

The anticancer drug tamoxifen is active against *Trypanosoma cruzi* in vitro but ineffective in the treatment of the acute phase of Chagas disease in mice

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The activity of the antineoplastic drug tamoxifen was evaluated against Trypanosoma cruzi. In vitro activity was determined against epimastigote, trypomastigote and amastigote forms of CL14, Y and Y benznidazole resistant T. cruzi strains. Regardless of the strain used, the drug was active against all life-cycle stages of the parasite with a half maximal effective concentration ranging from 0.7-17.9 μM. Two experimental models of acute Chagas disease were used to evaluate the in vivo efficacy of treatment with tamoxifen. No differences in parasitemia and mortality were observed between control mock-treated and tamoxifen-treated mice.

Key words: chemotherapy - Chagas disease - selective oestrogen receptor modulator

Chemotherapy of Chagas disease remains a major challenge in the Americas. As a result of sustained campaigns of vector control, the number of new infections in endemic areas has decreased, but the disease still afflicts 10-15 million people in Latin America (Schofield et al. 2006). No satisfactory treatment for *Trypanosoma cruzi* infection is available (Pérez-Molina et al. 2009); currently used drugs are toxic and have disputable efficacy in the treatment of chronic infections (Marin-Neto et al. 2009).

Tamoxifen is a triphenylethylene that has been in clinical use for the treatment and chemoprevention of breast cancer since 1971 (Jordan 2003). It has been shown to inhibit the growth of promastigotes and intracellular amastigotes of several species of *Leishmania* (Miguel et al. 2007, 2009). Tamoxifen was also effective against the parasite in vivo, as shown in various experimental rodent models of visceral and cutaneous leishmaniasis. In all models tested, parasite burden and/or lesion progression decreased significantly after intraperitoneal administration of 20 mg/kg/day tamoxifen for 15 days (Miguel et al. 2008, 2009).

Leishmania and *Trypanosoma* belong to the Trypanosomatidae family, are genetically related and share many biochemical pathways, as shown by comparative analysis of their respective genomes (El-Sayed et al.

2005) and by comparative biochemical studies (Michels et al. 2006, Landfear 2008, Stuart et al. 2008). In this study, we evaluated the activity of tamoxifen against *T. cruzi* in vitro and in vivo.

The *T. cruzi* strains used were CL-14 (Brenner & Chiari 1963), Y (Silva & Nussenzweig 1953) and a benznidazole-resistant line derived from the Y strain (BZR) (Murta & Romanha 1998). Epimastigotes were grown in liver infusion-tryptose medium (LIT) supplemented with 10% fetal calf serum (FCS) and maintained at 25°C (Camargo 1964). The activity of tamoxifen (Sigma-Aldrich) against epimastigotes was assayed by counting the number of motile forms in a hemocytometer after incubating parasites with increasing concentrations of the drug for 24 h.

To analyze the effect of tamoxifen on intracellular parasites, metacyclic trypomastigotes harvested from LIT cultures at the stationary growth phase were used to infect LLC-MK2 (rhesus monkey kidney, ATCC-CCL7) cells at a ratio of 50:1 parasites/host cell. Cultures were maintained at 34°C and 5% CO₂ in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich), supplemented with 2% FCS for 24 h, washed to remove free parasites and then incubated for 48 h with increasing concentrations of tamoxifen. Infected cells were maintained at 34°C in an atmosphere of 5% CO₂ and the medium was replaced every 24 h. After drug exposure, the untreated and treated infected cell cultures were fixed and stained with the Instant ProV kit (Newprov, Pinhais, Brazil). The number of infected host cells was determined using at least 400 host cells for two independent experiments run in triplicate.

