

Tamoxifen is effective against *Leishmania* and induces a rapid alkalinization of parasitophorous vacuoles harbouring *Leishmania (Leishmania) amazonensis* amastigotes

Danilo C. Miguel¹, Jenicer K. U. Yokoyama-Yasunaka¹, Walter K. Andreoli², Renato A. Mortara² and Silvia R. B. Uliana^{1*}

¹Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo 05508-900 SP, Brazil; ²Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo 04023-062 SP, Brazil

Received 8 February 2007; returned 6 April 2007; revised 7 May 2007; accepted 24 May 2007

Objectives: This study was performed to investigate the activity of tamoxifen, an antioestrogen widely used in the treatment of breast cancer, against *Leishmania*.

Methods: Drug activity was assessed *in vitro* against axenically grown promastigotes and amastigotes through cell counting or by measuring the cleavage of MTT, and against intracellular amastigotes by treating infected macrophage cultures and evaluating the number of intracellular parasites. Intravacuolar pH changes induced inside parasitophorous vacuoles of *Leishmania (Leishmania) amazonensis*-infected macrophages were evaluated using the fluorescent probes SNAFL-calcein and Acridine Orange.

Results: Tamoxifen killed *L. (L.) amazonensis* promastigotes and amastigotes with 50% inhibitory concentrations (IC₅₀) of 16.4 ± 0.2 and 11.1 ± 0.2 µM, respectively. The drug was also effective against *Leishmania (Viannia) braziliensis*, *Leishmania (Leishmania) major*, *Leishmania (Leishmania) chagasi* and *Leishmania (Leishmania) donovani* with IC₅₀ values ranging from 9.0 to 20.2 µM. Tamoxifen induced a rapid and long-lasting alkalinization of the vacuolar environment. We also provide evidence that tamoxifen is more effective against promastigotes and amastigotes at pH 7.5 when compared with cultures at pH 4.5.

Conclusions: Tamoxifen effectively kills several *Leishmania* species and its activity against the parasite is increased by a modulation of the host cell intravacuolar pH induced by the drug.

Keywords: chemotherapy, pH, oestradiol, Acridine Orange, SNAFL-calcein

Introduction

Human leishmaniasis is an endemic disease in 88 countries distributed in tropical and subtropical regions of the world. Clinical manifestations of this parasitic illness vary from self-healing cutaneous ulcers to very aggressive and disfiguring cases of diffuse or mucocutaneous diseases or to the life-threatening visceral form. More than 90% of cutaneous leishmaniasis cases occur in Iran, Afghanistan, Syria, Saudi Arabia, Brazil and Peru.¹ In Brazil, *Leishmania (Viannia) braziliensis* is the most common agent for cutaneous and mucocutaneous leishmaniasis, whereas *Leishmania (Leishmania) amazonensis* is the most

important causative agent of the diffuse form of the disease. Chemotherapy of leishmaniasis relies mainly on the administration of pentavalent antimonials that are toxic and poorly tolerated, require daily injections and are becoming ineffective due to the proliferation of resistant parasites.^{2,3} Therefore, alternative drugs are in pressing need.

Leishmania life cycle consists of two distinct forms: promastigotes, flagellated extracellular parasites of the digestive tract of sand flies, and amastigotes, non-flagellated, non-motile stages that live inside parasitophorous vacuoles (PVs) in macrophages of mammalian hosts. The PV encloses a strongly hydrolytic, acid environment—pH around 5.0—and the amastigote does not

*Corresponding author. Tel: +55-11-30917334; Fax: +55-11-30917417; E-mail: srbulian@icb.usp.br

Antileishmanial activity of tamoxifen

attenuate this hostile milieu.^{4–6} Amastigotes have been shown to carry out vital processes (i.e. cellular respiration, nutrient uptake and DNA and RNA synthesis) under these conditions.⁷

Tamoxifen is a triphenylethylene widely used in the treatment and in the prevention of breast cancer due to its activity as an oestrogen receptor modulator.⁸ However, it has become evident that many biological effects of tamoxifen are independent of the oestrogen machinery, such as modulation of calmodulin, caspases and kinases,⁹ interference in ceramide metabolism,^{10,11} inhibition of the acidification of intracellular organelles^{12–14} and partitioning into lipids where it exerts membrane fluidizing and antioxidant activities.¹⁵

While oestrogen receptor modulated responses have not been described in *Leishmania*, some of the effects observed with tumour cell lines suggested a potential activity of tamoxifen against these parasites. Particularly interesting to us were the findings of Altan *et al.*¹³ showing that adriamycin resistance in breast cancer cells was reversed by tamoxifen as a result of the redistribution of cellular adriamycin from acidic organelles to the nucleus. These authors established that tamoxifen was able to inhibit the acidification of organelles in several cell types in an oestrogen-independent way. Since *Leishmania* amastigotes are well adapted to survive in acidic PV, it seemed plausible to test the activity of tamoxifen against these parasites. In this paper, we describe the activity of tamoxifen against promastigotes and amastigotes of *Leishmania in vitro*. We also show that tamoxifen is able to modify the intravacuolar pH of *L. (L.) amazonensis*-infected macrophages inducing a state where the drug activity against the parasite is increased.

Materials and methods

Cells

Promastigotes of *L. (L.) amazonensis* (MHOM/BR/1973/M2269), *L. (V.) braziliensis* (MHOM/BR/1975/M2903), *Leishmania (Leishmania) chagasi* (MHOM/BR/1974/M2682), *Leishmania (Leishmania) donovani* (LD-15/MHOM/SD/00) and *Leishmania (Leishmania) major* (MHOM/IL/1981/Friedlin) were grown in Medium 199 (Invitrogen) supplemented with 10% or 20% heat-inactivated fetal calf serum (FCS; Invitrogen) and incubated at 25°C. Liquid cultures of *L. (V.) braziliensis* and *L. (L.) chagasi* were also supplemented with 2% sterile male human urine. *L. (L.) amazonensis* amastigotes were obtained from experimentally infected BALB/c mice as described previously.¹⁶ Amastigotes purified from lesions were kept at 33°C in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS in an atmosphere of 5% CO₂. Parasites retained the typical amastigote morphology and were viable in these conditions for at least 48 h.

Resident peritoneal macrophages were obtained by peritoneal lavage of 25- to 30-day-old female BALB/c mice with 3–5 mL of RPMI 1640 medium repeated three times. The collected cell suspension was centrifuged at 300 g for 10 min at 4°C, the pellet was resuspended in RPMI 1640 medium supplemented with 10% FCS and gentamicin 50 mg/L and cultivated at 37°C in an atmosphere of 5% CO₂.

