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Tamoxifen as a potential antileishmanial agent: efficacy in the treatment of *Leishmania braziliensis* and *Leishmania chagasi* infections

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***Objectives*: The aim of this study was to evaluate the efficacy of tamoxifen *in vivo* in experimental models of cutaneous (CL) and visceral leishmaniasis (VL) caused by *Leishmania braziliensis* and *Leishmania chagasi*, respectively.**

***Methods*: Drug activity was assessed against intracellular amastigotes by treating infected macrophage cultures and evaluating the number of infected cells. *In vivo* efficacy of tamoxifen was tested in *L. braziliensis*-infected BALB/c mice and in *L. chagasi*-infected hamsters. Treatment with 20 mg/kg/day tamoxifen was administered for 15 days by the intraperitoneal route. Efficacy was evaluated through measurements of lesion size, parasite burden at the lesion site or liver and spleen and survival rate.**

***Results*: Tamoxifen killed *L. braziliensis* and *L. chagasi* intracellular amastigotes with 50% inhibitory concentrations (IC_{50}) of 1.9 ± 0.2 and 2.4 ± 0.3 μM, respectively. Treatment of *L. braziliensis*-infected mice with tamoxifen resulted in significant reductions in lesion size and 99% decrease in parasite burden, compared with mock-treated controls. *L. chagasi*-infected hamsters treated with tamoxifen showed significant reductions in liver parasite load expressed as Leishman–Donovan units and 95% to 98% reduction in spleen parasite burden. All animals treated with tamoxifen survived while 100% of the mock-treated animals had died by 11 weeks after the interruption of treatment.**

***Conclusions*: Tamoxifen is effective in the treatment of CL and VL in rodent models.**

Keywords: chemotherapy, cutaneous leishmaniasis, visceral leishmaniasis, selective oestrogen receptor modulator, SERM

Introduction

Leishmania braziliensis is the most common causative agent of cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis in the New World and the main cause for the high annual incidence of the disease in Brazil.[1] Visceral leishmaniasis (VL), caused by *Leishmania chagasi* in Latin America, is the most severe form of the disease and may lead to death if untreated.[1]

Leishmaniasis treatment is based on parenteral administration of highly toxic drugs, including pentavalent antimonials, amphotericin B and pentamidine. Resistance to antimonials is widespread in India and oral administration of miltefosine has emerged as an alternative approach, having been approved for management of VL.[2] Despite current reports validating miltefosine as a satisfactory chemotherapeutic compound in the treatment of *L. braziliensis*-infected patients in Bolivia, low effectiveness against *L. braziliensis*-infections has been reported in Guatemala.[2] Therefore, the investigation of alternative leishmanicidal drugs remains imperative.

Tamoxifen, a classical oestrogen receptor antagonist in breast tissue, has been in clinical use for the treatment of breast cancer since 1971.[3] We have previously shown that tamoxifen is active

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against several species of *Leishmania in vitro*.[4] Recently, we have also demonstrated that treatment of *L. amazonensis*-infected BALB/c mice with tamoxifen results in significant reductions in lesion and ulcer sizes, as well as in a sharp decrease in parasite burden.[5]

In this work, we have focused on the investigation of tamoxifen efficacy in the treatment of leishmaniasis using two distinct rodent models to mimic CL and VL caused by *L. braziliensis* and *L. chagasi*, respectively.

Materials and methods

The strains used were *L.* (*Viannia*) *braziliensis* (MHOM/BR/2001/BA788) and *L.* (*Leishmania*) *chagasi* (MHOM/BR/1972/LD).

Drug cytotoxicity and activity against intracellular amastigotes were performed as described previously,[4] except that BALB/c bone marrow-derived macrophages were used. Assays with the reference drug meglumine antimoniate (kindly donated by Sanofi-Aventis) were performed in parallel as described previously.[6] Experiments were repeated at least three times.

