight every 24 h.
[e]Hd, hyphae or hyphal elements detected by histopathological analysis, nPCR from organs, and microbiological culture.
[f]ND, not detected.
[g]One animal showed signs of claudication, which did not progress to paralysis.

In this study, azithromycin, but not miltefosine, showed efficacy in the treatment of vascular/disseminated pythiosis. The results should be carefully interpreted, and although further studies are needed to ascertain its clinical relevance, we suggest that azithromycin must be considered in future *in vivo* experimental studies that evaluate the treatment of pythiosis.

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