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Efficacy of tamoxifen and miltefosine combined therapy for cutaneous leishmaniasis in the murine model of infection with *Leishmania amazonensis*

Cristiana T. Trinconi, Juliana Q. Reimão, Adriano C. Coelho and Silvia R. B. Uliana*

Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, 05508-000 São Paulo, Brazil

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

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Objectives: The objective of this study was to characterize *in vitro* interactions and evaluate the antileishmanial activity of tamoxifen and miltefosine combinations.

Methods: Interactions between drugs were evaluated *in vitro* against *Leishmania amazonensis* promastigotes and intracellular amastigotes by a modified isobologram method. Four different drug ratios were used to calculate the FIC index (FICI) and the mean sum of FICI. Treatment of *L. amazonensis*-infected BALB/c mice was initiated 4 weeks post-infection. Mice were treated with the half-maximal effective dose (ED_{50}) or half the ED_{50} of tamoxifen and miltefosine orally for 15 days. Efficacy was evaluated by lesion growth and parasite burden measured through luciferase detection at the end of treatment and 30 days later. Characterization of growth curves and stepwise increase in drug concentrations *in vitro* were used to measure survival and resistance selection of parasite populations submitted to combination treatment.

Results: No *in vitro* interactions between tamoxifen and miltefosine were found. In infected mice, the combination of tamoxifen and miltefosine at doses corresponding to half the ED_{50} was more effective than monotherapy with either tamoxifen or miltefosine. When the ED_{50} was employed, the efficacy of the combination was equivalent to miltefosine monotherapy. *In vitro*, tamoxifen was able to retard or suppress the growth of parasites treated with miltefosine.

Conclusions: *In vitro* and *in vivo* studies revealed no interaction between tamoxifen and miltefosine. Tamoxifen was able to hinder the emergence of miltefosine resistance.

Introduction

Leishmaniasis is a neglected disease caused by >20 species of protozoan parasites of the *Leishmania* genus. This infection causes a complex of diseases with clinical manifestations ranging from localized cutaneous lesions to severe diffuse tegumentary forms or to fatal visceral disease in untreated patients. About 12 million people are infected worldwide in a widespread geographical distribution that places 350 million people at risk of acquiring this parasitic infection.[1,2]

Treatment of visceral and cutaneous leishmaniasis relies on a limited drug arsenal with substantial limitations regarding efficacy, tolerability, cost and, more recently, problems derived from the emergence of parasite resistance. Miltefosine, an alkyl phospholipid, has emerged as an effective drug against visceral leishmaniasis[3,4] and was incorporated into the therapeutic routine in South Asia (India, Nepal and Bangladesh). Its use for the treatment of cutaneous leishmaniasis is still under investigation.[5–7]

In visceral-leishmaniasis patients, treatment failures,[8] as well as the identification of clinical strains resistant to miltefosine, have already been reported.[9,10] Relapses after treatment with miltefosine, on the other hand, are not always associated with decreased parasite susceptibility to the drug.[8,10,11] It also has to be noted that *in vitro* selection of miltefosine resistance can be easily achieved.[12–15]

According to the WHO, strategies to improve leishmaniasis therapy include the search for new drugs, repurposing of existing drugs and combination therapy.[16] Combination therapy is employed extensively for other infectious diseases, such as malaria, AIDS and TB,[17–19] and the need to consider drug combinations in antileishmanial therapy has been pointed out by several authors.[20–22]

The interactions of miltefosine with other standard antileishmanial drugs have been investigated *in vitro* and *in vivo*[23,24] and are being tested in clinical trials for visceral leishmaniasis.[25–27]

Tamoxifen, an antitumoral oral compound, was recently proposed as a potential drug candidate for visceral- and cutaneous-leishmaniasis chemotherapy.[28,29] In a cutaneous-leishmaniasis experimental model of extreme susceptibility, exemplified by the infection of BALB/c mice with *Leishmania amazonensis*, tamoxifen given for 15 days at doses of 20 mg/kg resulted in significant decreases in lesion size and parasite burden.[28] Moreover, multiple attempts to generate tamoxifen-resistant *L. amazonensis in vitro* and *in vivo* upon drug selection were unsuccessful, indicating that the drug's mode of action probably involves multiple targets.[30]

Considering the present risks of selecting miltefosine resistance in the field, we decided to characterize the properties of combinations of tamoxifen and miltefosine when used in the chemotherapy of cutaneous leishmaniasis. Therefore, the aim of this work was to investigate the efficacy of combinations of miltefosine and tamoxifen against *L. amazonensis in vitro* and in infected mice.

Materials and methods

Parasites and macrophages

Promastigotes of an *L. amazonensis* (MHOM/BR/1973/M2269) transgenic line expressing luciferase (La-LUC)[31] were grown in M-199 medium supplemented with 10% heat-inactivated FCS (Gibco™, Invitrogen Corporation), 25 mM HEPES (pH 6.9), 12 mM $NaHCO_3$, 7.6 mM haemin, 50 U/mL penicillin, 50 mg/L streptomycin and 32 mg/L G418 at 25°C. Bone marrow-derived macrophages (BMDMs) were obtained from BALB/c mice as previously described.[31]

Drugs

Tamoxifen, tamoxifen citrate and miltefosine were purchased from Sigma-Aldrich (St Louis, MO, USA). Tamoxifen and miltefosine stock solutions (10 mM) were prepared in ethanol and sterile water, respectively, and subsequent dilutions were done in culture media. Stock solutions were kept at −20°C. For *in vivo* experiments, stock solutions of miltefosine were prepared in sterile water, while tamoxifen citrate was diluted in 45% Cremophor®A25 (Sigma-Aldrich) solution as vehicle. Both drugs were prepared daily immediately before the treatment.