In vivo experiments were approved by the Ethics Committee for Animal Experimentation. Female BALB/c mice ($n=6-8$) were infected in the left ear with 1×10^5 *L. braziliensis* promastigotes as described previously.[7] Three weeks post-infection, groups were randomized according to the lesion size and 20 mg/kg/day tamoxifen (6 mg/mL tamoxifen citrate solutions in 150 mM NaCl), 20 mg/kg/day meglumine antimoniate or sterile saline was administered by the intraperitoneal (ip) route for 15 days. Lesion size was recorded as the difference between infected and non-infected ear thickness. Parasite burden was determined 6 weeks after infection using the limiting dilution method.[5] Body weight was recorded before and after treatment.

Golden hamsters (male or female, $n=6-12$) were infected with 1×10^8 *L. chagasi* amastigotes ip. Four weeks post-infection, animals were treated as described for BALB/c mice. At the end of the treatment, parasite burden was determined in the liver as Leishman–Donovan units (LDU) and in the spleen by the limiting dilution assay.[5] Parasite quantification was obtained for half of the animals in each group. The survival rate of the remaining animals was followed up for 3 months after the interruption of treatment. Serum concentrations of urea and creatinine were determined in hamsters at the end of treatment, using sets of commercial reagents (Doles Reagentes e Equipamentos para Laboratórios, Ltda., Brazil). Each *in vivo* experiment was repeated independently at least twice.

L. braziliensis and *L. chagasi* promastigotes differentiated from amastigotes recovered from treated animals were used to test drug sensitivity by determination of tamoxifen IC_{50} through cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as previously described.[4]

Statistical analysis was performed using one-way ANOVA followed by Dunnett's *post hoc* test (GraphPad Prism, CA, USA). A P value of <0.05 was considered statistically significant.

Results

Prior to the determination of tamoxifen activity against intracellular amastigotes, cytotoxicity assays were performed. The IC_{50} of tamoxifen for bone-marrow macrophages was higher than 20 μM. The treatment of *L. braziliensis*- or *L. chagasi*-infected macrophages with increasing concentrations of tamoxifen for 48 h allowed the determination of IC_{50}s for intracellular amastigotes (Table 1) and indicated that infection was completely abrogated with 9 μM tamoxifen. The reference drug meglumine antimoniate was assayed in parallel with tamoxifen and IC_{50} values were within the expected range (Table 1).

L. braziliensis-infected BALB/c mice were treated with saline, tamoxifen or meglumine antimoniate ip for 15 days. The treatment was initiated 3 weeks post-inoculation allowing the establishment of infection and development of lesions. At the end of the treatment, no statistically significant differences were detected in body weight between groups (tamoxifen, 22.6 ± 0.7 g; meglumine antimoniate, 23.9 ± 1.6 g; control, 23.3 ± 2.0 g).

Seven days after the end of treatment, all the animals that received saline showed erythema and swelling at the infection site. A significant decrease in the average lesion size was observed for tamoxifen and meglumine antimoniate-treated mice compared with mock-treated animals (Figure 1a). The average size of lesions at this timepoint was smaller in mice treated with tamoxifen than with meglumine antimoniate but this difference was not statistically significant.

Parasite burden in the lesion was evaluated at 6 weeks after inoculation of parasites (Figure 1b). Significant reductions of 99.0% and 99.9% were observed in tamoxifen- and meglumine antimoniate-treated mice, respectively (Figure 1b).

The treatment of *L. chagasi*-infected golden hamsters was initiated 4 weeks after infection. At the end of treatment, significant decreases in the liver (Figure 2a) and spleen (Figure 2b) parasite burden were detected in hamsters treated with tamoxifen compared with the control group. Tamoxifen was as effective as meglumine antimoniate with a 95% to 98% reduction in parasite load.

Table 1. *In vitro* activity of tamoxifen and meglumine antimoniate against *Leishmania* intracellular amastigotes

	Tamoxifen (μM)[a]		Meglumine antimoniate (μg/mL)[b]	
	IC_{50} (95% CI)	IC_{90}	IC_{50} (95% CI)	IC_{90}
L. (*V.*) *braziliensis*	1.9 ± 0.2 (1.7–2.1)	6.0	77.3 ± 12.1 (63.6–87.8)	404.9
L. (*L.*) *chagasi*	2.4 ± 0.3 (2.1–2.7)	7.9	259.3 ± 44.5 (214.8–303.8)	1024.7

[a]Values are expressed as means ± SD; 95% confidence intervals are shown in parentheses.
[b]Values are expressed as pentavalent antimony [Sb^V].