The continuous line of human foreskin fibroblasts (HFF) was grown in RPMI 1640 supplemented with 10% FCS and cultivated at 37°C in an atmosphere of 5% CO₂.

Drugs

Tamoxifen, 17 β -oestradiol and amphotericin B were purchased from Sigma-Aldrich, St Louis, MO, USA. Stock solutions of tamoxifen and 17 β -oestradiol were prepared in ethanol and amphotericin B stock was prepared in DMSO (5 mM final concentration). Tamoxifen and amphotericin B were stored at –20°C and 17 β -oestradiol at room temperature.

In vitro antiproliferative activity assays

Cell viability was evaluated *in vitro* by cultivating promastigotes (5×10^6) or amastigotes (1×10^7) in 199 medium or RPMI 1640, respectively. Parasites were incubated in the presence of increasing concentrations of tamoxifen (2.5–30 μ M), amphotericin B (as a control drug, 0.05–0.30 μ M) or 17 β -oestradiol in 24-well culture dishes (Corning Life Sciences, Corning, NY, USA) for 24 h. Quantification of viable cells was assessed either by cell counting or by measuring the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) by metabolically active cells as described previously.¹⁷ In brief, cells were incubated in 3-(*N*-morpholino) propanesulfonic acid (MOPS)-buffered saline [30 mM MOPS (pH 7.2), 116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM D-glucose] containing MTT 5 mg/mL for 2 h (at 25°C for promastigotes and 33°C for amastigotes). MTT cleavage was assessed by using a multiwell scanning spectrophotometer (Labsystems; Multiskan EX) with a reference wavelength of 690 nm and a test wavelength of 595 nm. Assays were performed in triplicate and results are expressed as the mean percentage reduction of parasite numbers compared with untreated control wells calculated for at least three independent experiments. The 50% and 90% inhibitory concentrations (IC₅₀ and IC₉₀) were determined from sigmoidal regression of the concentration–response curves using Scientific Graphing and Analysis Software ORIGIN 7.5.

Alternatively, cell viability and growth was analysed by incubating promastigotes (5×10^6) in the presence of increasing concentrations of tamoxifen for 24 h; parasites were then washed in PBS [10 mM Na₂PO₄, 1.76 mM KH₂PO₄ (pH 7.4), 137 mM NaCl and 2.7 mM KCl] and grown in 199 medium for additional 24 h in the absence of drug. At the end of this period, cell density was determined by counting aliquots of the cultures in a haemocytometer.

In vitro cytotoxicity was evaluated by cultivating 5×10^5 HFF in 24-well plates for 24 h in the presence of increasing concentrations of tamoxifen (5–80 μ M). Cell viability was assessed by the MTT assay as described above and results are expressed as percentage reduction in cell viability compared with untreated control cultures. The 50% cytotoxic concentration (CC₅₀) was determined as described above for IC₅₀ values.

Drug activity against intracellular amastigotes

Killing of intracellular *L. (L.) amazonensis* amastigotes was assayed by analyses of the number of infected cells in macrophage monolayers. Peritoneal macrophages were plated on round glass coverslips inside the wells of 24-well culture dishes, at a density of 4×10^5 cells per coverslip, in RPMI 1640 supplemented with 10% FCS and gentamicin 50 mg/L. After 2 h of incubation at 37°C in an atmosphere of 5% CO₂, *L. (L.) amazonensis* stationary-phase promastigotes (in a ratio of 7 parasites:1 macrophage) or lesion-derived amastigotes (1:1) were added to the wells and the cultures were incubated at 33°C in a 5% CO₂ atmosphere. After 3 h, parasites were removed by extensive washing with RPMI medium without FCS and infected cultures were treated with increasing concentrations of tamoxifen (5–20 μ M) or amphotericin B

(0.01–0.30 μM) for 48 h or with ammonium chloride (NH_4Cl , 5 or 10 mM) for 24 h. The monolayers were washed, fixed and stained with the Instant Prov kit (Newprov, Pinhais, Brazil). The percentage of infected macrophages was assessed by light microscopy observation by counting 100 cells in triplicate coverslips. IC_{50} and IC_{90} were calculated from data obtained in three independent experiments.

Intravacuolar pH alterations in response to tamoxifen

Intracellular and intravacuolar pH were evaluated using two different fluorescent probes: SNAFL-calcein and Acridine Orange (AO) (Sigma-Aldrich).

Peritoneal BALB/c macrophages (8×10^5) were cultivated on 0.15 mm thick coverslips in Delta T dishes (Bioprotechs, Butler, PA, USA) in RPMI 1640 medium at 37°C in a 5% CO_2 atmosphere. After 3 h, amastigotes of *L. (L.) amazonensis* purified from mice lesions were used for *in vitro* macrophage infections. Infected cultures were kept at 33°C in a 5% CO_2 atmosphere and samples were treated with 10 μM tamoxifen, ethanol (0.25%) or left untreated. Cells were loaded with the pH-sensitive ratiometric probe SNAFL-calcein (Molecular Probes).¹⁸ Experiments were performed as previously described.¹⁹ Lasershar software version 3.2 TC was used for image acquisition and basic processing.

Peritoneal BALB/c macrophages, plated on round glass coverslips as described above, were infected with *L. (L.) amazonensis* amastigotes. Infected and uninfected cells were incubated in RPMI 1640 medium with or without drugs for the times indicated in the figure legends. Tamoxifen treatment was performed at 10 μM for 30 min. For time-lapse experiments, tamoxifen-treated cultures (30 min) were washed and incubated with RPMI without drug for 30 min, 4 or 24 h. NH_4Cl was used at 5 or 10 mM concentrations for 30 min, 4 or 24 h. Cells were then incubated with 10 μM AO in PBS for 10 min at room temperature. Culture samples were immediately analysed using a Nikon-Microphot SX Fluorescence Microscope. ACT-1 Control Software was used for image acquisition.

pH effect on parasite viability and tamoxifen activity

L. (L.) amazonensis promastigotes (5×10^6) or freshly purified lesion-derived amastigotes (1×10^7) were incubated in RPMI medium pH 4.5 or 7.5 supplemented with 5% FCS for 4 or 24 h in the absence or in the presence of increasing concentrations of tamoxifen. Quantification of cell growth was evaluated by counting aliquots of the cultures in a haemocytometer.