Evaluation of in vitro antileishmanial activity

To determine the half-maximal effective concentration (EC_{50}) in *L. amazonensis* promastigotes, cells were plated in 96-well plates (Costar® Corning Incorporated, Corning, NY, USA) at a density of 2×10^7/mL (final volume of 200 μL) and incubated in the presence of increasing concentrations of miltefosine (from 3.75 to 44 μM) or tamoxifen (from 3.4 to 27 μM) for 24 h at 25°C. The viability of promastigotes was verified by the MTT assay as previously described.[32] Data analysis and calculation of EC_{50} were performed with GraphPad Prism 5.0 software.

For assays with intracellular amastigotes, BMDMs were distributed in 96-well plates (Costar®) at 8×10^4 macrophages per well. The cells were incubated for 24 h at 37°C in the presence of 5% CO_2. Thereafter, macrophages were infected with La-LUC stationary-phase promastigotes in a ratio of 20:1 promastigotes per macrophage. Infected macrophage cultures were kept at 33°C in the presence of 5% CO_2 for 3 h in RPMI 1640 medium containing 10% FCS and then washed twice with sterile PBS to remove free promastigotes. Treatment of infected BMDMs was performed for 48 h with different concentrations of tamoxifen (from 1.5 to 12 μM) and/or miltefosine (from 1.9 to 15 μM). At the end of the experiment, quantification of macrophage infection was performed with the ONE-Glo™ Luciferase Assay System (Promega Corporation), according to the manufacturer's instructions. Briefly, in each well the medium was replaced by 100 μL of PBS plus 20 μL of ONE-Glo reagent at room temperature, followed by homogenization. Luminescence was evaluated in a microplate reader (Polarstar Omega, BMG Labtech). The luminescence reading from treated wells was used to calculate sigmoidal regression curves using untreated infected macrophages as controls. Experiments were performed in triplicate.

Determination of drug interactions

The interactions between drugs were evaluated *in vitro* by a modified isobologram method.[23,33] Top concentrations of individual drugs were determined, ensuring that the EC_{50} was at the serial dilution midpoint. Top concentrations were prepared in proportions of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 of tamoxifen and miltefosine, respectively, followed by serial dilutions (base 2) until the seventh well of the microplate. For each ratio, an EC_{50} was calculated for each of the drugs. Two independent experiments in triplicate were performed for each drug combination and susceptibility assay.

Determination of FIC index (FICI) and isobologram construction

The FICI at the EC_{50} was calculated as EC_{50} when in combination/EC_{50} of drug alone. The sum of FICI ($\sum$FICI) was calculated as $\sum$FICI = FICI drug A + FICI drug B.[23] The mean $\sum$FICI ($x\sum$FICI) was calculated as the average of $\sum$FICI. Isobolograms were built by plotting the FICI for each drug ratio. The $\sum$FICI was used to classify the interaction as recommended by Odds:[34] synergy for $x\sum\text{FICI} \leq 0.5$, no interaction for $x\sum\text{FICI} > 0.5-4$ and antagonism for $x\sum\text{FICI} > 4$.

Evaluation of drug interactions in vivo

In vivo experiments were performed in BALB/c mice (4–5 weeks old) inoculated with 1×10^6 stationary-phase La-LUC promastigotes at the base of the tail (final volume 30 μL). Four weeks after infection, lesion size was evaluated and mice were distributed in homogeneous groups of five or six animals, with equivalent mean lesion sizes. The assignment of treatment to a given group was random. Animals received treatment by oral gavage in 100 μL final volume for 15 consecutive days. Tamoxifen was given at 6.5 or 13 mg/kg/day and miltefosine at 2 or 4 mg/kg/day. These doses correspond approximately to half of the half-maximal effective dose (ED_{50}) and to the ED_{50} for the two drugs, respectively. Miltefosine was also used at doses of 13 mg/kg/day in dose-finding studies. Combination schemes were given as an ED_{50} dose scheme (4 mg/kg/day miltefosine plus 13 mg/kg/day tamoxifen) and $0.5ED_{50}$ dose scheme (2 mg/kg/day miltefosine plus 6.5 mg/kg/day tamoxifen). Animals were also treated with the vehicle used to dissolve tamoxifen citrate. Treatment outcome was determined through lesion growth and parasite burden at the end of treatment and 30 days after the end of treatment. Lesion growth was expressed as the mean of the tail-base diameters in horizontal and vertical directions minus the tail measurement value obtained 1 day before the initiation of treatment. Measurements were obtained using a calliper (Mitutoyo Corp., Japan). Parasite load was determined through luciferase detection by bioimaging (IVIS Spectrum, Caliper Life Sciences, Inc., MA, USA) as previously described.[31] Briefly, bioimaging was obtained 20 min after luciferin intraperitoneal administration (75 mg/kg of VivoGlo™ Luciferin, Promega). Images were taken in high-resolution mode with 2 min of time exposure from a fixed-size region of interest. Results were analysed with Living Image software version 4.3.1 (Caliper Life Sciences, Inc.) and expressed as the number of photons/s/cm^2/steradian.