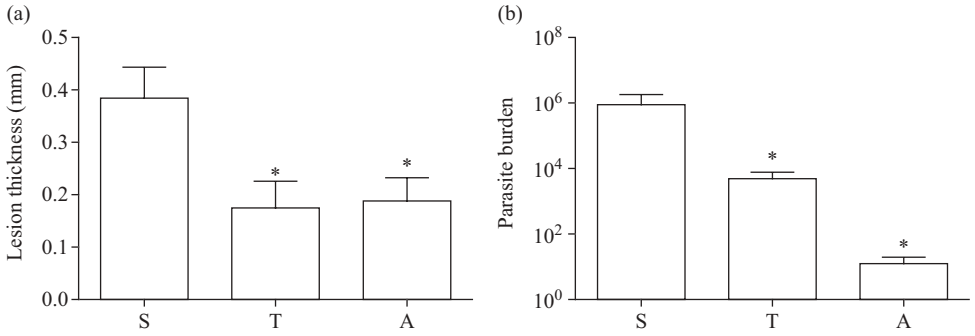

Figure 1. *In vivo* efficacy of tamoxifen in *L. braziliensis* infection. Female BALB/c mice were infected with *L. braziliensis* promastigotes in the left ear. Three weeks after infection, treatment was initiated with saline (S), 20 mg/kg/day tamoxifen (T) or meglumine antimoniate (A) ip for 15 days. (a) Lesion thickness recorded 6 weeks after infection ($*P < 0.05$). (b) Parasite burden quantified by limiting dilution 7 days post-treatment ($*P < 0.05$). The results correspond to the mean of three independent experiments, each of them with $n = 6$ per group.

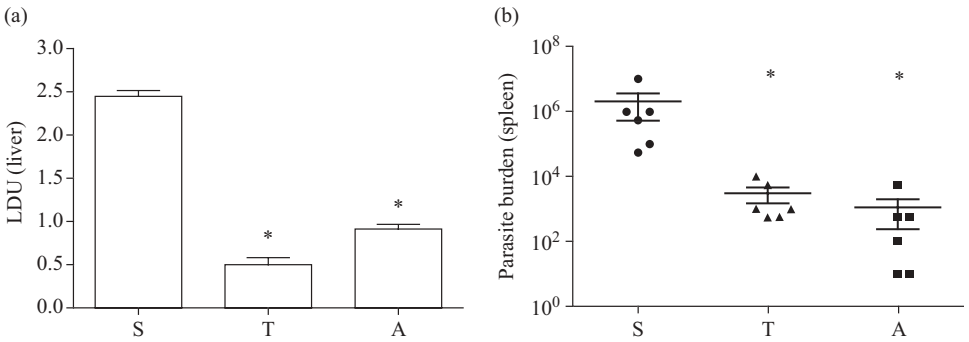

Figure 2. *In vivo* efficacy of tamoxifen in *L. chagasi* infection. Golden hamsters were infected with *L. chagasi* amastigotes. Four weeks post-infection, treatment was initiated with saline (S), 20 mg/kg/day tamoxifen (T) or meglumine antimoniate (A) ip for 15 days. (a) LDU measured by the number of amastigotes/cell×liver weight (mg) ($*P < 0.0001$). (b) Parasite burden quantified by limiting dilution at the end of the treatment (7 weeks post-infection) ($*P < 0.05$). The results correspond to one of two independent experiments, $n = 12$ per group.

To evaluate nephrotoxicity after treatment, serum levels of urea and creatinine were determined. No statistically significant alterations were detected between groups (data not shown). Additionally, at the end of treatment, the average body weight showed no significant variation between untreated (138.7 ± 12.4 g), tamoxifen-treated (127.8 ± 9.3 g) and meglumine antimoniate-treated animals (139.4 ± 15.9 g).

After the interruption of treatment, the survival rate was also assessed. Death in saline-treated animals was detected from week 12 post-infection. By week 18, all control hamsters had died, while a 100% survival rate was registered in tamoxifen and meglumine antimoniate-treated groups.

