Antifungal activity of ajoene on experimental murine paracoccidioidomycosis

Marcia L. F. Maluf, Gisele Takahachi, Terezinha I. E. Svidzinski, Patricia Xander, Raphael Apitz-Castro, Ciomar A. Bersani-Amado, Roberto K. N. Cuman

Division of Medical Mycology, State University of Maringá, Maringá, Paraná, Brazil

Summary The natural compound ajoene (4,5,9- trithiadodeca-1,6,11-triene 9-oxide) is capable of controlling infection by *Paracoccidioides brasiliensis* in experimental models. Swiss mice were inoculated with 5.0 x 10^6 cells of the fungus *Paracoccidioides brasiliensis* Pb18 by intraperitoneal route and treated with ajoene. In weeks 2, 6, 10 and 13 of treatment, levels of anti-Pb antibodies were measured by the ELISA test and the animals were put down and their lungs, livers and spleens removed for histopathological analysis and determination of the number of viable fungus. The results show that experimental murine paracoccidioidomycosis was well established and that ajoene was capable of controlling the evolution of the disease, as it significantly reduced the levels of antibodies from the 10th week of treatment.

Key words Ajoene, *Paracoccidioides brasiliensis*, Treatment, ELISA

Actividad antifúngica del ajoeno en la paracoccidioidomicosis experimental en ratones

Resumen El producto natural ajoeno (4, 5, 9-trithiadodeca-1,6,11-trieno 9-óxido) fue capaz de controlar parcialmente la infección por *Paracoccidioides brasiliensis* en un modelo experimental murino. Fueron inoculados por vía intraperitoneal ratones Swiss con 5,0 x 10^6 células de la cepa Pb 18 de *Paracoccidioides brasiliensis*. Los animales fueron divididos en cuatro grupos, uno fue tratado con ajoeno, otro con itraconazol, un tercero no recibió tratamiento y el cuarto grupo no fue infectado y actuó como control. Se midieron los niveles de anticuerpos anti-Pb por ELISA a las dos, seis, 10 y 13 semanas de evolución. Los animales fueron sacrificados y se estudiaron pulmones, hígados y bazos, mediante exámenes histopatológicos y determinación de unidades formadoras de colonias. Los resultados observados permiten establecer que este modelo de paracoccidioidomicosis experimental produjo una infección progresiva y que el ajoeno fue capaz de controlar la evolución de la misma, como lo demuestra la reducción de la carga fúngica en hígados y bazos y la disminución significativa de los títulos de anticuerpos a la décima semana de tratamiento.

Palabras clave Ajoeno, *Paracoccidioides brasiliensis*, Tratamiento, ELISA

Paracoccidioidomycosis (PCM) is a systemic mycosis caused by the dimorphic fungus *Paracoccidioides brasiliensis*. This disease is endemic in Brazil, Venezuela and Colombia, where it is considered the most important mycosis [7]. It is a serious disease that when not properly diagnosed and treated often results in death. In Brazil, it is the eighth most common cause of death following chronic or recurrent types of infectious and parasitic diseases, and has the highest death rate among the systemic mycoses at 1.45/million inhabitants [4]. Treatment of PCM is slow and consists of an initial phase followed by a maintenance phase [13]. The most frequently used medicines are sulphonamides, azole derivatives and amphotericin B [24] with the latter being reserved for serious cases because of its high toxicity. Among the azole derivatives, itraconazole has been the most commonly used drug in the treatment of PCM due to its efficacy, tolerance and low toxicity. In this regard, terbinafine has also shown promising results both in vitro [9] and in vivo [16]. However, the use of these two drugs is still limited by their high cost. Although second-generation triazole derivatives, like voriconazole and posaconazole, have been shown to be effective in the treatment

Address for correspondence:
Dra. Terezinha I.E. Svidzinski
Division of Medical Mycology
State University of Maringá, Bloco J90, sala 11
Av. Colombo 5790
87020-900 Maringá
Paraná, Brazil
Tel.: +44 3261 4809
Fax: +44 32614860
E-mail: tiesvidzinski@uem.br

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of some systemic mycoses, their anti-*P. brasiliensis* activity still needs to be better evaluated. The echinocandins are a new class of drugs that may also be efficaceous, considering their action on the cell wall, however these drugs have not yet demonstrated efficacy against *P. brasiliensis* [6,19]. Furthermore, the high cost of these drugs prohibits their use in public health services.