Trypomastigotes were obtained from the blood of infected mice. To obtain sufficient numbers of parasites for drug susceptibility tests, these trypomastigotes were used to infect Vero cell cultures, which were maintained at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS, as previously described (Santos 2008). Trypomastigotes released on the

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culture supernatant were recovered after 6–8 days, resuspended in RPMI without phenol red and incubated with increasing concentrations of tamoxifen for 24 h at 37°C. The number of viable parasites was then assessed using the vital stain Alamar Blue (Invitrogen). Parasites were incubated with 10% Alamar Blue for 24 h at 37°C and the reduction of resazurin was monitored at 570 nm using 600 nm as a reference wavelength (Santos 2008). The percentage of viable cells was determined by comparison with control untreated cultures.

The half maximal effective concentration (EC_{50}) values for the *T. cruzi* populations were determined from sigmoidal regression of the concentration-response curves using Scientific Graphing and Analysis Software Origin 5.0. Experiments were performed in triplicate and repeated at least twice.

In vivo experiments were approved by the Ethical Committee for Animal Experimentation of the Institute of Biomedical Sciences of São Paulo University (approval certificates 033/07 and 067/08). Animals were bred, handled and maintained according to the Brazilian rules and regulations of animal experimentation. Acute infections were performed as described (Filardi & Brener 1987). BALB/c and Swiss Webster female and male mice were used (8–10 animals/experimental group). Infections were initiated by intraperitoneal inoculation of 5,000 *T. cruzi* Y strain tissue culture trypomastigotes/Swiss Webster mouse and 500 parasites/BALB/c mouse, due to the increased sensitivity of BALB/c mice to infection. Tamoxifen treatment was initiated on the 4th day of infection for Swiss Webster mice and on the 2nd day for BALB/c mice. Tamoxifen citrate was tested at 10, 20, 25 and 50 mg/kg/day of drug equivalent for 15–20 days. Benznidazole (BZ) (Rochagan, Roche Co) was used as a reference drug at 100 mg/kg/day. Infected animals that received only saline were used as control groups. Parasitemia was assessed every other day from the 2nd day of infection by counting the trypomastigotes in a 5 μ L blood sample obtained from the tail vein. Surviving animals were monitored for 60 days and then subjected to hemoculture, used as a criterion of cure (Brener 1962). Tamoxifen citrate (6 mg/mL) and BZ (6.25 mg/mL) solutions were prepared in 150 mM NaCl, pH 7.2–7.4.

The table shows the in vitro activity of tamoxifen against *T. cruzi* strains and life stages. The drug was active against all *T. cruzi* life stages tested, with increased susceptibility noted for the infective mammalian stages, trypomastigotes and amastigotes. EC_{50} values calculated for epimastigotes of CL14, Y and BZR strains were similar, indicating homogeneous sensitivity between different strains and showing that resistance to BZ does not have an impact on sensitivity to tamoxifen.

The efficacy of tamoxifen in the control of acute infections was initially tested in female Swiss mice (Figs 1–3). Parasitemia peaks were reached at the 8th day after infection. The treatment of infected mice with 10, 25 or 50 mg/kg/day of tamoxifen for 15–20 days did not alter the parasitemia curves as compared to control mock-treated groups, whereas parasitemia was undetectable in BZ-treated mice (Figs 1–3). Previous experiments in leishma-

niasis experimental models did not identify any differences between male and female responses to treatment with tamoxifen (Miguel et al. 2008). However, other research (de Souza et al. 2001) suggests that oestradiol may interfere in the outcome of *T. cruzi* infection in C57BL/6 female mice and, in a less pronounced way, in BALB/c and C3H mice. Therefore, we decided to evaluate tamoxifen effectiveness in infected male Swiss mice (Fig. 4). No differences were detected in the response of male vs. female mice and tamoxifen treatment was completely ineffective in the control of parasitemia.

Acute *T. cruzi* infections in another mouse model were also performed. BALB/c mice infected with *T. cruzi* Y also developed parasitemia peaks at the 8th day post-infection. Male or female BALB/c mice treated with 20 mg/kg/day tamoxifen for 15 days showed no reduction in parasitemia levels when compared to control animals. In contrast, parasitemia reached a peak in BZ-treated BALB/c mice around the 7th–8th day post-infection and became negative by the 23rd day post-infection (Fig. 5).