L. amazonensis growth curve in the presence of tamoxifen and/or miltefosine

L. amazonensis promastigotes at late logarithmic phase were seeded in medium 199 at a concentration of 2×10^5 cells/mL. Cells were incubated with 7 or 14 μM tamoxifen and 18 or 24 μM miltefosine, or with

combinations of the two drugs. The number of promastigotes was determined daily by microscopic counting in a Neubauer haemocytometer.

Miltefosine resistance selection in combination with tamoxifen

Selection of resistant parasites was performed using stepwise selection as previously reported.[15,30] For stepwise selection with miltefosine, drug concentrations applied to *L. amazonensis* promastigotes were 20 μM followed by 40 and 80 μM. Combined stepwise selection was performed in the presence of fixed concentrations of tamoxifen of 2, 4, 6, 8 or 14 μM and varying concentrations of miltefosine. Selection was performed with at least three successive passages for each dose in combination or not. At each step, miltefosine's and tamoxifen's EC_{50} for the selected parasites were determined.

Ethics statement

Animal experiments were approved by the Ethics Committee for Animal Experimentation (Protocol 178/138/02) in agreement with the guidelines of the Sociedade Brasileira de Ciência de Animais de Laboratório (SBCAL) and of the Conselho Nacional de Controle da Experimentação Animal (CONCEA).

Statistical analysis

Data were analysed for statistical significance by one-way ANOVA followed by the Tukey post test. Statistical analyses were performed using GraphPad Prism 5 software.

Results

Interaction between tamoxifen and miltefosine in vitro

To evaluate the behaviour of combinations of tamoxifen and miltefosine, we first established the EC_{50} for each drug against the *L. amazonensis* line expressing luciferase (La-LUC), to confirm that drug susceptibility was unchanged compared with the parental line. The EC_{50} of tamoxifen was calculated as 13.51 ± 0.10 and 4.25 ± 0.80 μM (Table 1) for promastigotes and amastigotes, respectively. Miltefosine's EC_{50} was determined as 16.82 ± 1.74 and 2.61 ± 0.30 μM for promastigotes and amastigotes, respectively (Table 1). For both drugs, EC_{50} values were in accordance with previously published data for the WT parasite and for La-LUC.[15,35] Tamoxifen and miltefosine were combined in four different ratios (4:1, 3:2, 2:3 and 1:4). The EC_{50} values obtained for each drug ratio were used to calculate the FICIs and ΣFICI, as shown in Table 1. For promastigotes and intracellular amastigotes, *x*ΣFICI was calculated as 1.32 and 0.63, respectively (Table 1). According to Odds,[34] these values are indicative of no interactions. The isobolograms representing tamoxifen and miltefosine interactions when used against promastigotes and amastigotes are shown in Figure 1.

Combination of tamoxifen and miltefosine in vivo

The *in vivo* drug combination assay was performed in a BALB/c mouse experimental model of infection with La-LUC. Tamoxifen's ED_{50} was previously determined in this model as 13.2 mg/kg/day.[35] Miltefosine was shown to be effective for the treatment of *L. amazonensis* infections in BALB/c mice when used at 13 mg/kg/day for 15 days.[15] To determine the dose–response curve for miltefosine in this model, mice were treated with 2, 4 and 13 mg/kg/day and the ED_{50} was calculated as 3.5 mg/kg/day (Figure 2).

The combined treatment schemes were designed using doses corresponding to the approximate ED_{50} of each drug (13 mg/kg/day tamoxifen plus 4 mg/kg/day miltefosine) or half the ED_{50} of each drug (6.5 mg/kg/day tamoxifen plus 2 mg/kg/day miltefosine), always given orally. Progression of disease was evaluated by measuring lesion growth and parasite burden. No differences were observed in these parameters when untreated mice were

Table 1. EC_{50} and FICI of tamoxifen/miltefosine combinations against *L. amazonensis*

	$EC_{50} \pm$ SD (μM)		FICI			
Drug ratio of tamoxifen:miltefosine	tamoxifen	miltefosine	tamoxifen	miltefosine	∑FICI	*x*∑FICI
Promastigotes[a]						
5:0	13.51 ± 0.10					1.32
4:1	13.49 ± 0.21	4.32 ± 1.00	1.00	0.26	1.26	
3:2	11.39 ± 0.13	9.43 ± 2.13	0.84	0.56	1.40	
2:3	7.27 ± 0.88	13.74 ± 1.87	0.54	0.82	1.36	
1:4	3.35 ± 0.56	17.20 ± 2.04	0.25	1.02	1.27	
0:5		16.82 ± 1.74				
Intracellular amastigotes[b]						
5:0	4.25 ± 0.80					0.63
4:1	2.36 ± 0.41	0.15 ± 0.03	0.56	0.06	0.61	
3:2	1.30 ± 0.27	0.68 ± 0.14	0.30	0.26	0.57	
2:3	0.42 ± 0.06	1.40 ± 0.19	0.10	0.53	0.63	
1:4	0.09 ± 0.01	2.55 ± 0.36	0.02	0.97	1.00	
0:5		2.61 ± 0.30				

[a]*In vitro* activities of tamoxifen and miltefosine against promastigotes were determined by the MTT assay.
[b]Activity against intracellular amastigotes was determined in infected macrophages by luminescence.

