The activity of azithromycin against *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* in the golden hamster model

A atividade da azitromicina contra a *Leishmania (Viannia) braziliensis* e a *Leishmania (Leishmania) amazonensis* no modelo *golden hamster*

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ABSTRACT

New therapeutic alternatives against leishmaniasis remain a priority. The activity of azithromycin against Leishmania *(*Leishmania) major *has been previously demonstrated. Different responses among species of* Leishmania *make species-specific drug screening necessary. The activity of azithromycin against* Leishmania (Viannia) braziliensis *and* Leishmania (Leishmania) amazonensis *was evaluated in golden hamsters infected through footpad injections of metacyclic promastigotes, and compared with untreated controls and animals treated with meglumine antimoniate. Footpad thickness, lesion cultures and dissemination sites were analyzed. Treatment of golden hamsters with oral azithromycin at 450mg/kg had no activity against infections with* Leishmania (Leishmania) amazonensis*. For infections due to* Leishmania (Viannia) braziliensis*, azithromycin demonstrated significant activity relative to untreated controls, but inferior to meglumine antimoniate, for controlling lesion size. Neither drug was able to totally eliminate parasites from the lesions. It was concluded that azithromycin has activity against* Leishmania (Viannia) braziliensis *but not against* Leishmania (Leishmania) amazonensis *in this model.*

Key-words: *Leishmania* Viannia braziliensis. Leishmania Leishmania amazonensis. *Azithromycin. Hamster.*

RESUMO

Novas alternativas terapêuticas contra a leishmaniose são ainda uma prioridade. A atividade da azitromicina contra a Leishmania (Leishmania) major *foi anteriormente demonstrada. Diferentes respostas entre as espécies de* Leishmania *fazem com que um* screening *de drogas específicas para espécies seja necessário. A atividade da azitromicina contra a* Leishmania (Viannia) braziliensis *e a* Leishmania (Leishmania) amazonensis *foi avaliada em* Golden hamsters *infectados a través de injeções de promastigotas metacíclicas e comparando com controles sem tratamento e animais tratados com antimoniato de N-metil-glucamina. Foram analisadas a espessura da pata, a cultura das lesões e disseminação para órgãos internos. A azitromicina oral em dose de 450mg/kg não teve atividade contra a infecção por* Leishmania (Leishmania) amazonensis*. Para infecções devidas à* Leishmania (Viannia) braziliensis*, a azitromicina teve uma atividade significativa em relação aos controles sem tratamento, mas foi inferior ao antimoniato de N-metil-glucamina quanto ao controle do tamanho das lesões. Nenhuma das drogas conseguiu eliminar totalmente os parasitos das lesões. Foi concluído que a azitromicina tem atividade contra* Leishmania (Viannia) braziliensis*, mas não tem atividade contra* Leishmania (Leishmania) amazonensis *neste modelo.*

Palavras-chaves: Leishmania Viannia braziliensis. Leishmania Leishmania amazonensis*. Azitromicina. Hamster.*

The search for new therapies against leishmaniasis in its different forms continues to be a clinical priority. *Leishmania (Viannia) braziliensis* has been identified as the causative agent of most cases of cutaneous and mucosal leishmaniasis in Argentina, although a role for *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) guyanensis* has recently been described in a minority of cases[3, 8]. Infection of golden hamsters with *Leishmania (Viannia) braziliensis* results in local lesions and dissemination[15]. In the case of infections with *Leishmania (Leishmania) amazonensis,* golden hamsters develop florid lesions at the inoculation site that tend to grow chronically with dissemination to metastatic foci. Treatments with

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meglumine antimoniate are able to control but not to sterilize the lesions caused by both *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*[2 11].

The activity of azithromycin against *Leishmania (Leishmania) major in vitro* and in BALB/cByJ mice has been previously demonstrated. *In vitro*, azithromycin significantly reduced parasite numbers in cell-free cultures, the number of amastigotes per macrophage and the number of infected macrophages. Treatment of BALB/cByJ mice with azithromycin caused diminished lesion size and parasite load[6 7].

Azithromycin has a benign toxicity profile, good tolerance in pediatric populations, oral and parenteral formulations and a lack of contraindications during pregnancy (FDA category B)[14]. This drug also has preferential active concentration in macrophages[5].

The objective of this study was to determine the effect of azithromycin on *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* infections of golden hamsters using experimental systems designed for each species.