Results

Activity of tamoxifen against *Leishmania* *in vitro*

Tamoxifen was tested for its effect on the viability of *L. (L.) amazonensis* promastigotes *in vitro*. Cell viability, evaluated through MTT cleavage, decreased as a function of drug concentration. After 24 h of incubation, the calculated IC_{50} against *L. (L.) amazonensis* was $16.4 \pm 0.2 \mu\text{M}$ (Table 1). As a control, activity of amphotericin B against *L. amazonensis* promastigotes was calculated as being $0.15 \pm 0.06 \mu\text{M}$, in agreement with previously published data.²⁰ These results were confirmed by cell counting. The effect of tamoxifen was irreversible since parasites incubated with the drug and left in normal culture media for 24 h did not recover (data not shown).

The activity of tamoxifen against other *Leishmania* species was analysed by testing promastigotes of *L. (V.) braziliensis* and

Table 1. *In vitro* activity of tamoxifen against *Leishmania* spp.

Parasites	IC_{50} (μM) ^a	CI 95% ^b	IC_{90} (μM)
<i>L. amazonensis</i> ^c	16.4 ± 0.2	16.32–16.48	21.3
<i>L. amazonensis</i> ^d	11.1 ± 0.2	11.02–11.18	17.5
<i>L. braziliensis</i> ^c	10.0 ± 0.1	9.96–10.04	20.9
<i>L. chagasi</i> ^c	17.7 ± 0.8	17.39–18.01	27.9
<i>L. donovani</i> ^c	19.9 ± 0.3	19.78–20.02	37.0
<i>L. major</i> ^c	9.3 ± 0.3	9.18–9.42	14.0

^aValues are expressed as means \pm SD of three independent experiments.

^b95% confidence interval.

^cPromastigotes.

^dAmastigotes.

L. (L.) major, aetiological agents of cutaneous leishmaniasis and *L. (L.) chagasi* and *L. (L.) donovani*, species that cause visceral leishmaniasis. A dose-related response was detected when promastigotes of all the species tested were incubated with increasing concentrations of tamoxifen. The sensitivity of different *Leishmania* species was uniform with IC_{50} values ranging from 9.0 to 20.2 μM as depicted in Table 1.

Viability of *L. (L.) amazonensis* amastigotes, obtained from lesions and axenically cultivated for 24 h at 33°C , was also inhibited by tamoxifen at an IC_{50} of $11.1 \pm 0.2 \mu\text{M}$ (Table 1).

Cytotoxicity assays were performed treating HFF cultures with increasing concentrations of tamoxifen, allowing the determination of a CC_{50} of $22.8 \pm 2.6 \mu\text{M}$. Treatment of HFF or peritoneal macrophage cultures with 10 μM tamoxifen for 24 h resulted in 100% and 85% survival, respectively.

Effect of tamoxifen on intracellular amastigote survival

The activity of the drug against the parasite in the intracellular environment was tested by treating peritoneal BALB/c macrophage cultures infected with *L. (L.) amazonensis*. The IC_{50} of tamoxifen against intracellular amastigotes was $5.46 \pm 0.12 \mu\text{M}$ and the IC_{90} was calculated as 6.73 μM (Figure 1 and data

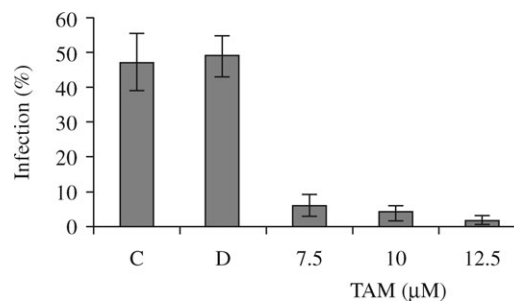


Figure 1. Activity of tamoxifen against intracellular *L. (L.) amazonensis* amastigotes *in vitro*. Macrophages were infected with *L. (L.) amazonensis* promastigotes for 3 h in a ratio of 7 parasites:1 macrophage. After infection, cultures were incubated in the presence of tamoxifen at final concentrations of 7.5, 10 or 12.5 μM for 48 h. Cells were fixed and stained and the percentage of infection was determined by counting 100 cells/coverslip. Cultures were tested in triplicates and the results shown are representative of three independent experiments. Bars represent percentage of infected cells in: control untreated cultures (C), cells incubated with the drug diluent ethanol (D), or treated with tamoxifen.

Antileishmanial activity of tamoxifen

not shown). Parasitism was unaltered in control-infected cells incubated in the presence of ethanol (the drug diluent). Testing of amphotericin B in the same assays allowed the calculation of an IC_{50} of $0.050 \pm 0.003 \mu\text{M}$ for intracellular amastigotes.

Oestradiol does not alter the viability of L. (L.) amazonensis promastigotes or the activity of tamoxifen

A search for oestrogen receptor homologues in the *L. (L.) major* genome database did not produce positive hits, indicating that tamoxifen's activity on these parasites is unlikely to be linked to the oestrogen receptor. To verify whether this was indeed the case, *L. (L.) amazonensis* promastigotes were incubated with increasing concentrations of 17β -oestradiol and with equimolar concentrations of oestradiol plus tamoxifen. 17β -Oestradiol-treated parasites did not exhibit changes in growth or survival while parasites incubated with the combination of equimolar concentrations of 17β -oestradiol and tamoxifen were as sensitive to the drug as parasites incubated with tamoxifen alone (Figure 2). The same was observed for lesion-derived *L. (L.) amazonensis* amastigotes which exhibited the same pattern of growth in the absence or in the presence of 17β -oestradiol while incubation with 17β -oestradiol did not modify the activity of tamoxifen (Figure 2), confirming that the leishmanicidal activity of tamoxifen is independent of the oestrogen receptor machinery.