Another parameter assessed was the survival rate. While no mortality was detected in BZ-treated groups up to 60 days after infection (in Swiss Webster or BALB/c mice), animals treated with tamoxifen did not survive longer than control untreated groups, confirming the lack of antitrypanosomal activity of tamoxifen in these models of infection (Figs 1–5). Because there were no survivors in tamoxifen-treated and untreated groups, only the BZ-treated Swiss Webster mice were subjected to hemoculture. The cure rate was 70%, as expected for the partially-resistant *T. cruzi* Y strain when infecting Swiss Webster mice.

The current chemotherapies of leishmaniasis and Chagas disease do not include drugs that are effective against both diseases. However, the novel anti-leishmanial activity of tamoxifen (Miguel et al. 2007, 2008, 2009) inspired curiosity about a possible common target between these organisms and led to the investigation of an anti-*Trypanosoma* effect. The results shown here indicate that tamox-

TABLE
In vitro activity of tamoxifen against
Trypanosoma cruzi strains and life stages

Strain	Life stage	$EC_{50} \pm SD$ (μ M) ^a
CL14	Amastigote	2.7 \pm 1.1
CL14	Epimastigote	17.9 \pm 3.3
Y	Epimastigote	12.3 \pm 2.1
BZR	Epimastigote	16.1 \pm 2.6
Y	Trypomastigote	0.7 \pm 0.1
BZR	Trypomastigote	0.7 \pm 0.1

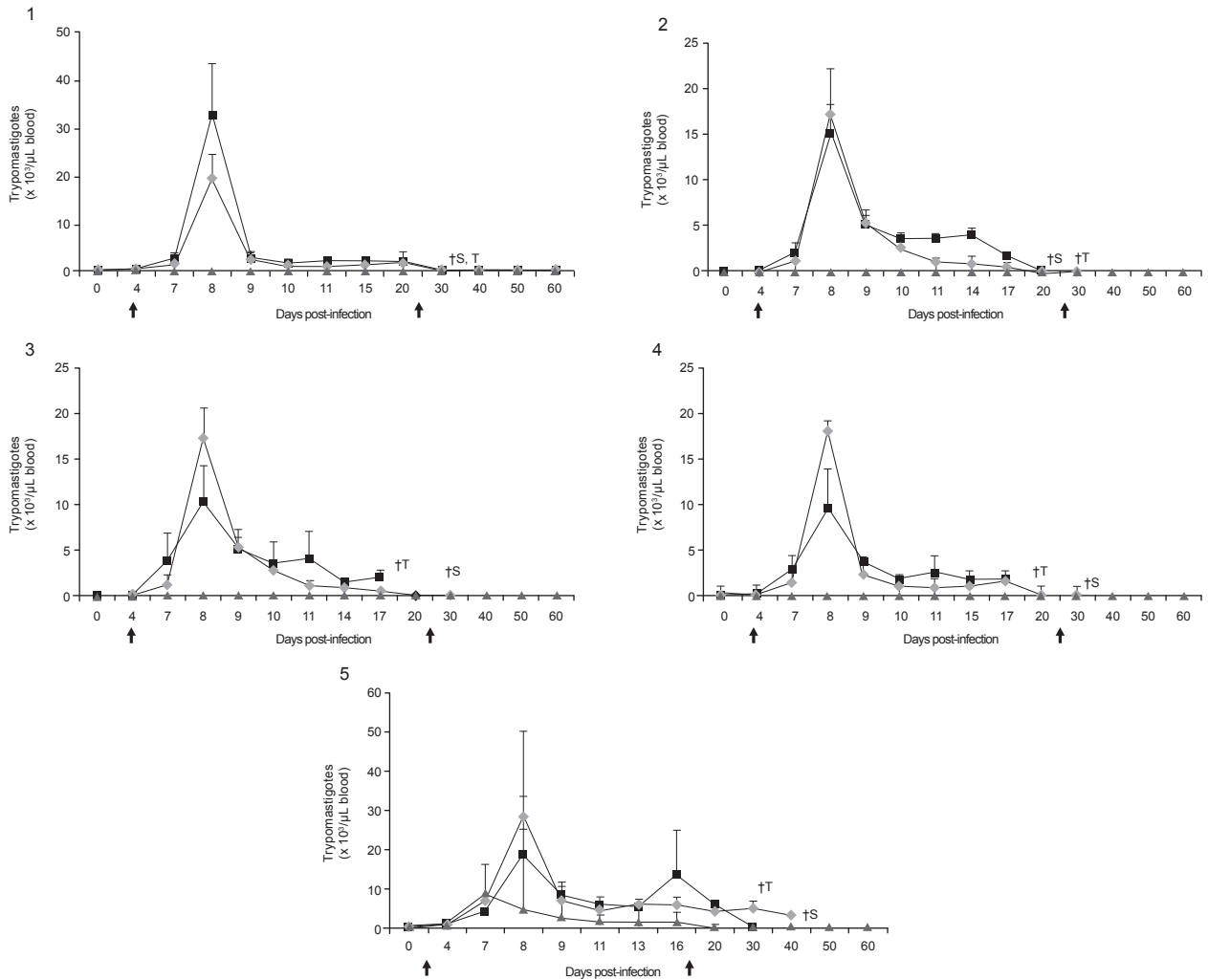
^a: values are expressed as means \pm standard deviation (SD) of at least two independent experiments performed in triplicates. EC_{50} : tamoxifen effective concentration that decreases the number of viable parasites by 50% (in 24 h for epimastigotes and trypomastigotes and in 48 h for amastigotes).