Therefore, the treatment of paracoccidioidomycosis remains a challenge that has still not been completely resolved [13]. Because of the limitations of the antifungal agents cited above, additional compounds are currently being investigated. Plants are important sources of natural, biologically-active products, many of which are models for the synthesis of a large number of drugs, mainly because products found in nature have great structural diversity and a great diversity of physicochemical and biological properties [27]. Ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide) is an organic sulphur compound derived from garlic *(Allium sativum)* with important antimicrobial properties [1,2]. Its anti-malarial [18], anti-trypanosomal [25] and anti-fungal activity [10,29], against both yeasts [8] and filamentous fungi [5], has already been demonstrated. Some studies have showed that ajoene is efficacious in the topical treatment of superficial mycoses [11,12] as well as subcutaneous disease [17]. In addition to its in vitro antifungal action [20,26], ajoene is also capable of blocking the transition of *P. brasiliensis* from the mycelial phase to the yeast-like phase [21].

Therefore, the objective of this study was to evaluate the effect of ajoene in controlling the evolution of experimental paracoccidioidomycosis in a mouse model.

Materials and methods

Ajoene. Synthetic ajoene, patent 4665088, May 1987 [1], was prepared as previously described [2], and provided by the Instituto Venezolano de Investigaciones Científicas, Caracas. The ajoene was dissolved in ethanol, and diluted with intralipid (Lipofundin MCT/LCT 10%- B. BRAUN). The final concentration of ethanol was less than 1% (vol/vol).

Animals. Four to six week-old male Swiss mice obtained from Biotério Central of the State University of Maringá, and weighing 25 to 30 g, were used. During the period of the experiments, the animals were kept in the animal facilities of the Laboratory of Inflammation in the Pharmacy and Pharmacology Department of this university, where they received water *ad libitum* and balanced food.

Fungus. The isolate *P. brasiliensis* 18 (Pb 18) from the Mycology Collection of the Pathology in the Botucatu School of Medicine (UNESP- Botocatu) was used. The yeast cells were cultered in BHI-A (Brain Heart Infusion - Agar) at 35 ºC for seven days. To prepare the inoculum, the cells were washed three times with sterile phosphate buffered saline (PBS) pH 7.2. The cell viability was determined using the Janus Green vital stain. Cultures with a minimum of 80% viability were used and the concentration was adjusted by counting in a Neubauer chamber. The mice were infected by the intraperitoneal route with 0.5 ml of the fungal suspension at a concentration of 1.0×10^7 cells/ml (5×10^6 yeast cells).

Animal experiments and treatment. A total of 160 (120 infected with Pb18 and 40 not infected) mice were utilized in this study which was divided into four groups: ajoene (40 animals infected and treated with ajoene); itraconazole (40 animals infected and treated with itraconazole); positive control (40 animals infected but not treated) and negative control (40 animals not infected and not treated). Mice were treated by intraperitoneal injection of ajoene (20 mg/kg) every other day, or itraconazole (Sporanox®, Janssen - lot 303191), (20 mg kg/day, intra-gastric route), diluted with polietilenoglicol [14]. The treatment with ajoene and itraconazole was started four weeks after infection.

Treatment evaluation. In weeks 2, 6, 10, 13 of the treatment, 10 mice from each group were anaesthetized (50 mg/kg Ketamine hydrochloride and 50 mg/kg Thiazine hydrochloride, intraperitoneal route), blood samples were collected to determine the level of total antibodies against *P. brasiliensis,* and three organs, lungs, liver and spleen, were removed for enumeration of viable organisms and histopathology analysis.

IgG anti-gp43 detection in mouse sera by ELISA. Polystyrene microtitre plates (Costar) were coated with gp43 (50 µl/well) in 0.1 mol/l carbonate buffer, pH 9.6. The plates were washed with PBS containing 0.05% Tween 20 and the wells were blocked with PBS-T 5% skim milk (PBS- T-M) for 1 h at 37 ºC. After washing with PBS-T, aliquots of 100 µl of the serum samples, diluted 1:400 in PBS 1% milk (PBS-M) were added and incubated at 37 ºC for 1 h. The plates were then washed as above and 100 µl of conjugate anti-mouse IgG - peroxidase (Sigma) was added to each well. Plates were then incubated at 37 ºC for 1 h. After washing with PBS-T, the substrate (H_2O_2/tetramethylbenzidine) was added, and the reaction was stopped by adding 50 µl of 4N H_2SO_4. Absorbance was measured at 492 nm with an ELISA reader (EIA reader Titertek, Multiskan, MCC340). Serum from a mouse immunized with *P. brasiliensis* was used as a positive control. The negative control was a pool of sera from uninfected mice. Sera with twice or more the absorbance of the negative control were considered positive.