Intravacuolar pH alterations in response to tamoxifen

In vitro infections were analysed using non-invasive ratiometric confocal microscopy with dual-label SNAFL-calcein-AM

probe before and after incubation with $10 \mu\text{M}$ tamoxifen. SNAFL-calcein-AM compartmentalizes into acid vacuoles and the fluorescence is emitted in distinct wavelengths according to H^+ concentrations—the more acidic the intravacuolar pH, the greener the organelle appears, whereas a more alkaline environment appear in tones towards red. As shown in Figure 3, images were obtained by merging the 550 and 640 nm emission channels. In infected and untreated macrophages, SNAFL-calcein-AM was detected mainly as yellow and green granules concentrated in the PV. Upon addition of tamoxifen and as incubation time elapsed, detection of SNAFL-calcein-AM in PVs was more intense in the 640 nm emission channel, resulting in red fluorescence (Figure 3). This suggested a rapid alkalization of the PVs, detectable as soon as 10 min after drug addition. The presence of parasites inside PVs was confirmed by the phase contrast images also shown in Figure 4. Infected macrophages treated with $10 \mu\text{M}$ tamoxifen for 24 h and incubated with SNAFL-calcein-AM exhibited large vacuoles devoid of amastigotes and more alkaline than PVs in untreated cells (data not shown).

Measurements of intravacuolar pH were also carried out in infected macrophages treated with ethanol in the concentration necessary to deliver the highest tamoxifen concentration used in the tests. Changes in pH were not observed in these cells (data not shown) indicating that the effect obtained with tamoxifen was not due to the drug diluent.

These findings were therefore consistent with the hypothesis of reduced acidification of intracellular compartments induced by tamoxifen, previously observed in other systems.

The effect of tamoxifen on intravacuolar acidification was also tested labelling peritoneal BALB/c macrophages infected with *L. (L.) amazonensis* amastigotes with AO, a weakly basic

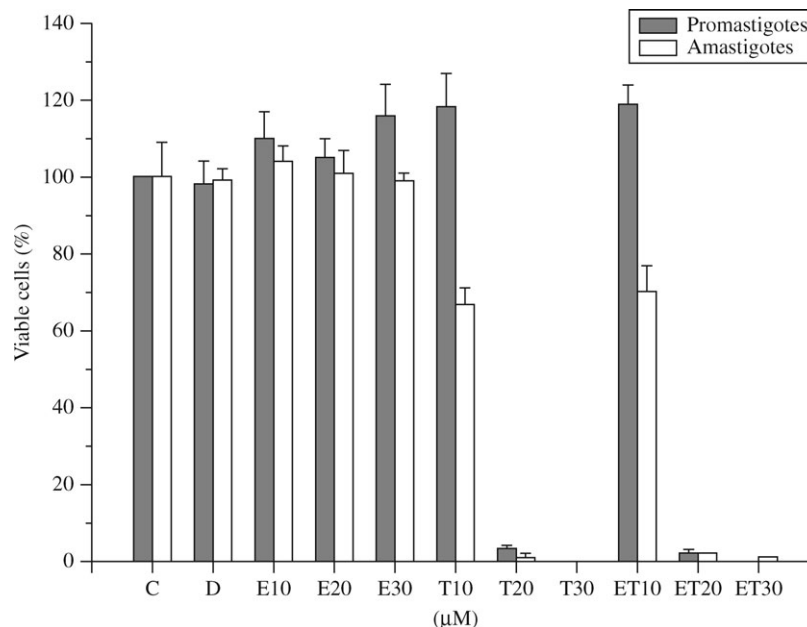


Figure 2. Effect of 17β -oestradiol, tamoxifen and 17β -oestradiol/tamoxifen on the survival of *L. (L.) amazonensis* promastigotes and amastigotes. Promastigotes (grey bars) or amastigotes (white bars) were incubated for 24 h with 10, 20 or $30 \mu\text{M}$ 17β -oestradiol (E10, E20, E30), 10, 20 or $30 \mu\text{M}$ tamoxifen (T10, T20, T30) or with the equimolar mixture of 17β -oestradiol and tamoxifen (ET10, ET20, ET30). Viability was evaluated by MTT and calculated as a percentage of control parasites incubated without drugs (C). Control cultures were also incubated in the presence of the drug diluent ethanol (D). Cultures were tested in triplicate and the results shown are the average of two independent experiments.

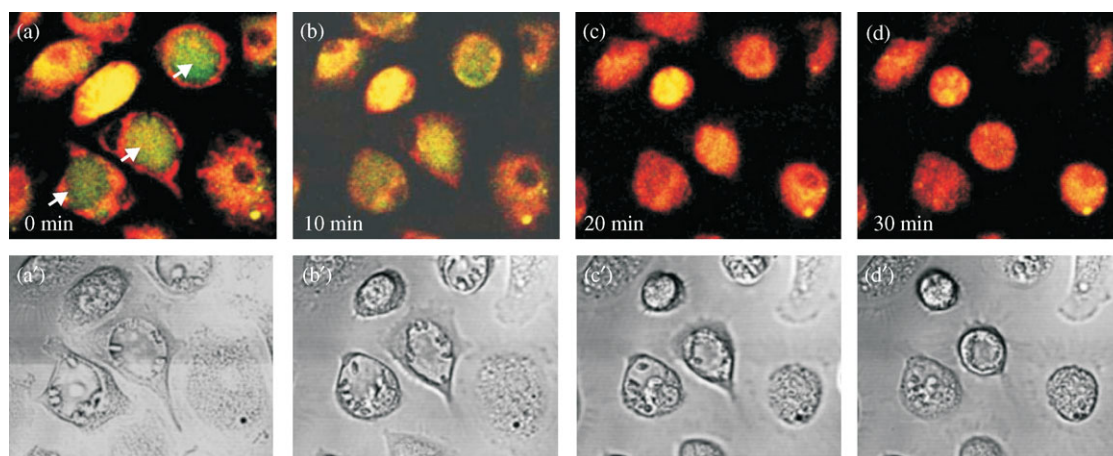


Figure 3. Confocal analysis of intravacuolar pH alteration in response to tamoxifen. *L. (L.) amazonensis* amastigote-infected macrophages were incubated with SNAFL-calcein-AM. All images were obtained by confocal microscopy (63×1.4) using a superposition of fluorescein (550 nm) and rhodamine (640 nm) emission channels (a–d) or by phase contrast (a'–d'). After the initial acquiring of the image (0 min), 10 μM tamoxifen was added. The compartmentalization of SNAFL-calcein-AM into macrophages was monitored at 10, 20 and 30 min. Arrows point to PV harbouring amastigotes.