MATERIAL AND METHODS

Drugs. Azithromycin (Zitromax™, Pfizer) powder was mixed in water to a dilution of 40mg/ml. Meglumine antimoniate (Lazar, Argentina) was used from vials containing 5ml of solution (1.5g of antimony, corresponding to 425mg of SbV).

Parasites. The strains IFLA/BR67/PH8 of *Leishmania (Leishmania) amazonensis* and MHOM/AR/90LEA4 of *Leishmania (Viannia) braziliensis* were used. For both strains, promastigotes in the stationary phase were obtained from six-day-old *in-vitro* cultures in Senekjie medium with RPMI1640 (GIBCO, USA) and 20% fetal bovine serum at 26ºC. The parasites in these cultures originated from lesions in golden hamsters. Promastigotes for inoculation were counted in Neubauer chambers.

Animals. Male and female 55 to 65-day-old golden hamsters (*Mesocriscetus auratus*) bred at the animal facilities of the *Mario Fatala Chabén* National Parasitology Institute were used in the experiments. Throughout the experiments, the animals were housed in cages of two to three animals with food and water *ad libitum*. In every experiment, each group included at least five animals.

Infection and treatment. Animals were subcutaneously inoculated in the right footpad with 1 x 10^6 metacyclic promastigotes of *Leishmania (Leishmania) amazonensis* or 5 x 10^5 metacyclic promastigotes of *Leishmania (Viannia) braziliensis* using a 25g 5/8" needle. Drug treatments were started immediately after infections, at doses of 450mg/kg/day for five days a week through an oral cannula for azithromycin and at doses of 60mg/kg/day intramuscularly for five days a week for meglumine antimoniate, based on previous efficacy reports for hamsters infected with *Leishmania (Viannia) panamensis*[15]. The dose of azithromycin was chosen based on the maximum dose that was well tolerated by the animals in a preliminary experiment. The treatment duration was four weeks for meglumine antimoniate; treatments with azithromycin lasted eight weeks for *Leishmania (Leishmania) amazonensis* and five weeks for *Leishmania (Viannia) braziliensis*. The differential duration of the treatments with azithromycin was defined in the experimental design based on the known duration and aggressiveness of lesions due to *Leishmania (Leishmania) amazonensis* compared with *Leishmania (Viannia) braziliensis*[13].

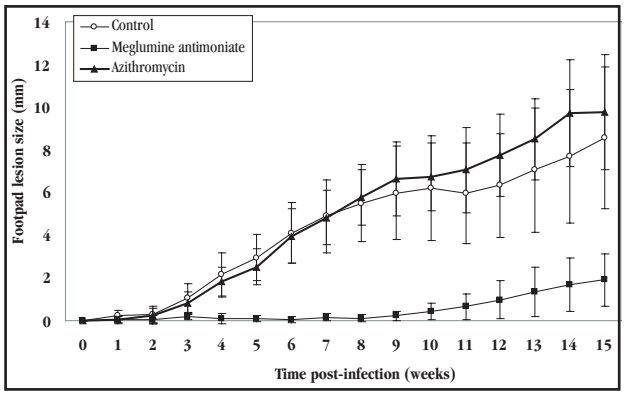
Lesion size was determined weekly by measuring the thickness of the infected and contralateral footpad using a digital caliper (Schwyz Model SC111101E, Switzerland), and the difference between the footpads was calculated. *Leishmania (Leishmania) amazonensis* infections were followed for up to 15 weeks and *Leishmania (Viannia) braziliensis* infections for up to 7 weeks.

Dissemination was studied by taking samples from homolateral popliteal lymph nodes, liver and/or spleen. These tissues were processed and cultured in Senekjie medium for 30 days, after which the presence of live parasites was determined. Semiquantitative parasite counts were made on biopsy tissue of approximately 10mg that was taken from the lesion at the end of the experiment. This tissue was weighed and a homogenate was prepared in a grinder with RPMI1640 containing 20% fetal calf serum. Serial tenfold dilutions were prepared in 96-well plates and incubated at 26ºC for 14 days. The highest dilution with moving parasites was the final titer for each sample. Each biopsy was cultured in duplicate.

Data analysis. All experiments were performed at least in duplicate; the data presented represent a summary from all experiments. The data were analyzed for statistical significance with the Mann-Whitney test or Student t test when appropriate. Differences were considered significant with P values of <0.05. The data analysis was performed using SPSS for Windows, version 12.0 (SPSS Inc, USA).