Assay for organ colony-forming units (CFU). The numbers of viable microorganisms in the lungs, liver and spleen from experimental and control mice were determined by counting the number of CFU. Aliquots (100 µl) of the cellular suspensions and its serial dilutions of each organ were plated on brain heart infusion (BHI) agar (Difco) supplemented with 4% (v/v) horse serum (Centro de Produção e Pesquisa de Imunobiológicos, Paraná, Brazil) and 5% Pb192 culture filtrate, the latter constituting a

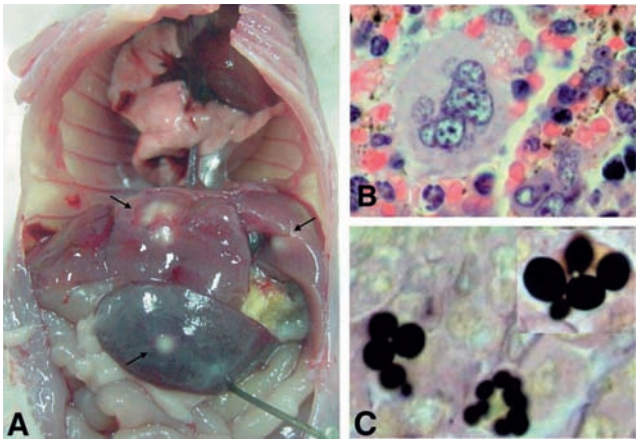

Figure 1. A. Swiss mice after laparotomy of the abdominal cavity, four weeks after inoculation with *P. brasileinsis* 18, showing accentuated hepatosplenomegaly: the arrows show the formation of paracoccidioic granuloma. B. Epithelioid granuloma with the presence of abundant giant, polymorphonuclear and mononuclear cells observed by stain with HE (x1000). C. Multiple budding yeast-like cells characteristic of *P. brasiliensis* in a histological cross-section of the spleen stained by Gomori-Grocott (x1000).

source of growth-promoting factor. The plates were incubated at 35 °C and colonies were counted daily until no increase in counts was observed.

Histopathology. Part of these organs were fixed in 10% neutral formalin (Sigma) and embedded in paraffin (Sigma). Serial sections (5 μm thick) were prepared and stained by hematoxylin- eosin (HE) and Gomori-Grocott methods.

Statistical Analyses for serologic results. The program GraphPad Prism® (Graphpad Software, inc.) was used for the statistical analysis. The results were expressed as mean ± the standard error of the mean (SEM). The different groups were analyzed in each week using analysis of variance (ANOVA) for multiple comparisons, followed by the Tukey test. The level of significance used was 0.05.

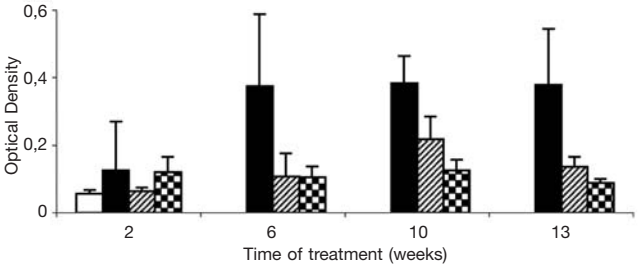

Figure 2. Levels of serum IgG anti-*P. brasiliensis* antibodies in Swiss mice infected, by intraperitoneal route, with 5.0 x 10^6 yeast-like cells in weeks 2, 6, 10 and 13 of treatment. Non-infected control ■ (n = 40), infected non-treated control □ (n = 40), treated with itraconazole, 20 mg/Kg ▨ (n = 40) and ajoene, 20 mg/kg ▩ (n = 40).
The results represent the mean ± SEM (standard error mean).

Results

In this study, murine paracoccidioidomycosis was established in 120 mice infected by the intraperitoneal route. After four weeks of infection, it was possible to observe typical symptoms of the infection. Important hepatosplenomegaly and paracoccidioic granulomas in the spleen, liver and abdominal cavity (Figure 1A) were observed, as well as alteration of the size, structure and coloration of the lungs. Histopathological analysis by HE stain demonstrated the presence of compact epithelioid paracoccidioic granulomas in the three organisms analyzed. The granulomas contained numerous giant cells, as well as polymorphonuclear and mononuclear cells (Figure 1B). The presence of typical structures of live parasitic fungi was also demonstrated in the tissues analysed by the Gomori-Grocott stain, as can be observed in the cross-section of the spleen (Figure 1C). Recovery of viable *P. brasiliensis* yeast cells counts (CFUs) in the lungs, spleen and liver of mice inoculated with Pb18 revealed that the infection had been established by the fourth week (Table).