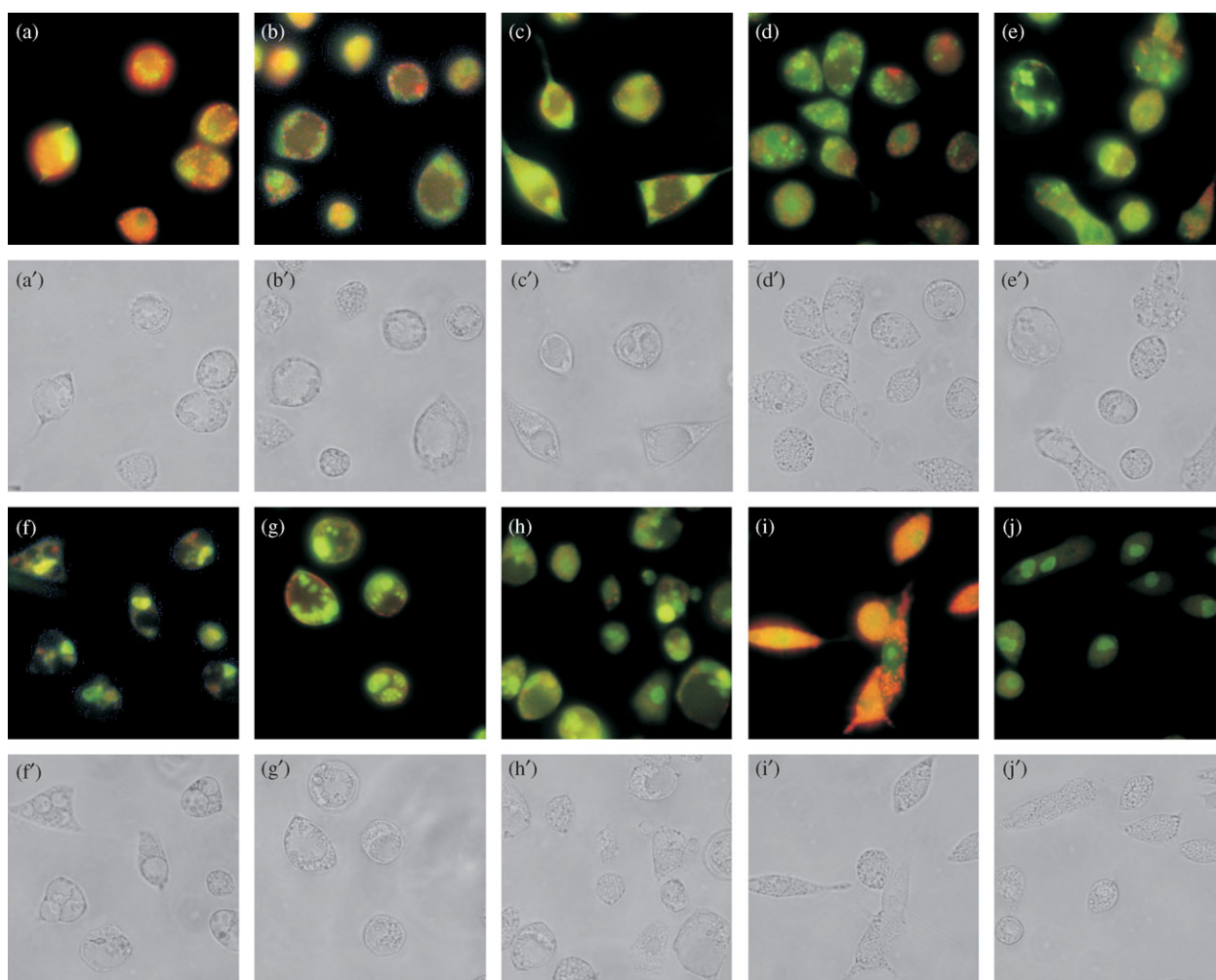


Figure 4. Analysis of AO accumulation in peritoneal macrophages upon treatment with tamoxifen or NH_4Cl . *L. (L.) amazonensis*-infected macrophages (a–h) or non-infected cells (i and j) were stained with AO after various treatments and fluorescence emissions of 530 and 640 nm acquired simultaneously: (a) untreated infected cells; (b) 10 μM tamoxifen for 30 min; (c), (d) and (e) 10 μM tamoxifen for 30 min followed by washing and subsequent incubation in medium without drug for 30 min (c), 4 h (d) and 24 h (e); (f) 10 mM NH_4Cl for 30 min; (g) 10 mM NH_4Cl for 4 h; (h) 10 mM NH_4Cl for 24 h; (i) untreated uninfected macrophages; (j) uninfected macrophages treated with 10 μM tamoxifen for 30 min. (a–j) Images acquired using fluorescence microscopy; (a'–j') respective phase contrast images. Magnification: $400\times$.

Antileishmanial activity of tamoxifen

fluorescent probe. AO is taken up by living cells and accumulates in acidic compartments. Fluorescence of AO is green at low concentrations, whereas at high concentrations the fluorescence changes to orange.²¹ Nuclei in infected or non-infected cells (Figure 4a and i) stained green. In the cytoplasm of infected cells, AO accumulated inside vacuoles containing amastigotes resulting in red fluorescence (Figure 4a). Upon addition of 10 μ M tamoxifen for 30 min, a decrease of the red AO fluorescence was observed in PVs of infected cells (Figure 4b). To verify whether this effect was lasting, infected macrophages treated with tamoxifen for 30 min were washed and kept at 33°C for 30 min, 4 or 24 h in culture media without drug (Figure 4c–e). Cells were then stained with AO for 10 min and examined. The pH in PVs of infected macrophages remained alkaline, even 24 h after drug removal, indicating that tamoxifen's effect on these cells was probably irreversible.

Tamoxifen's alkalinizing effect was not exclusive to infected cells, since non-infected macrophages also showed a remarkable reduction of orange-stained microvesicles (Figure 4i and j).

Alkalinization of the PV is not sufficient to control the infection

To verify whether parasites were killed by tamoxifen's direct action or by an indirect effect of the drug on the host cell, two different approaches were taken. The alkalinizing agent NH_4Cl was used to treat infected macrophages for 30 min, 4 or 24 h. As expected, 5 or 10 mM NH_4Cl induced a pH shift observed in infected cells as AO stained PVs green (Figure 4f–h and data not shown). However, the treatment of *Leishmania*-infected macrophages with 5 or 10 mM NH_4Cl for 30 min or 24 h did not induce any reduction in the infection rates (Figure 4f–h and Table 2), indicating that the alkaline PV is not sufficient to arrest the infection.

The second approach used to test whether tamoxifen's direct effect was required was to pre-treat uninfected macrophage cultures with tamoxifen for 24 h. Cells were then washed, infected and the cultures were grown for additional 24 h in the absence of tamoxifen. Pre-treated macrophages were as permissive to the infection as naive cells and there was no reduction in the percentage of infection compared with control untreated cells (Table 2), indicating that tamoxifen is directly active against the intracellular parasite.