RESULTS

Effect of azithromycin on *Leishmania (Leishmania) amazonensis* infections. Footpad lesions developed in untreated infected golden hamsters within one week of infection. Treatment of infected animals with azithromycin had no effect on the size of the lesions (Figure 1). Semiquantitative analysis of parasite load in the lesion and cultures of regional lymph nodes at 10 weeks also showed no effect compared with untreated animals (Table 1). Meglumine antimoniate was effective for controlling the development of lesions for 10 weeks, after which the lesions increased in size until the end of the experiment (Figure 1). In a significant number of animals treated with meglumine antimoniate, at 10 weeks postinfection, parasites were not recovered from the lesions and dissemination was blocked (Table 1). However, at 15 weeks postinfection there was uniform positivity of lesion and popliteal lymph node cultures in all groups. Dissemination to the liver was observed in one of the six animals in the untreated control group and in none of the treated animals (five with meglumine antimoniate and five with azithromycin) at 10 weeks postinfection,



P <0.05 between meglumine antimoniate and the other groups from week 4 until the end of the experiment.

Figure 1 - Effect of treatment with 450mg/kg of oral azithromycin for 8 weeks or 60mg/kg of subcutaneous meglumine antimoniate on the development of footpad swelling caused by infections with 1 x 10^6 metacyclic promastigotes of* Leishmania (Leishmania) amazonensis *in Golden hamsters. Ten animals per group were followed until week 10, when half were sacrificed and the rest were followed until week 15. The values represent the mean ± standard deviation difference between the thickness of the infected and the contralateral footpad.

but this difference did not reach statistical significance. Similarly, dissemination to the spleen was observed in two of the ten untreated animals but in none of those treated with meglumine antimoniate (nine) or azithromycin (ten).

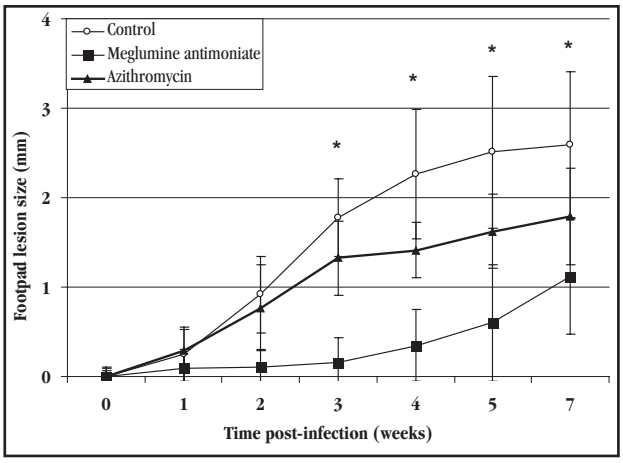
Table 1 - Positive cultures of lesions (skin), regional lymph nodes, liver and spleen at 10 weeks post infection in animals infected with* Leishmania (Leishmania) amazonensis *IFLA/BR67/PH8 and treated with meglumine antimoniate for 4 weeks or azithromycin for 8 weeks. Data represents number of positive animals/number of animals in the group.

	Control	Meglumine antimoniate	Azithromycin
Lesion	10/10	5/10*	9/9
Lymph node	8/10	2/10**	6/8
Liver	1/6	0/5	0/5
Spleen	2/10	0/10	0/9

*p<0.01 compared to other 2 groups.

**p=0.01 compared to control and 0.02 compared to azithromycin.

Effect of azithromycin on *Leishmania (Viannia) braziliensis* infections. Statistically significant increases in footpad thickness developed at one week postinfection in untreated animals and in animals treated with azithromycin. At three weeks postinfection, the animals treated with meglumine antimoniate developed lesions. Treatments with meglumine antimoniate resulted in lesions with delayed appearance and lower peak swelling (Figure 2). The animals treated with azithromycin had lesions that were significantly smaller than those in untreated controls and larger than those in golden hamsters treated with meglumine antimoniate, from three weeks postinfection (p = 0.002) until the end of the experiment (Figure 2). Recovery of viable parasites from the lesions at seven weeks postinfection was performed without evidence of significant differences between groups. At seven weeks, dissemination was confirmed in popliteal lymph nodes homolateral to the lesions in all groups, without evidence of significant differences between them (Table 2). Dissemination of parasites to the liver was detected in one out of 18 animals, in the meglumine antimoniate group, at seven weeks postinfection (Table 2).



* p <0.05 for comparisons between all three groups.

