

Combination Therapy with Tamoxifen and Amphotericin B in Experimental Cutaneous Leishmaniasis

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Leishmaniasis chemotherapy remains very challenging. The high cost of active drugs, along with the severity of their side effects and the increasing failure rate of the current therapeutic schemes, calls for the discovery of new active drugs and schemes of treatment. The use of combination therapy has gained much attention in recent years as a possible strategy for overcoming the various shortcomings in the present arsenal. We recently described the effectiveness of tamoxifen in murine models of leishmaniasis, and here, we investigated the interactions between tamoxifen and amphotericin B, one of the most potent drugs used in leishmaniasis treatment. The *in vitro* interactions were indifferent for the association of tamoxifen and amphotericin B. The association was also assayed *in vivo* in *Leishmania amazonensis*-infected BALB/c mice and was found to yield at least additive effects at low doses of both drugs.

Leishmaniasis affects 12 million people worldwide in 98 countries. Clinical presentations are highly variable, ranging from localized cutaneous lesions to severe diffuse tegumentary forms or to the life-threatening visceral form. The treatment of both cutaneous and visceral leishmaniasis depends heavily on expensive and toxic drugs, most of which have to be administered by the parenteral route. The first-line treatment is still based on pentavalent antimonials in many areas, but parasite resistance is now widespread in some regions of the Indian subcontinent. Evidence of decreased efficacy of antimonials in other regions of the world (1–3) and of geographical variations in parasite susceptibility to drugs is also emerging (4). In regions with a high incidence of antimonial resistance, amphotericin B and miltefosine became first-choice drugs, but there are also difficulties with these drugs, related to toxicity or to the possibility of selecting resistant strains (5).

Difficulties in treatment of the various clinical forms of leishmaniasis and the spread of resistance to drugs, mainly observed in areas where anthroponotic transmission is predominant, led to the present trend of utilizing combination therapy, as has been the case for many other infectious diseases. In leishmaniasis, this trend is justified because the available drugs belong to different chemical classes and are believed to have different targets on the parasite and combined therapy has the potential to reduce collateral effects and therefore increase treatment compliance. Furthermore, combination therapy may improve the efficacy of therapeutic schemes in patients with concomitant infections, as is the case in HIV-infected patients, who generally do not respond well to classical therapy.

Several combinations have been tried or are under trial in India and East Africa. For example, a shorter therapeutic scheme of combined sodium stibogluconate (SSG) and paromomycin has been tried in Africa and shown to be as effective as a longer scheme of SSG alone (6). A trial is under way in East Africa to determine the efficacy of miltefosine in combination with SSG and liposomal amphotericin B (7). However, there is concern about the emergence of resistance even for combination therapy (8), since it has been shown that stepwise *in vitro* selection is an efficient method to obtain resistant lines of *Leishmania donovani* against all drugs in use today, including

amphotericin B (9). Amphotericin B resistance has also been detected in clinical isolates (10).

Therefore, the search for new alternative drugs useful for treating leishmaniasis is still necessary. We have previously shown that the selective estrogen receptor modulator (SERM) tamoxifen is effective in the treatment of cutaneous and visceral leishmaniasis in animal models (11, 12). Here we investigated the interactions of tamoxifen and amphotericin B, one of the drugs most widely used for the treatment of leishmaniasis.

MATERIALS AND METHODS

Parasites and macrophages. *Leishmania amazonensis* (MHOM/BR/1973/M2269) promastigotes were grown in M-199 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 25 mM HEPES (pH 6.9), 12 mM NaHCO₃, 7.6 mM hemin, 50 U/ml penicillin, and 50 μg/ml streptomycin at 25°C. Promastigotes of a *L. amazonensis* transgenic line expressing luciferase (La-LUC) (13) were grown in the same medium supplemented with 32 μg/ml G418 and incubated at 25°C. Bone marrow-derived macrophages (BMDM) were obtained from BALB/c mice as described previously (14).

Drugs. Amphotericin B deoxycholate was a gift from Cristália (São Paulo, Brazil). Tamoxifen and tamoxifen citrate were purchased from Sigma-Aldrich. Stock solutions of amphotericin B (10 mM) were prepared in sterile water and kept at –20°C. Tamoxifen stock solutions (10 mM) were prepared in ethanol. Dilutions from the stock solutions were done in culture media. For *in vivo* experiments, stock solutions of tamoxifen citrate and amphotericin B were prepared in saline every day.

Evaluation of *in vitro* antileishmanial activity. Promastigotes were counted in a Neubauer hemocytometer and seeded at 2×10^6 /well in a final volume of 200 μl. The plates were incubated for 24 h at 25°C, and the

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viability of promastigotes was verified by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (15). Data analysis and 50% effective concentration (EC₅₀) determinations were performed with Graph-Pad Prism 5.0 software.