Treatment with ajoene for 10 weeks enabled the reversal of these symptoms, as demonstrated by the significant decline ($p < 0.05$) of the levels of IgG anti-*P. brasiliensis* antibodies in the animals treated with ajoene in relation to the non-treated group (Figure 2). Among the animals that received itraconazole, a decline in the levels of antibodies should also be observed; however, there was no significant difference when compared to the non-treated group. After treatments with ajoene and itraconazole were started, no viable cells of *Paracoccidioides brasiliensis* were found in either the spleen or liver of the infected animals. On the other hand, the fungal load in the lungs of control mice was similar to that found at weeks 4, 8 and 10 after infection.

Discussion

The establishment of experimental models for the study of PCM has permitted a good correlation with the human disease, clarifying the understanding of the parasite-host relation, of therapeutic actions and of the establishment of the prognosis of the disease. The intraperitoneal route is often used due to its easy performance and good reproductivity [23]. Figure 1 shows clinical alterations in infected mice, proving that an infection was established. Others findings reinforce this affirmation: the inflammatory infiltrates seen by the histopathological examination, typical structures of *P. brasiliensis* found in the organs, and the presence of viable microorganisms in cultures obtained in the fourth week of infection.

Among the three criteria for documenting the evolution of murine PCM, the best evidence was obtained with the determination of the antibody levels. Viable fungal cells and the histopathological alterations in the control group (infected and not treated) disappeared quickly, probably due to natural resistance of Swiss mice to the PCM. Apparently the animals themselves had been capable of controlling the infection by week 10 after inoculation. However it is important to remember that, as occurs in humans, the absence of viable cells of *P. brasiliensis* in the lesions does not imply a cure, and a much longer monitoring period would be required

The determination of the antibody levels is very useful in the serodiagnosis of PCM as well as in the evolution of the disease under antifungal treatment, as it acts as an important marker of active disease. High antibody levels are related to the severity of the disease in humans, increasing in relapses and declining during effective treatment or after cure. Camargo and Franco [3] have encouraged the use of ELISA for patients' follow up, as

Table. Numbers of viable Pb18 cells in the lungs, spleen and liver from experimental and control mice intraperitoneally infected with 5x10^6 yeast cells.

Group	[2]Time of infection	Organ[1]		
		Lung	Apleen	Liver
Positive control	4	294.875 ± 61.28	7.500 ± 3.13	0.625 ± 0.2630
	8	283.286 ± 93.44	90.000 ± 44.67	1.4286 ± 1.403
	10	240.396 ± 62.34	26.754 ± 32.78	0.5360 ± 0.451
Itraconazole	4	280.258 ± 47.82	21.6480 ± 0.71	0.9938 ± 0.243
Ajoene	4	279.712 ± 52.64	22.0740 ± 0.69	0.9182 ± 0.4571

[1]Number of cfu/g of tissue. The results represent the mean ± SEM (standard error of the mean) of six to eight mice.
[2]Time in weeks, in both treated groups (itraconazole and ajoene). No CFU were obtained in the spleen and liver at weeks 8 and 10 after infection, that is to say at weeks 4 and 6 after the treatment had been started.

the method is relatively simple to perform, gives quantitative results, is highly sensitive, and the reagents have a long shelf life. Furthermore, different classes of immunoglobulins can be determined, thus allowing a more precise evaluation of the humoral immune response. Recently ELISA was used in patients with chromoblastomycosis caused by *Cladophialophora carrionii* [15].The antibody levels in this study were determined before, during and after treatment with either ajoene or itraconazole.

The results obtained in our study show that the production of anti-*P. brasiliensis* antibodies by the animals of the non-treated group increased from the 14th week of infection, suggesting it was the most severe period of the disease. This period corresponds to 10 weeks of treatment and to the period in which ajoene promoted a significant reduction in the levels of anti-*P. brasiliensis* antibodies. In the 10th week of treatment, when the disease was found to be most active, a significant decline in the levels of antibodies and the disappearance of the fungi in the tissues of the animals that received ajoene compared to the non-treated group mentioned was observed. Therefore, the natural product ajoene was effective in the control of the disease, with better results than those observed in the group treated with itraconazole. In the 13th week of treatment, the reduction of the levels of antibodies observed in the group of animals treated with ajoene was comparable to those that had received itraconazole, a medicine with proven antifungal action and considered in Brazil the reference drug for the treatment of PCM. The results obtained in this paper show that ajoene has antifungal activity against *P. brasiliensis* in the dose tested, as it caused the elimination of the fungi and significantly reduced the levels of anti-*P. brasiliensis* antibodies, with a similar or slightly better effect than that found in the group treated with itraconazole; therefore suggesting control of the disease. Research in this direction with novel compounds shows promise, as there is a consensus that new treatment modalities which are more potent, less toxic, and less expensive, may be found among new classes of drugs [28].

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