Tamoxifen's activity is increased at neutral pH

To verify whether a neutral–alkaline pH in the PV would have any impact on drug activity, promastigotes and freshly lesion-purified amastigotes were submitted to tamoxifen treatment for 4 and 24 h at pH 4.5 or 7.5. A striking increase in drug activity was observed against promastigotes grown at pH 7.5 as compared with cultures kept at pH 4.5 (Figure 5a and b). A less pronounced but nonetheless clear increase in effect was also observed against amastigotes kept at neutral pH conditions (Figure 5c and d). The increased parasite killing at neutral pHs was not due to the pH itself since, after 4 and 24 h, both control promastigotes and amastigotes, grown in the absence of drugs, maintained unaltered their metabolic rate and viability in either pH as judged by similar optical densities in the MTT test (data not shown).

Table 2. Influence of PV alkalinization on parasitism

	Treatment with NH_4Cl^a	Pre-treatment with tamoxifen ^b
Control	100 \pm 0 ^c	92 \pm 4
5 mM (30 min)	98 \pm 1	—
10 mM (30 min)	100 \pm 0	—
5 mM (24 h)	98 \pm 2	—
10 mM (24 h)	97 \pm 2	—
5 μ M	—	94 \pm 6
7.5 μ M	—	89 \pm 6
10 μ M	—	92 \pm 7

^aInfected cells were incubated with 5 or 10 mM NH_4Cl for 30 min or 24 h.

^bPrior to the infection, macrophages were incubated in medium with increasing concentrations of tamoxifen for 24 h.

^cAll the values represent means \pm SD of the percentage of infected cells calculated from triplicate coverslips.

—, not determined.

This confirms that tamoxifen, with a pK_a of 6.9, is more effective in its neutral form, attained at neutral and alkaline pHs. Consequently, activity against intracellular amastigotes is probably intensified by the increase in PV pH verified after addition of tamoxifen.

Discussion

We have shown that tamoxifen is effective against *Leishmania*. Susceptibility of microorganisms to tamoxifen *in vitro* has only been described for *Candida albicans*²² and *Coccidioides immitis*.²³ There are, as yet, no reports available regarding the activity of tamoxifen against protozoan parasites.

The effect of tamoxifen against *Leishmania* is remarkable, given the absence of response to oestrogen in these organisms. On the other hand, several studies have reported biological properties of tamoxifen that cannot be explained by oestrogen receptor binding, such as the inhibition of acidification of organelles reported in tumour cells.¹⁴ Using resident peritoneal macrophages, we demonstrated that 10 μ M tamoxifen reduced parasitism by more than 90% in 24 h. We have also shown that PVs of *L. (L.) amazonensis*-infected macrophages undergo a rapid pH shift upon addition of tamoxifen.

The rapid change in PV pH, however, is not directly responsible for killing amastigotes, as shown by the evaluation of parasite survival in pH 4.5 or 7.5. It does, however, have a major impact on tamoxifen's effectiveness against the parasite. That is most likely explained by the properties of tamoxifen: the neutral molecule partitions into the membrane and flips more easily into the interior than would the charged molecule. It is tempting to speculate that, once inside the acid PV, tamoxifen gets protonated, the pH shifts and this is followed by an increase in the influx of Cl^- , as shown in microsomes isolated from MCF-7/ADR cells.¹⁴ That would bring about the regeneration of neutral tamoxifen that may now combine with the amastigote membrane.

Besides the ability to accumulate in the lipid phase, tamoxifen has also been shown to be a potent inhibitor of ATP-dependent membrane acid transport.^{24,25} Vacuoles containing