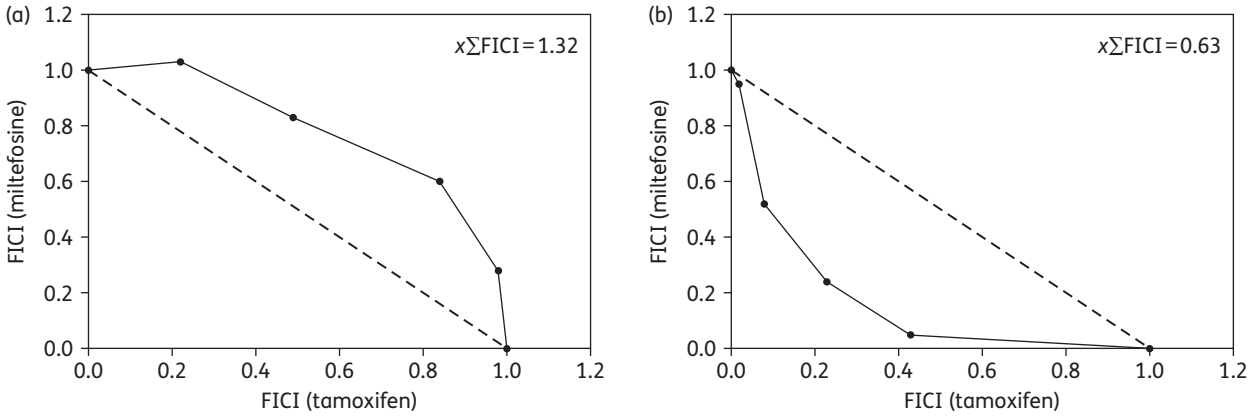

Figure 1. *In vitro* drug interactions between tamoxifen and miltefosine. Drug activity was measured against *L. amazonensis* promastigotes (a) and intracellular amastigotes (b). Isobolograms represent activity at the EC_{50}. Results shown are from one experiment performed in triplicate, representative of at least two independent experiments. Plots were compared with a theoretical line that produced $\sum FICI = 1$ at all ratios tested (broken line), which represents no interaction. $x\sum FICI$ for all ratios tested is shown in the upper right corner.

compared with the control group treated with the vehicle alone (data not shown).

At the end of the treatment, the mean lesion growth in the group treated with the $0.5ED_{50}$ dose scheme (6.5 mg/kg/day tamoxifen plus 2 mg/kg/day miltefosine) was significantly reduced when compared with the untreated group (Figure 3a). Lesions in this combination group were also reduced, although not at statistically significant levels, when compared with animals treated with each of the drugs alone. Mice treated with the $0.5ED_{50}$ combination scheme also displayed a significant decrease in parasite burden in comparison with the untreated group (Figure 3b). As observed for lesion size, the decrease in parasite burden observed in animals treated with the combination at the $0.5ED_{50}$ dose was larger than in animals treated with monotherapy at the same doses (Figure 3b), but the difference was not statistically significant. The combination employing the approximate ED_{50} of each drug (13 mg/kg/day tamoxifen plus 4 mg/kg/day miltefosine) was as effective as miltefosine alone at the end of treatment, considering clinical and parasitological parameters (Figure 3a and b).

To assess the long-term efficacy of the combination treatment, lesion growth and parasite load were determined 30 days after the end of treatment. At this time, lesions and parasite burden in animals treated with the combination schemes remained significantly reduced compared with the control untreated group (Figure 3c and d). The $0.5ED_{50}$ combination scheme resulted in better clinical and parasitological outcomes than miltefosine or tamoxifen as single drugs (Figure 3c and d). Animals treated with the ED_{50} combination scheme presented a clinical and parasitological outcome equivalent to miltefosine single therapy (Figure 3c and d). The parasite burden through luciferase quantification of representative animals for each group at the end of treatment and 30 days later is shown in Figure 4(a and b).

Influence of sub-effective doses of tamoxifen on selection of resistance to miltefosine

In vitro selection of miltefosine-resistant parasites can be easily obtained in different species of *Leishmania* by a stepwise increase in drug concentration.[12–15] Having shown that tamoxifen and miltefosine can be used in combination without any undesirable antagonism, we next tried to verify whether the use of the combination would have an impact on restraining or delaying the selection of miltefosine-resistant parasites. With this aim, we evaluated the survival and growth of parasites in the presence of different concentrations of miltefosine or tamoxifen used alone or in combination. Promastigotes grown in the presence of 18 μM miltefosine (the approximate EC_{50}) achieved the stationary phase at day 8, while untreated cultures achieved the stationary phase at day 7, at a maximum density 25% higher than miltefosine-treated cultures (Figure 5). A higher miltefosine dose (24 μM) resulted in a similar growth profile to that presented by parasites cultured in the presence of 18 μM miltefosine.