Lastly, we investigated whether parasites recovered from tamoxifen-treated mice or hamsters presented reduced sensitivity to the drug. Tamoxifen IC_{50} values for *L. braziliensis* and *L. chagasi* promastigotes recovered from tamoxifen-treated animals were not different from those determined for parasites isolated from control mice (17.7 ± 0.4 and 18.4 ± 0.7 μM for treated and control *L. braziliensis* and 16.1 ± 1.9 and 14.7 ± 2.9 μM for treated and control *L. chagasi*, respectively).

Discussion

Tamoxifen, a triphenylethylene derivative, has been shown to be cytotoxic to several neoplastic cell types.[8] *In vitro* effects of tamoxifen have also been established for fungal cells[9] and *Leishmania* parasites.[4] Having demonstrated the *in vitro* activity of tamoxifen against several species of *Leishmania*, we now show its effectiveness *in vitro* and *in vivo* against an *L. braziliensis* isolate obtained from a Brazilian patient with CL and on a *L. chagasi* reference strain.

For *in vivo* efficacy tests, the tamoxifen dose was chosen based on previous reports that established the drug levels in mouse serum after ip administration of 25–100 mg/kg/day tamoxifen.[10] The dosage scheme used in the present experiments did not lead to alteration of body weight or other toxic effects.

An ideal experimental model for *L. braziliensis* infections would be characterized by initial cutaneous lesions followed by spreading to mucocutaneous sites. Unfortunately, such a model is unavailable as yet. BALB/c mice infected in the ear dermis behave as a model of localized CL with erythema and oedema developing at the inoculation site after 3 weeks of infection and evolving to spontaneous healing within 10 weeks of infection. Consequently, to evaluate treatment efficacy, we compared lesion sizes and parasite burden at the lesion peak, 6 weeks after infection. Meglumine antimoniate's effect was more pronounced than tamoxifen's but both drugs significantly reduced the number of parasites at the site of infection. As expected, treatment with meglumine antimoniate did not lead to sterile cure, as observed previously in animal models as well as in humans.[11]

We have also established that tamoxifen, when administered to hamsters infected with *L. chagasi*, significantly reduces parasite numbers in both the liver and the spleen. Parasite burden quantified after treatment with tamoxifen was equivalent to that observed in animals receiving meglumine antimoniate. Outstandingly, the percentage of survival was identical between drug-treated groups, whereas saline-treated animals did not survive 18 weeks post-infection. Hamsters infected with *L. chagasi* develop a progressive disease closely mimicking active human VL. This is, therefore, an appropriate model to test the effect of tamoxifen on *L. chagasi* infection and confirm its effective leishmanicidal action.

The anti-oestrogen tamoxifen is one of the most prescribed anticancer drugs in the world. Indicated for treatment or prevention of breast cancer, it is used continuously for 5 years with daily doses of 20–40 mg.[12] Clinical trials have extensively examined the side effects of tamoxifen and the most worrying consequence of prolonged use of this drug is the potential development of endometrial carcinoma.[3] In this work, tamoxifen was effective as a short-term treatment. We have previously observed that this scheme does not induce changes in uterine weight or histopathology in BALB/c mice[5] (data not shown).

The efficacy of tamoxifen was the same in male and female hamsters. As the majority of clinical experience with tamoxifen derives from its use in women, concerns could be raised about its toxicity in men. Interestingly, clinical trials have been conducted to determine tamoxifen's usefulness as an antimanic agent.[13,14] Daily tamoxifen doses of 10–80 mg/day for up to 3 weeks were well tolerated in a controlled test treating 29 patients diagnosed as having bipolar disorder.[13] Another double-blind placebo-controlled study reported that 20–140 mg/day tamoxifen for 3 weeks did not induce any severe adverse effect in men or women undergoing the treatment. Decreased appetite was the only statistically significant event with increased frequency in the tamoxifen group compared with placebo.[14] These data indicate that tamoxifen could be safely used in men as well.

The data presented here together with our previously reported findings on tamoxifen's efficacy in *L. amazonensis*-infected mice[5] provide the grounds to extend the tests to other models of *Leishmania* infection. This pioneering alternative may work as a novel chemotherapeutic approach to treat leishmaniasis.

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Transparency declarations

None to declare.

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