International Journal of Antimicrobial Agents 48 (2016) 292–297

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Evaluation of the efficacy of antifungal drugs against *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii* in a *Galleria mellonella* model

Junya de Lacorte Singulani, Liliana Scorzoni, Ana Carolina Alves de Paula e Silva, Ana Marisa Fusco-Almeida, Maria José Soares Mendes-Giannini *

Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista (UNESP), Rodovia Araraquara—Jaú Km 1, Araraquara, São Paulo CEP: 14801-902, Brazil

ARTICLE INFO

Article history:
Received 21 January 2016
Accepted 22 May 2016

Keywords:
Alternative animal model
Antifungals
Efficacy
Paracoccidioidomycosis

ABSTRACT

Paracoccidioides brasiliensis and *P. lutzii* belong to a group of thermodimorphic fungi and cause paracoccidioidomycosis (PCM), which is a human systemic mycosis endemic in South and Central America. Patients with this mycosis are commonly treated with amphotericin B (AmB) and azoles. The study of fungal virulence and the efficacy and toxicity of antifungal drugs has been successfully performed in a *Galleria mellonella* infection model. In this work, *G. mellonella* larvae were infected with two *Paracoccidioides* spp. and the efficacy and toxicity of AmB and itraconazole were evaluated in this model for the first time. AmB and itraconazole treatments were effective in increasing larval survival and reducing the fungal burden. The fungicidal and fungistatic effects of AmB and itraconazole, respectively, were observed in the model. Furthermore, these effects were independent of changes in haemocyte number. *G. mellonella* can serve as a rapid model for the screening of new antifungal compounds against *Paracoccidioides* and can contribute to a reduction in experimental animal numbers in the study of PCM.

1. Introduction

Paracoccidioides brasiliensis belongs to a group of thermodimorphic fungi that is found in the form of mycelia at room temperature (25–27 °C) and in yeast form at body temperature (37 °C). Phylogenetic studies showed the existence of three different phylogenetic species of *P. brasiliensis* (S1, PS2 and PS3) [1]. More recently, genomic studies showed that *P. brasiliensis* strain 01 could not be included in these groups and was considered a new species, termed *P. lutzii* [2,3]. The individual characteristics of each species have implications in the diagnosis, clinical manifestations and treatment of the disease [4].

Paracoccidioides spp. causes paracoccidioidomycosis (PCM), a human systemic mycosis whose clinical importance has increased due to the frequency, severity of their anatomical and clinical forms, and mortality rate. PCM is endemic in South and Central America, among which Brazil, Venezuela, Colombia and Argentina are the countries with the highest number of cases. In these regions, the annual incidence rate of new cases ranges from 1 to 3 per 10^5 inhabitants. In Brazil, PCM is the eighth most important cause of mortality from chronic infectious diseases, with 1.65 deaths per 10^6 inhabitants. In non-endemic regions such as the USA, Europe and Asia, some imported cases have been reported. The marked predominance of clinical disease is in adult men, rural workers and the immunocompromised [5–7].

Paracoccidioides infection occurs upon inhalation of propagules by the host. These structures then invade terminal airways, where they differentiate into yeast forms. Temperature is essential for this transformation. The lung is the first site of infection, but the yeast can spread to other organs. It is speculated that modulation of host cell apoptosis is advantageous for the fungi because the micro-organisms can evade the killing activity of the phagocytic antimicrobial machinery in tissues; this would prevent their death, and the fungi could disseminate through the host's circulatory system and stimulate the inflammatory response to invade tissues as a consequence of these injures [8]. There is evidence that *P. brasiliensis* is able to modulate the chronic inflammatory response through nitric oxide (NO), which improves tissue degradation and/or decreases extracellular matrix synthesis by controlling inflammatory and immune mediators. In the later phase of PCM, the deleterious effects of NO may be associated with loose granulomas and high fungal dissemination [9,10]. Treatment of PCM takes place over long periods of time and depends on the severity in each patient: azoles (itraconazole, fluconazole and voriconazole) and sulfonamides are options for mild-to-moderate clinical forms; and amphotericin B (AmB) is used for severe and disseminated cases [6,7,11].

* Corresponding author. Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista (UNESP), Rodovia Araraquara—Jaú Km 1, Araraquara, São Paulo CEP: 14801-902, Brazil. Fax: +55 16 3322-0073.
E-mail addresses: gianninimj@gmail.com; giannini@fcfar.unesp.br (M.J.S. Mendes-Giannini).

http://dx.doi.org/10.1016/j.ijantimicag.2016.05.012
0924-8579/

Virulence studies of different fungi such as *P. brasiliensis*, *P. lutzii*, *Histoplasma capsulatum* [12,13], *Cryptococcus neoformans* [14], *Fusarium* spp. [15], *Candida* [16,17] and *Trichosporon* spp. [18] have been successfully performed in a *Galleria mellonella* infection model. It has important advantages as a fungal host study model: larvae can be incubated at temperatures between 25 °C and 37 °C, enabling the habitat and conditions of infection in mammals to be simulated; exact amounts of pathogens can be administered; and the viability of the larvae can easily be detected by the lack of movement and melanisation response after infection [19]. Furthermore, larvae have six important types of cells in the defence system called haemocytes, which are classified as prohaemocytes, plasmatocytes, granulocytes, coagulocytes, spherulocytes and oenocytoids. The plasmatocytes and granulocytes participate in phagocytosis, nodule formation, encapsulation and defence against microbial pathogens [20,21]. *G. mellonella* has also been used to test the efficacy and toxicity of commercial and new antifungals [22–24].