On the other hand, in the presence of 14 μM tamoxifen (the approximate EC_{50}), there was no growth until the experimental endpoint (60 days) (Figure 5 and data not shown). When cultured in

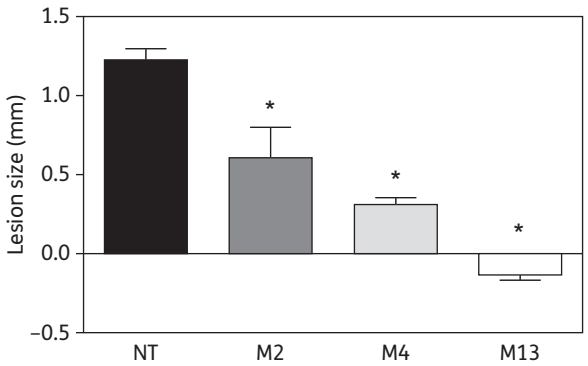

Figure 2. Miltefosine's ED_{50} in a cutaneous leishmaniasis animal model. BALB/c mice were infected with 1×10^6 promastigotes of *L. amazonensis* at the base of the tail. Starting 4 weeks post-infection, animals were treated with 2, 4 or 13 mg/kg/day miltefosine for 15 consecutive days by oral gavage. To determine the ED_{50} of miltefosine, normalized lesion growth at the end of treatment was plotted and analysed as sigmoidal dose–response curves with variable slopes using GraphPad Prism 5 software. NT, untreated; M2, M4 and M13, treated with 2, 4 and 13 mg/kg/day miltefosine, respectively. *$P<0.0001$ versus untreated group.

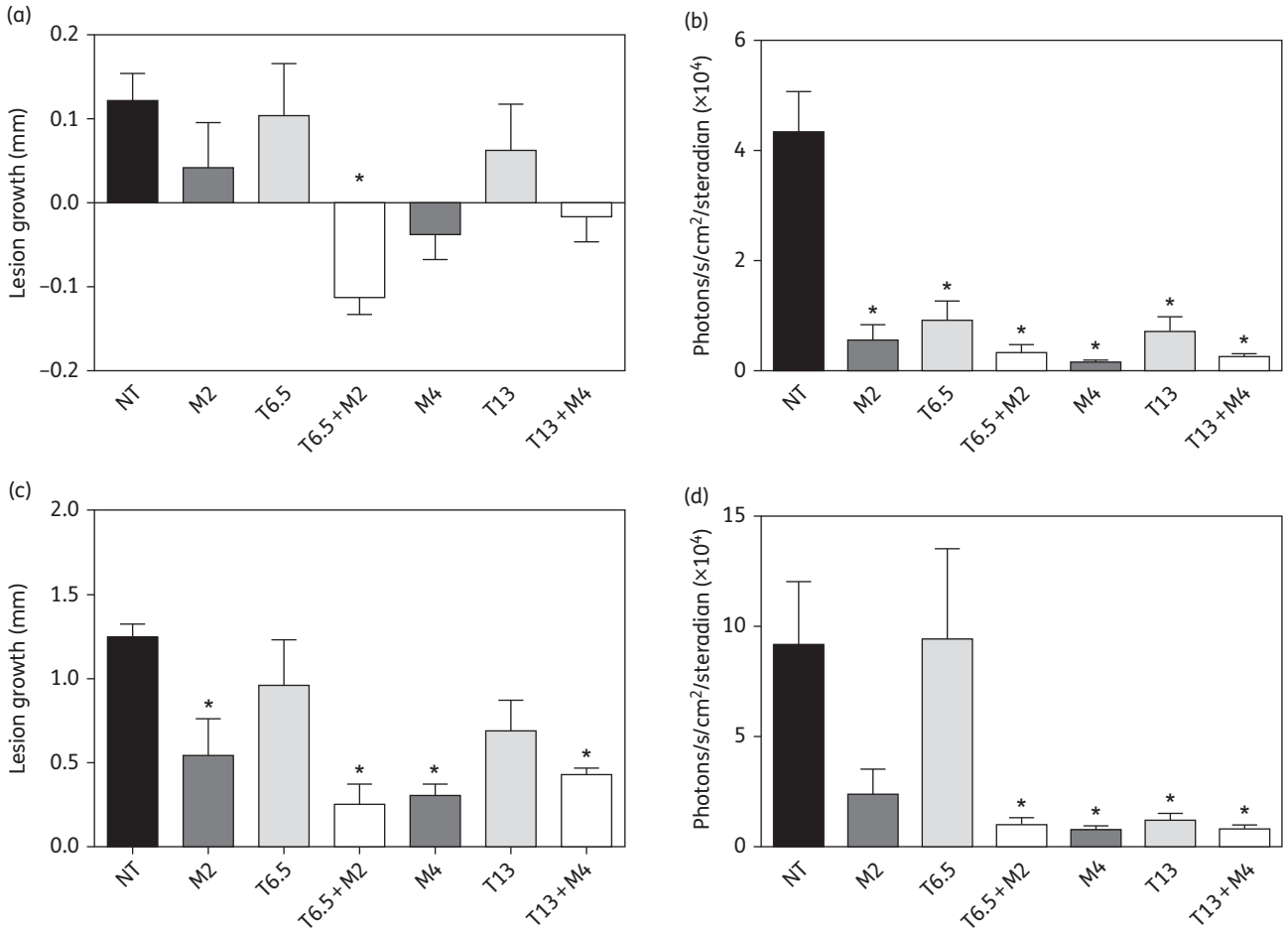

Figure 3. Efficacy of tamoxifen and miltefosine combined therapy in *L. amazonensis* infections. Evaluation of lesion growth (a and c) and parasite burden (b and d) of La-LUC-infected BALB/c mice treated with tamoxifen (6.5 or 13 mg/kg/day) and/or miltefosine (2 or 4 mg/kg/day) at the end of treatment (a and b) and 30 days after the end of the treatment (c and d). NT, untreated animals; T6.5 and T13, animals treated with 6.5 and 13 mg/kg/day tamoxifen; M2 and M4, animals treated with 2 and 4 mg/kg/day miltefosine. *$P < 0.005$ versus untreated group.

a lower concentration of tamoxifen (7 μM), promastigotes presented delayed growth and achieved the stationary phase at day 8. *L. amazonensis* promastigotes were also grown in the presence of both drugs. When parasites were kept in the presence of 7 μM tamoxifen plus 18 or 24 μM miltefosine, a delay in the parasite's growth was observed, with the stationary phase being reached after 16 or 20 days, respectively. As expected, parasites cultured in the presence of 14 μM tamoxifen plus 24 μM miltefosine did not grow until the 60th day of incubation (Figure 5 and data not shown).