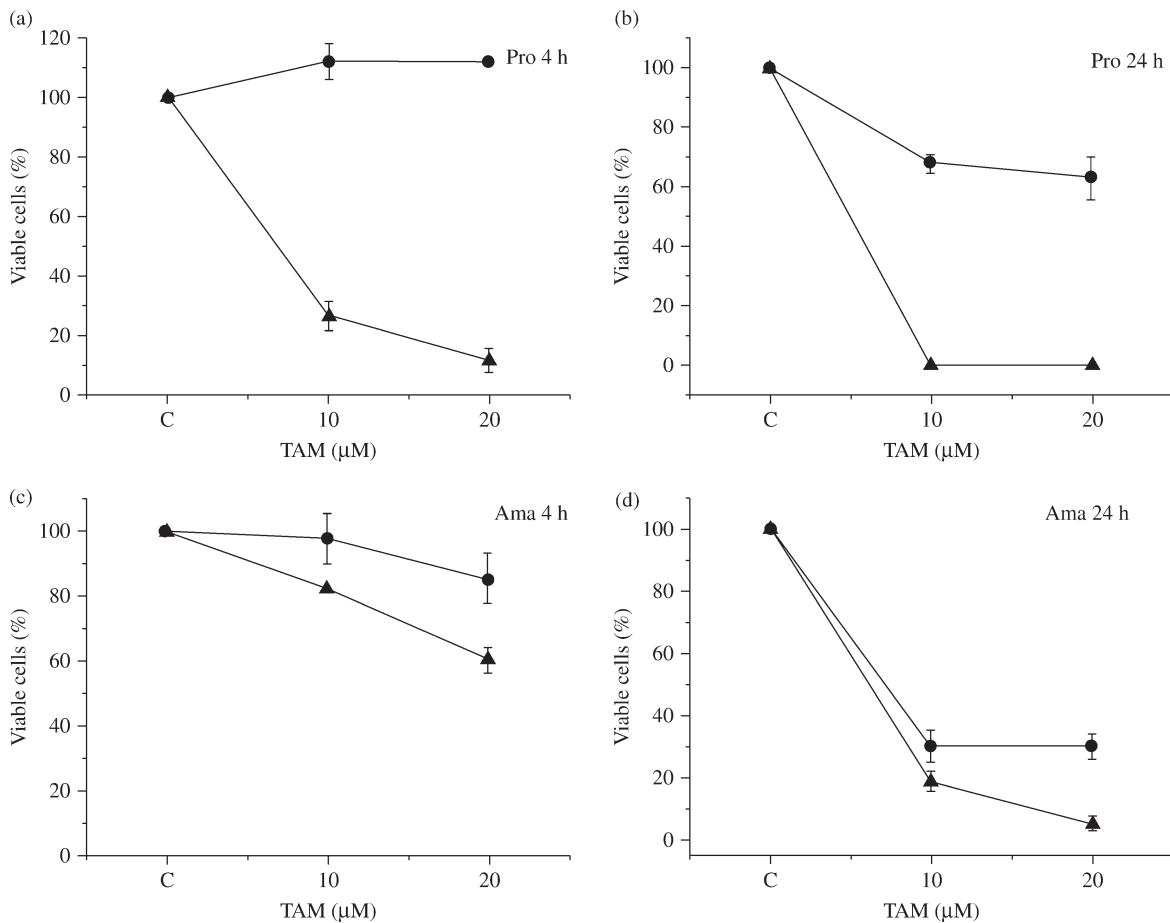


Figure 5. Effect of tamoxifen on the viability of *L. (L.) amazonensis* promastigotes (Pro) and amastigotes (Ama) at distinct pHs. Promastigotes (a and b) or amastigotes purified from lesions (c and d) were cultivated in RPMI medium with or without tamoxifen at densities of 1×10^7 /well at pH 4.5 (filled circles) or 7.5 (filled triangles) for 4 h (a and c) or 24 h (b and d). The viability of parasites was evaluated after incubation with different concentrations of tamoxifen by counting aliquots of cultures in a haemocytometer. The survival of treated cultures is represented as a percentage of control parasites incubated without drug (C). Results represent the mean and standard deviation of cultures tested in triplicates.

Leishmania are rapidly acidified and reach pH 5.0 in <30 min after phagocytosis.²⁶ The acidic luminal pH is sustained by a vacuolar H⁺-ATPase of macrophage origin in the PV membrane. Amastigotes also express a P-type H⁺-ATPase in their plasma membrane and may take part in the PV acidification.²⁷ This P-type H⁺-ATPase is vital for the parasite to maintain a neutral internal pH.²⁸ H⁺ ATPases both at the PV membrane and at the amastigote surface could be the targets of tamoxifen's action resulting initially in vacuole alkalization followed by disruption of the ionic metabolic steady-state of the parasite. Omeprazole, a potent ATPase inhibitor at acidic pHs, has been suggested as a potential antileishmanial drug. It has been shown to kill intracellular *L. donovani* *in vitro* through inhibition of the parasite K⁺, H⁺ ATPase.²⁹

Interestingly, not only intracellular amastigotes were killed by tamoxifen but also extracellular promastigotes. In fact, we showed that tamoxifen inhibits the survival of promastigotes of a wide range of *Leishmania* species, aetiological agents of distinct clinical forms of leishmaniasis, in a low micromolar concentration range. Plasma membrane P-type H⁺ ATPases have also been shown to regulate intracellular pH in *L. (L.) amazonensis* promastigotes.³⁰ Further studies are needed to verify

whether tamoxifen's mechanism of action against *Leishmania* depends on ATPase inhibition.

Tamoxifen is highly lipophilic and has been shown to be strongly incorporated in biomembranes, reported to disrupt the structure of model and artificial membranes and to induce the permeabilization of mitochondria.³¹ It is also possible, therefore, that the effect on *Leishmania* is derived from drug partition into the parasite membranes. It has been observed that the partition of tamoxifen into membranes is modulated by several factors, including membrane composition: for example, partition of tamoxifen into membranes decreases as cholesterol concentration is raised.³² *Leishmania* do not synthesize cholesterol. Ergosterol makes up the main sterol in the plasma membrane in these parasites.³³ Amastigotes have been shown to be able to incorporate cholesterol from their hosts but they are also able to synthesize ergosterol (D. C. Arruda and S. R. B. Uliana, unpublished results). Distinct parasite and host cell membrane compositions could result in preferential partitioning into the parasite's membrane. Further studies are necessary to verify whether tamoxifen is indeed incorporated into *Leishmania* membranes and if the presence of ergosterol favours the partition of tamoxifen.

Antileishmanial activity of tamoxifen

Breast cancer accounts for 31% of all incident cancers in women in North America. It is estimated that more than 214 000 new cases of breast cancer were diagnosed in the USA in 2005³⁴ and tamoxifen represents the cornerstone of breast cancer treatment in women with tumours bearing hormone receptors. Since these patients are treated with tamoxifen for at least 5 years, much clinical experience has been gained on the safety and undesirable effects of the drug. Tamoxifen is well tolerated and side effects, of which the most serious is an increased risk for endometrial cancer, appear only after prolonged use. The doses usually administered for the treatment of breast cancer, of 20 mg/kg body weight, achieve serum concentrations of 0.2–0.8 μM .^{35,36} Due to the high hydrophobicity of tamoxifen, in human tissues concentrations are 10- to 60-fold higher than in serum, with particularly high levels detected in the liver and lung.³⁷ Tamoxifen also accumulates in the skin and bone tissue.³⁷ This distribution pattern is relevant considering parasite localization in cutaneous and visceral leishmaniasis. Tissue concentrations attainable with currently used regimens are at least equivalent to the effective concentrations that kill *Leishmania* intracellular amastigotes in our study. Furthermore, it is to be expected that antileishmanial therapy with tamoxifen would not require such prolonged use. In conclusion, our results suggest that tamoxifen has a great potential to be tested as a therapeutic agent against leishmaniasis.

Acknowledgements

We are grateful to Dr Alejandro M. Katzin for providing tamoxifen for the initial experiments, and to Alexandre Santos de Moura, Dr A. Tania Bijovsky and Dr Maria Julia M. Alves for helping with the microscopy and for the critical reading of the manuscript.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). D. C. M. is the recipient of a post-graduate fellowship from FAPESP.

Transparency declarations

None to declare.

References

1. Special Programme for Research and Training in Tropical Diseases (TDR). <http://www.who.int/tdr/diseases/leish/> (3 May 2007, date last accessed).
2. Croft SL, Coombs GH. Leishmaniasis: current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol* 2003; **19**: 502–8.
3. Murray HW. Treatment of visceral leishmaniasis (kala-azar): a decade of progress and future approaches. *Int J Infect Dis* 2000; **4**: 158–77.
4. Burchmore RJS, Barret MP. Life in vacuoles—nutrient acquisition by *Leishmania* amastigotes. *Int J Parasitol* 2001; **31**: 1311–20.