Figure 2 - Effect of treatment with 450mg/kg of oral azithromycin for 5 weeks or 60mg/kg of subcutaneous meglumine antimoniate for 4 weeks on the development of footpad swelling caused by infections with 5 x 10^5 metacyclic promastigotes of* Leishmania (Viannia) braziliensis *in Golden hamsters. Ten animals per group were followed throughout each of the 2 repetitions of the experiment. The values represent the mean ± standard deviation difference between the thickness of the infected and the contralateral footpad.

Table 2 - Positive cultures of lesions (skin) regional lymph nodes and liver at 7 weeks post infection in animals infected with* Leishmania (Viannia) braziliensis *MHOM/AR/90LEA4 and treated with meglumine antimoniate for 4 weeks or azithromycin for 5 weeks. No significant differences between groups were identified. Data represents number of positive animals/number of animals in the group.

	Control	Meglumine antimoniate	Azithromycin
Lesion	16/16	14/18	13/14
Lymph node	12/16	10/18	11/14
Liver	0/16	1/18	0/14

DISCUSSION

Our results show that *Leishmania (Leishmania) amazonensis* infections in golden hamsters are resistant to azithromycin, such that the treated animals developed infections indistinguishable from untreated controls. Meglumine antimoniate was used as a positive control and demonstrated its efficacy in limiting infections. Control over the parasite was however not absolute, in that lesions and local lymph nodes were infected with parasites 15 weeks postinfection (11 weeks after treatment completion). This finding is consistent with previous reports regarding treatment of *Leishmania (Leishmania) amazonensis* and other species of *Leishmania* with antimony compounds, in hamsters[2].

The lesion size in the animals infected with *Leishmania (Viannia) braziliensis* was significantly reduced by treatment with azithromycin and meglumine antimoniate. Azithromycin was unable to totally block the development of foot pad swelling, although the lesions were significantly smaller in treated animals than in untreated controls. These results mirror those seen in BALB/cByJ mice infected with *Leishmania (Leishmania) major* and treated with azithromycin[6], in which animals were treated subcutaneously using regimens of 100 to 200mg/kg/day for

eight weeks. Meglumine antimoniate was significantly superior to oral azithromycin for controlling infection by *Leishmania (Viannia) braziliensis*. However, as in the case of *Leishmania (Leishmania) amazonensis*, lesions developed in the mice treated with meglumine antimoniate at the end of the experimental period. This observation confirms previous reports that meglumine antimoniate does not eliminate all *Leishmania* parasites from golden hamsters using similar treatment regimens[15].

The use of positive and negative controls gives support to the conclusions and makes it unlikely that the differences in the results between the two species described in this report might be related to the different inoculum used for each species ($1x10^6$ for *Leishmania (Leishmania) amazonensis* and $5x10^5$ for *Leishmania (Viannia) braziliensis*). The effects of azithromycin and meglumine antimoniate at higher and lower inoculum doses or in different therapeutic schedules were not investigated.

The mechanism of action of azithromycin against *Leishmania* is unknown. Azithromycin has been shown to have immunomodulatory effects in humans with bronchiolitis obliterans[4], and treatment of macrophages with azithromycin enhanced the elimination of fungal pathogens[16]. Alternatively, it is possible that azithromycin has direct antimicrobial activity on the parasites. In the case of pentavalent antimonials, with over 50 years of use for treating leishmaniasis, their mechanism of action is also still uncertain: both direct and immune-mediated mechanisms have been implicated[9].

In addition to the preclinical studies performed on mice for evaluating azithromycin against *Leishmania* parasites[6], the clinical experiences include an uncontrolled study that involved 20 patients with cutaneous leishmaniasis in an area endemic for *Leishmania (Viannia) braziliensis* treated with variable doses of azithromycin for two to ten days with a cure rate of 85%[10]. Contrasting with these findings, in an area endemic for *Leishmania (Leishmania) major* in Syria, no cures were recorded among 45 patients with cutaneous leishmaniasis using ten-day cycles of azithromycin[1]. Three cases of mucosal leishmaniasis with contraindications for the use of antimonials were successfully treated with three cycles of ten days of azithromycin in Brazil[12].

Based on the discrepant responses of these species to azithromycin, clinical trials aimed at testing the findings from the current study must take into consideration the species involved in the treated patients. In conclusion, azithromycin has activity against *Leishmania (Viannia) braziliensis* but not against *Leishmania (Leishmania) amazonensis* in golden hamsters. This activity is inferior to meglumine antimoniate for lesion control, and yet both drugs failed to eliminate all parasites from the lesions. Further studies should evaluate the clinical use of azithromycin as an oral option for treating American cutaneous leishmaniasis caused by *Leishmania (Viannia) braziliensis*.

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