To evaluate whether resistance to miltefosine could be selected in the presence of tamoxifen, a classical strategy for drug resistance selection was used. Parasites were incubated initially with 20 μM miltefosine, followed by increases to 40 and 80 μM. Miltefosine's EC_{50} values against selected parasites, determined after at least three passages at 20, 40 or 80 μM, were 67.2 (64.4–70.1), 106.6 (100.9–112.6) and 117.9 (91.0–152.9) μM, respectively (Table 2). On the other hand, and confirming previously reported data,[30] selection with tamoxifen at 2, 4, 6 and 8 μM did not result in altered EC_{50} values in selected parasites when compared with the EC_{50} prior to selection (Table 2 and data not shown). Furthermore, the recovery of living parasites was not possible when tamoxifen concentrations were raised to 14 μM (Figure 5 and Table 2).[30]

To evaluate whether tamoxifen would impair the selection of miltefosine-resistant parasites, a miltefosine stepwise selection was performed in the presence of fixed concentrations of tamoxifen. In these experiments, parasites were seeded in the presence of 2, 4, 6, 8 or 14 μM tamoxifen and 20 μM miltefosine. When these cultures reached stationary phase, they were subcultured in the same drug concentrations twice. When the third stationary phase was reached, these parasites were seeded in medium containing tamoxifen and 40 μM miltefosine. For each step, time to register culture growth and the susceptibility to miltefosine and tamoxifen of selected parasites were evaluated (Table 2 and data not shown).

Selection of miltefosine resistance in the presence of tamoxifen was possible in the presence of tamoxifen concentrations up to 8 μM. In these circumstances, miltefosine-resistant parasites were eventually selected, but after longer periods than in the absence of tamoxifen (Table 2). However, when these parasites grew and reached stationary phase, their susceptibility to miltefosine was decreased in the same manner as parasites selected in