For assays with intracellular amastigotes, macrophages were seeded in 96-well plates (8×10^4 macrophages per well) containing RPMI 1640 medium with 10% FCS and allowed to adhere overnight at 37°C and 5% CO₂. Infections were initiated by adding La-LUC stationary-phase promastigotes at a ratio of 20 promastigotes per macrophage. Infected macrophage cultures were kept at 33°C and 5% CO₂ for 3 h in RPMI 1640 medium with 10% FCS and then were washed twice with sterile phosphate-buffered saline (PBS) to remove free promastigotes. Infected BMDM were treated for 48 h. Enzyme detection was performed with the One-Glo luciferase assay system (Promega Corporation) according to the manufacturer's instructions. Briefly, medium was removed without disturbing the adherent cells and replaced by 100 μ l of PBS. To each well, 20 μ l of One-Glo reagent was added at room temperature followed by homogenization by pipetting the mixture up and down six times. Luminescence units were determined immediately after adding the substrate in a POLARstar Omega reader (BMG Labtech, Ortenberg, Germany). The luminescence reading from treated wells was used to calculate sigmoidal regression curves using untreated infected macrophages as a control.

Determination of drug interactions. The interactions between drugs were evaluated *in vitro* by a modified isobologram method (16, 17). EC₅₀s were used to determine the maximum concentrations of individual drugs, ensuring that the EC₅₀ was at the midpoint of the serial dilution. The highest concentrations of solutions were prepared in proportions of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 of tamoxifen and amphotericin B, respectively, which were serially diluted (base 2) to the seventh well of the microplate in triplicate. Sigmoidal regression curves were used to determine the EC₅₀ as described above. For each ratio, an EC₅₀ was calculated for each of the drugs. Two or three independent experiments were performed for each drug combination and susceptibility assay.

Determination of FIC index and isobologram construction. Fractional inhibitory concentrations (FICs) at the EC₅₀ were calculated as [EC₅₀ when in combination]/[EC₅₀ of drug alone]. The sum of the FICs (Σ FIC) was calculated with the equation Σ FIC = FIC drug A + FIC drug B (17). The mean sum of the FICs ($x\Sigma$ FIC) was calculated as the average of the Σ FICs. Isobolograms were built by plotting the FIC for each drug ratio. The Σ FIC was used to classify the interaction as recommended by Odds (18). Interactions were considered synergic for an $x\Sigma$ FIC of ≤ 0.5 , indifferent with an $x\Sigma$ FIC between 0.5 and ≤ 4 , and antagonistic for an $x\Sigma$ FIC of >4 .

Evaluation of drug interactions *in vivo*. Animal experiments were approved by the Ethical Committee on Animal Experimentation (protocol 178/2012). Female BALB/c mice (4 to 5 weeks old) were inoculated with 1×10^6 stationary-phase La-LUC promastigotes at the base of the tail. Five weeks after infection, mice were randomly assigned to experimental groups ($n = 6$). Doses used *in vivo* were 6.5 or 26 mg/kg of body weight/day tamoxifen and 1.2 or 4 mg/kg/day amphotericin B. Combinations were tested using a low-dose scheme (1.2 mg/kg/day amphotericin B plus 6.5 mg/kg/day tamoxifen) and a high-dose scheme (4 mg/kg/day amphotericin B plus 26 mg/kg/day tamoxifen). Treated groups received intraperitoneal injections containing the drugs in a 200- μ l final volume. All animals received 20 doses of the assigned scheme, given on weekdays.

Disease progression was evaluated once a week by recording the average diameter of the tail measured as the mean of tail base diameters in horizontal and vertical directions. Measurements were taken with a caliper (Mitutoyo Corp., Japan). The treatment outcome was evaluated at the end of the treatment (9 weeks postinfection) through luciferase detection by bioimaging (IVIS Spectrum; Caliper Life Sciences, Inc., MA) as described previously (13). Briefly, prior to imaging, mice received 75 mg/kg luciferin (VivoGlo Luciferin; Promega) intraperitoneally. Imaging was