ifen is indeed active against both parasites. Data from in vitro tests, which demonstrated activity against the different *T. cruzi* life forms in the same order of magnitude as encountered for *Leishmania* spp, were initially very encouraging. However, despite the marked in vitro activity against two *T. cruzi* strains, tamoxifen did not prolong life or decrease parasitemia in experimental models of acute infection with *T. cruzi*. The therapeutic schemes in the present work included doses up to 2.5-fold greater than the effective doses for the treatment of leishmaniasis (Miguel et al. 2008, 2009) and data presented here were obtained independently in two laboratories.

While the in vitro results indicate the shared presence of the tamoxifen biochemical target(s) in both organisms, undetermined factors prevent tamoxifen activity in vivo against *T. cruzi*. Whereas *Leishmania* preferentially para-

sitises macrophages in the mammalian host and localizes preferentially in organs of the mononuclear phagocytic system, *T. cruzi* can inhabit most types of differentiated cells and has a wide organ distribution, especially in the acute form of the disease. Tamoxifen has been shown to accumulate in organs such as liver, bone marrow and adipose tissue, while only a minor portion of the drug remains in the serum (Lien et al. 1991). Differential tissue permeability and accumulation could explain the different outcomes of tamoxifen treatment. We cannot exclude that other factors, such as interference in immune-mediated defence mechanisms, are also of importance.

Beyond demonstrating that tamoxifen is ineffective against *T. cruzi* infections, these results also highlight the importance of using in vivo studies to validate new candidates for chemotherapy.



Figs 1-5: tamoxifen treatment of *Trypanosoma cruzi* acute infections in mice. Swiss Webster female (1-3) or male (4) mice and BALB/c female and male mice (5) were infected with *T. cruzi* Y strain. Treatment was started on the 4th day (1-4) or on the 2nd day (5) post-infection and parasitemia evaluated every other day by tail vein puncture. Animals were treated with tamoxifen (squares) at 10 (1), 25 (2), 50 (3,4) or 20 mg/kg/day (5) for 15-20 days. Parasitemia curves from mock-treated animals (diamonds) and from animals that received 100 mg/kg/day benznidazole (BZ) (triangles) were also obtained. Results are expressed as the mean of two independent experiments. Standard deviation is shown as a plus sided bar. The arrows indicate the period of treatment with tamoxifen or BZ. †S, †T: death of all animals from saline or tamoxifen-treated groups, respectively (n = 8-10 animals per group).

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