5. Rivas L, Chang KP. Intraparasitophorous vacuolar pH of *Leishmania mexicana* infected macrophages. *Biol Bull* 1983; **165**: 536.
6. Antoine JC, Prina E, Jouanne C *et al.* Parasitophorous vacuoles of *Leishmania amazonensis*-infected macrophages maintain an acidic pH. *Infect Immun* 1990; **58**: 779–87.
7. Mukkada AJ, Meade JC, Glaser TA *et al.* Enhanced metabolism of *Leishmania donovani* amastigotes at acid pH: an adaptation for intracellular growth. *Science* 1985; **13**: 1099–101.
8. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. *Nat Rev Drug Discov* 2003; **2**: 205–13.
9. Mandlekar S, Kong AN. Mechanisms of tamoxifen-induced apoptosis. *Apoptosis* 2001; **6**: 469–77.
10. Lavie Y, Cao H, Volner A *et al.* Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. *J Biol Chem* 1997; **272**: 1682–7.
11. Cabot MC, Giuliano AE, Volner A *et al.* Tamoxifen retards glycosphingolipid metabolism in human cancer cells. *FEBS Lett* 1996; **394**: 129–31.
12. Altan N, Chen Y, Schindler M *et al.* Defective acidification in human breast tumor cells and implications for chemotherapy. *J Exp Med* 1998; **187**: 1583–98.
13. Altan N, Chen Y, Schindler M *et al.* Tamoxifen inhibits acidification in cells independent of the estrogen receptor. *Proc Natl Acad Sci USA* 1999; **96**: 4432–7.
14. Chen Y, Schindler M, Simon SM. A mechanism for tamoxifen-mediated inhibition of acidification. *J Biol Chem* 1999; **274**: 18364–73.
15. Wiseman H, Cannon M, Arnstein HR. The protective effect of ascorbate on the inhibition of growth, RNA and protein synthesis by tamoxifen in yeast is time dependent. *Biochem Soc Trans* 1990; **18**: 1167–8.
16. Uliana SRB, Goyal N, Freymüller E *et al.* *Leishmania*: overexpression and comparative structural analysis of the stage-regulated *meta 1* gene. *Exp Parasitol* 1999; **92**: 183–91.
17. Barcinski MA, Schechtman D, Quintao LG *et al.* Granulocyte-macrophage colony-stimulating factor increases the infectivity of *Leishmania amazonensis* by protecting promastigotes from heat-induced death. *Infect Immun* 1992; **60**: 3523–7.
18. Zhou Y, Marcus EM, Haugland RP *et al.* Use of a new fluorescent probe, seminaphthofluorescein–calcein, for determination of intracellular pH by simultaneous dual-emission imaging laser scanning confocal microscopy. *J Cell Physiol* 1995; **164**: 9–16.
19. Andreoli WK, Mortara RA. Acidification modulates the traffic of *Trypanosoma cruzi* trypomastigotes in Vero cells harbouring *Coxiella burnetii* vacuoles. *Int J Parasitol* 2003; **33**: 185–97.
20. Tanaka AK, Valero VB, Takahashi HK *et al.* Inhibition of *Leishmania (Leishmania) amazonensis* growth and infectivity by aureobasidin A. *J Antimicrob Chemother* 2007; **59**: 487–92.
21. Barasch J, Kiss B, Prince A *et al.* Defective acidification of intracellular organelles in cystic fibrosis. *Nature* 1991; **352**: 70–3.
22. Beggs WH. Drug protonation and pH in relation to the lethal action of tamoxifen on *Candida albicans*. *J Antimicrob Chemother* 1996; **37**: 841–2.
23. Drutz DJ, Huppert M, Sun SH *et al.* Human sex hormones stimulate the growth and maturation of *Coccidioides immitis*. *Infect Immun* 1981; **32**: 897–907.
24. Williams JP, McDonald JM, McKenna MA *et al.* Differential effects of tamoxifen-like compounds on osteoclastic bone degradation, H⁺-ATPase activity, calmodulin-dependent cyclic nucleotide phosphodiesterase activity, and calmodulin binding. *J Cell Biochem* 1997; **66**: 358–69.

25. Williams JP, Blair HC, McKenna MA *et al.* Regulation of avian osteoclastic H⁺-ATPase and bone resorption by tamoxifen and calmodulin antagonists. Effects independent of steroid receptors. *J Biol Chem* 1999; **271**: 12488–95.
26. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P *et al.* Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase. *Science* 1994; **263**: 678–81.
27. Zilberstein D, Shapira M. The role of pH and temperature in the development of *Leishmania* parasites. *Annu Rev Microbiol* 1994; **48**: 449–70.
28. Jiang S, Anderson SA, Winget GD *et al.* Plasma membrane K⁺/H⁺-ATPase from *Leishmania donovani*. *J Cell Physiol* 1994; **159**: 60–6.
29. Jiang S, Meadows J, Anderson SA *et al.* Antileishmanial activity of the antiulcer agent omeprazole. *Antimicrob Agents Chemother* 2002; **46**: 2569–74.
30. Marchesini N, Docampo R. A plasma membrane P-type H⁺-ATPase regulates intracellular pH in *Leishmania mexicana amazonensis*. *Mol Biochem Parasitol* 2002; **119**: 225–36.
31. Custódio JB, Moreno AJ, Wallace KB. Tamoxifen inhibits induction of the mitochondrial permeability transition by Ca²⁺ and inorganic phosphate. *Toxicol Appl Pharmacol* 1998; **152**: 10–7.
32. Custódio JB, Almeida LM, Madeira VC. A reliable and rapid procedure to estimate drug partitioning in biomembranes. *Biochem Biophys Res Commun* 1991; **176**: 1079–85.
33. Goad LJ, Holz GG, Jr, Beach DH. Sterols of *Leishmania* species. Implications for biosynthesis. *Mol Biochem Parasitol* 1984; **10**: 161–70.
34. Jemal A, Siegel R, Ward E *et al.* Cancer statistics, 2006. *CA Cancer J Clin* 2006; **56**: 106–30.
35. Kemp JV, Adam HK, Wakeling AE *et al.* Identification and biological activity of tamoxifen metabolites in human serum. *Biochem Pharmacol* 1983; **13**: 2045–52.
36. Kisanga ER, Gjerde J, Guerrieri-Gonzaga A *et al.* Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. *Clin Cancer Res* 2004; **10**: 2336–43.
37. Lien EA, Solheim E, Ueland PM. Distribution of tamoxifen and its metabolites in rat and human tissues during steady-state treatment. *Cancer Res* 1991; **51**: 4837–44.