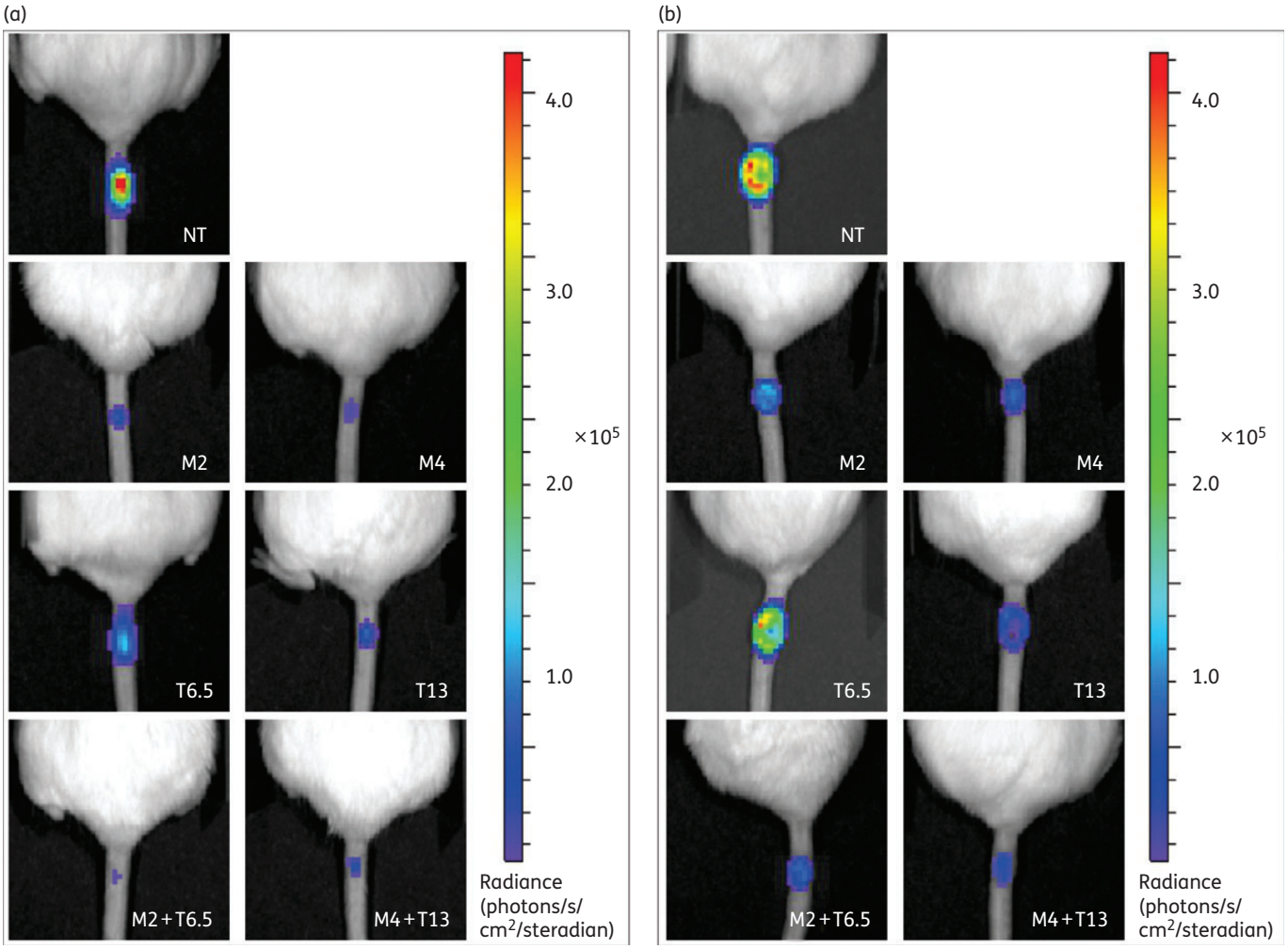

Figure 4. Parasite burden after combined therapy with tamoxifen and miltefosine, evaluated by bioluminescence. Representative animals of groups treated with single and combined schemes of tamoxifen and miltefosine at the end of treatment (a) and 30 days after the end of treatment (b). NT, untreated animals; T6.5 and T13, animals treated with 6.5 and 13 mg/kg/day tamoxifen, respectively; M2 and M4, animals treated with 2 and 4 mg/kg/day miltefosine, respectively. The bar on the right shows a pseudo-colour scale representing light intensities. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

the absence of tamoxifen. For example, the EC_{50} against selected promastigotes grown with 20, 40 and 80 μM miltefosine in combination with 8 μM tamoxifen was 65.4 (64.3–66.6), 113.4 (110.9–116.0) and 127.0 (112.9–142.7) μM, respectively. Tamoxifen's EC_{50} against these parasites was not different from values observed against the WT promastigotes (Table 2 and data not shown). On the other hand, when we attempted to select for miltefosine resistance in the presence of tamoxifen at approximately the EC_{50} (14 μM), no growth was observed, indicating that tamoxifen at its half-effective concentration can prevent selection of miltefosine-resistant parasites *in vitro*.

Discussion

Together with complete compliance, combined therapy is the most important strategy to increase efficacy and prevent drug resistance in infectious diseases. Its advantages include increased effectiveness, reduction in the administered doses and reduction in the length of treatment, with consequent reduction of undesirable side effects and cost. The possibility of delaying the selection of resistant parasites is another interesting advantage of combination therapy.[22]

In this paper, we have evaluated the effects of combinations of tamoxifen and miltefosine. In amastigotes, a shift from right to left in the sigmoidal dose–response curves, indicating a decrease in EC_{50} values, was observed when drugs were administered in combination (data not shown). Isobologram and FICI determinations indicated no interaction for the combination. The use of combined therapy with tamoxifen and miltefosine *in vivo*, employing doses corresponding to half the EC_{50} of each drug, resulted in better clinical and parasitological outcomes than monotherapy with either drug, both at the end of treatment and 30 days after treatment interruption. The EC_{50} combination scheme was clinically and parasitologically as effective as single therapy with miltefosine. While the combination was not synergic,

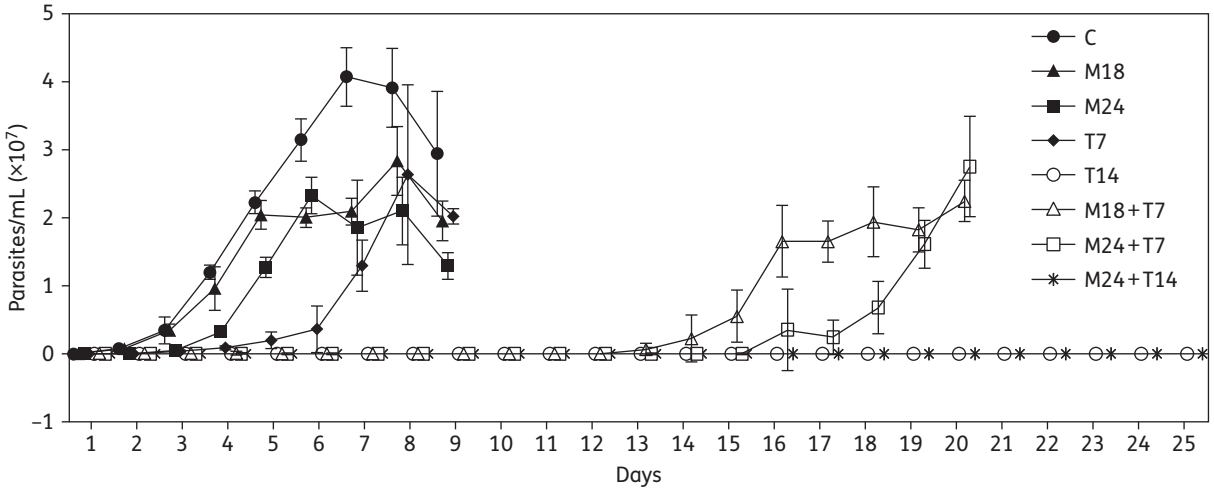

Figure 5. *L. amazonensis* growth curves in the presence of tamoxifen and/or miltefosine. *L. amazonensis* promastigotes (2×10^5 cells/mL) were treated with 7 or 14 μM tamoxifen and 18 or 24 μM miltefosine, as single or combined schemes. Parasite numbers were determined daily by microscopic counting in a Neubauer haemocytometer. C, control group; M18 and M24, promastigotes treated with 18 and 24 μM miltefosine, respectively; T7 and T14, promastigotes treated with 7 and 14 μM tamoxifen, respectively. Results shown are from one experiment performed in triplicate, representative of two independent experiments.