In this work, *G. mellonella* was infected with two species of *Paracoccidioides* and the efficacy and toxicity of AmB and itraconazole were evaluated in this model for the first time.

2. Materials and methods

2.1. Fungi

P. brasiliensis isolate 18 (chronic PCM; São Paulo, Brazil) and *P. lutzii* Pb01-like strain ATCC MYA-826 (acute PCM; Goiânia, Brazil) were grown in Fava Netto medium (prepared in-house) at 37 °C for 4–5 days. Fungi were transferred to brain–heart infusion (BHI) (Neogen, Lansing, MI) broth supplemented with 1% glucose (Hexapur, Solon, OH) and were grown on an shaker (New Brunswick, Edison, NJ) for 3–4 days at 150 rpm and 37 °C. For the experiment, yeast cells were washed three times with phosphate-buffered saline (PBS) (prepared in-house) containing 20 mg/L ampicillin (Sigma-Aldrich, St Louis, MO) to prevent bacterial contamination.

2.2. Insects

Eggs of *G. mellonella* (Lepidoptera: Pyralidae) were provided by Dr Carlos Eduardo Winter (Universidade de São Paulo, São Paulo, Brazil) to initiate the culture of this insect in our laboratory. Larvae were kept in plastic boxes and were reared on honeybee wax and pollen at 25 °C in darkness. Larvae of ca. 150 mg were selected for the experiments and were kept without food in Petri plates at 37 °C in the dark for 24 h prior to use.

2.3. Survival analysis

Before injection, the pro-leg area was cleaned with 70% ethanol. Each larvae group was inoculated using a Hamilton syringe through the last left pro-leg with 10 μL of 1×10^5, 5×10^5, 5×10^6 or 5×10^6 *P. brasiliensis* or *P. lutzii* yeast cells. The inoculum of 5×10^6 yeast cells/larva was used for the remaining studies. A group of uninfected larvae and a group of uninfected larvae inoculated with PBS were used as controls in all experiments. Larvae were incubated in Petri plates at 37 °C and were assessed at 7 days for lack of physical movement. A total of 16 larvae were used for each condition, and each experiment was replicated three times.

2.4. Efficacy and toxicity assays

At 1 h after infection with *Paracoccidioides* spp., larvae were injected with 10 μL of AmB (Sigma-Aldrich) at 0.5, 1 or 2 mg/kg or with itraconazole (Sigma-Aldrich) at 5.5, 11, 22 and 44 mg/kg through the last right pro-leg. Stock solutions of AmB and itraconazole were prepared in dimethyl sulfoxide (DMSO) (Labsynth, Diadema, SP, Brazil) and were diluted in PBS to a DMSO concentration of 5%. Groups of uninfected larvae were treated with antifungals alone and with the vehicles to test their toxicity. Larvae were incubated at 37 °C and were assessed for 7 days for lack of physical movement.

2.5. Fungal burden

At 1, 48 and 96 h post-treatment with AmB or itraconazole, larvae from each group were surface-sterilised in 70% ethanol. A group of infected larvae without treatment was also used. Each larva was cut into small pieces with a scalpel and was suspended in 1 mL of PBS with 20 mg/L ampicillin. The tissues were transferred to conical tubes with glass beads and were homogenised using a vortex mixer (Norte Científica, Araraquara, SP, Brazil). Each sample was diluted 100× in PBS and then 100 μL of the resulting dilution was plated on BHI agar supplemented with 4% horse serum, 5% *P. brasiliensis* 339 culture filtrate and 40 mg/L gentamicin [25]. The plates were incubated at 37 °C for 10 days and then CFU of *P. brasiliensis* or *P. lutzii* were counted.

2.6. Histological evaluation

At 48 h post-treatment with AmB or itraconazole, larvae from each group were fixed by immersion in phosphate-buffered 4% formalin. A group of uninfected larvae and a group of infected larvae without treatment were also used. Samples were embedded in paraffin, were serially sectioned at a thickness of 5 μm and were stained using Periodic acid-Schiff (PAS) solution (Sigma-Aldrich). Images were analysed using an optical microscope (ZEISS AxioCam HRc; Carl Zeiss Microscopy GmbH, Jena, Germany) at 40×.

2.7. Haemocyte density

At 1 h and 48 h post-treatment with AmB or itraconazole, haemolymph samples were collected by puncturing the larval abdomen and were diluted in ice-cold PBS (1:20). Then, 10 μL aliquots of the haemocyte suspension were added to a Neubauer chamber (haemocytometer) and cells were counted in four main squares under a brightfield microscope. As the larvae without treatment were debilitated or dead at 96 h, it was not possible to acquire sufficient amount of haemolymph for analysis.

2.8. Statistical analysis

Survival curves were analysed using the log-rank (Mantel–Cox) test in GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Fungal burden and haemocyte density were analysed using analysis of variance (ANOVA) with the Bonferroni post-test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Killing of G. mellonella by Paracoccidioides spp. and the effect of antifungal agents

To determine whether *G. mellonella* larvae were a suitable model for studying *Paracoccidioides* pathogenesis, its infection characteristics were first defined. Larval killing was dependent on the number of *Paracoccidioides* cells injected. Very few *G. mellonella* larvae were killed with $\leq 1 \times 10^6$ yeast cells/larva within the period of infection (data not shown), whereas most of the *G. mellonella* larvae were killed with 5×10^6 yeast cells/larva, which was the concentration selected for testing the antifungal treatments.

At the end of 7 days, 11% and 4% of the larvae infected with 5×10^6 yeast cells/larva of *P. brasiliensis* or *P. lutzii* survived, respectively. All doses of AmB (0.5, 1 and 2 mg/kg) protected *G. mellonella* from