Table 2. Tamoxifen and miltefosine selection applied to *L. amazonensis* as single drugs or combined

	EC_{50} (95% CI) (μM)[b]		Time to stationary phase (days)[c]		
Parasite[a]	tamoxifen	miltefosine	M20	M40	M80
WT	14.4 (12.5–16.6)	18.2 (17.5–19.0)			
T8	14.6 (13.6–15.5)				
T14	NS				
M20		67.2 (64.4–70.1)	10		
M40		106.6 (100.9–112.6)		27	
M80		117.9 (91.0–152.9)			39
T8+M20	16.0 (14.8–17.3)	65.4 (64.3–66.6)	48		
T8+M40	ND	113.4 (110.9–116.0)		71	
T8+M80	ND	127.0 (112.9–142.7)			81
T14+M20	NS	NS			

NS, no survival; ND, not determined.
[a]WT or parasites selected by exposure to tamoxifen (T) and/or miltefosine (M).
[b]EC_{50}, 50% effective concentration against promastigotes, representative of at least two independent experiments.
[c]Time in days, counted from the beginning of selection, required for the cultures to reach the stationary phase on the third passage when submitted to drug concentrations of 20 (M20), 40 (M40) or 80 (M80) μM miltefosine.

these results clearly demonstrated that tamoxifen does not disrupt miltefosine efficacy.

The approval of miltefosine as an antileishmanial drug brought much hope to endemic areas because of its oral administration and remarkable efficacy.[4] However, parasite resistance to miltefosine can be easily detected upon drug selection.[12,14] Moreover, and perhaps for unrelated reasons, a reduction in clinical efficacy has already been observed in visceral-leishmaniasis patients, with relapse rates of 7%–10% observed within 6 months after treatment in India and Nepal.[8,36] Twelve months after the end of treatment, relapse rates of up to 20% were seen in Nepal.[8,36] While most of the relapses identified so far are not due to changes in the parasite's susceptibility to miltefosine, treatment failure can also be associated with drug resistance, as described in a miltefosine-resistant *Leishmania infantum* isolated from an HIV-coinfected patient previously treated with the drug[9] or in *Leishmania braziliensis* patients.[37] Therefore, it seems obvious that miltefosine should not be used as a single drug in the field.

Tamoxifen is a safe, low-cost, orally administered drug that has been widely used as a therapeutic and prophylactic agent in breast cancer therapy for >40 years.[38] The drug has proved to be effective in the treatment of established cutaneous and visceral leishmaniasis in animal models.[28,29] It has also been shown to be effective in combination with amphotericin B.[35]

The antileishmanial mechanisms of action of these two drugs have not been completely elucidated, but data available so far suggest that they have different targets. Our preliminary results indicate that tamoxifen alters parasite-membrane properties and interferes in sphingolipid metabolism (C. T. Trinconi, D. C. Miguel, J. Q. Reimão, N. Heise, A. Alonso and S. R. B. Uliana, unpublished results). Miltefosine's leishmanicidal mode of action is also not completely understood, but its activity has been linked to apoptosis[39,40] and to fatty-acid and sterol metabolism.[41,42] Miltefosine is metabolized mainly by cell phospholipases, generating choline.[43] With a half-life of ~7 days[44] miltefosine is widely distributed in the body, with the highest drug concentrations found in the spleen, adrenal glands, kidneys and skin.[43,45] After oral administration, peak plasma concentrations of tamoxifen are attained in 3–4 h.[46] Tamoxifen is metabolized in the liver by cytochrome P450 and the main active metabolites, 4-hydroxy-tamoxifen and endoxifen, are more potent anti-oestrogens.[47] Due to its high hydrophobicity, tamoxifen (and its metabolites) distributes extensively into peripheral tissues and only a minor portion of the drug is present in the serum.[46,48] In human tissues, tamoxifen concentrations are 10- to 60-fold higher than in the plasma, with levels particularly high in the liver and lungs. Tamoxifen also accumulates in the skin and bones.[48] These data indicate that both miltefosine and tamoxifen could be targeting parasites in the skin and draining lymph nodes in cutaneous-leishmaniasis patients.

Even in the absence of synergic interactions between tamoxifen and miltefosine *in vivo*, the use of this combination offers advantages, such as oral administration, low cost and a multi-target mode of action, and it is likely to have a strong impact in preventing the emergence of drug resistance. As mentioned previously, miltefosine resistance in *Leishmania* can be easily selected upon drug pressure. Selection of miltefosine-resistant *Leishmania* seemed to be possible even when the drug was combined with amphotericin B or paromomycin.[49] However, we have been unable to select for tamoxifen resistance, even after mutagenesis or selection *in vivo*.[30] Taking advantage of tamoxifen's activity, we also showed that miltefosine resistance could not be selected if parasites were grown in the presence of tamoxifen at its EC_{50}.

Taken together, the data presented herein indicate that tamoxifen can be a good clinical partner to miltefosine therapy once it can hinder the selection of miltefosine-resistant parasites. Furthermore, this combination presented good efficacy when used in a challenging experimental animal model, indicating that it deserves further testing.

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Transparency declarations

None to declare.

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