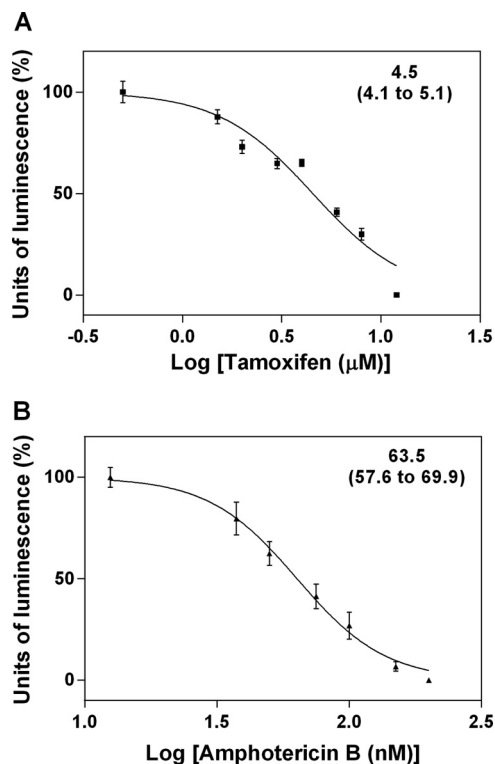


FIG 1 EC₅₀ for *L. amazonensis* intracellular amastigotes. Infected BMDM were treated with tamoxifen (A) or amphotericin B (B) for 48 h at 33°C. The numbers of amastigotes were determined by luciferase quantification. Relative light units for control untreated cells were considered 100%. Values shown are the means and standard deviations of triplicates from one experiment representative of at least three independent experiments. The EC₅₀s and 95% confidence intervals are indicated in the upper right corners.

performed 20 min later, through the high-resolution mode from a fixed-size region of interest. Results were quantified with Living Image software, version 4.3.1 (Caliper Life Sciences, Inc.), and results are expressed as the number of photons/s/cm².

Statistical analysis. Data were analyzed for statistical significance by one-way analysis of variance (ANOVA), followed by the Fisher posttest. Statistical analyses were performed using GraphPad Prism 5 software.

RESULTS

***In vitro* associations of amphotericin B and tamoxifen.** Wild-type or luciferase-expressing parasites were used to determine the EC₅₀s against *L. amazonensis* promastigotes and intracellular amastigotes, respectively. The transgenic parasites express luciferase constitutively and show the same infectivity *in vitro* and *in vivo* as well as susceptibility to drugs as the parental line (13). Tamoxifen EC₅₀s against promastigotes and amastigotes were 13.3 and 4.5 μ M, respectively. Amphotericin B EC₅₀s were determined to be 118.5 and 63.5 nM for promastigotes and amastigotes, respectively (Fig. 1 and data not shown).

The associations between tamoxifen and amphotericin B against *L. amazonensis* promastigotes and intracellular amastigotes were evaluated using the fixed-ratio isobologram method (16, 17). This experimental setup allowed the determination of FIC values for each combination (Table 1). The $x\Sigma$ FICs for all combinations were calculated as 1.23 when tested against promastigotes and 0.74 against intracellular amastigotes. Accord-

TABLE 1 EC₅₀s and FICs of tamoxifen-amphotericin B combinations against *L. amazonensis*

Form	Combination ratio (%) ^a		EC ₅₀ (95% CI) of ^b :		FIC of ^c :		ΣFIC ^d
	Tam	AmB	Tam (μM)	AmB (nM)	Tam	AmB	
Promastigotes ^e	100	0	13.3 (12.6–14.1)				
	80	20	13.2 (12.5–13.9)	23.6 (22.5–24.7)	0.99	0.20	1.19
	60	40	11.2 (10.4–12.1)	53.0 (50.0–56.2)	0.84	0.45	1.29
	40	60	7.7 (6.8–8.7)	82.7 (75.1–91.0)	0.58	0.70	1.28
	20	80	3.7 (3.1–4.3)	105.9 (91.6–122.5)	0.27	0.90	1.17
	0	100		118.5 (111.3–126.2)			
Intracellular amastigotes ^f	100	0	4.5 (4.0–5.1)				
	80	20	2.6 (2.1–3.3)	2.2 (1.7–2.8)	0.57	0.04	0.61
	60	40	1.7 (1.4–1.9)	11.7 (10.0–13.8)	0.37	0.20	0.57
	40	60	0.8 (0.7–1.0)	36.7 (29.6–45.6)	0.18	0.63	0.81
	20	80	0.1 (0.1–0.2)	55.3 (49.6–61.8)	0.03	0.95	0.98
	0	100		63.5 (57.6–70.0)			

^a Tam, tamoxifen; AmB, amphotericin B.

^b CI, confidence interval.

^c FIC, fractional inhibitory concentration at the indicated EC₅₀.

^d ΣFIC, sum of FICs.

^e *In vitro* activities of tamoxifen and amphotericin B against promastigotes was determined by the MTT assay.

^f Activity against intracellular amastigotes was determined in infected macrophages by luminescence.

ing to Odds (18), this indicates an indifferent interaction. This observation was confirmed by plotting the individual FICs in isobolograms (Fig. 2), where all points are close to the additivity line.

Tamoxifen in combination with amphotericin B *in vivo*. The *L. amazonensis*-infected BALB/c mouse was the experimental model employed for *in vivo* efficacy studies. The half-maximal effective dose (ED₅₀) was initially determined for each of the drugs given alone. Tamoxifen and amphotericin B ED₅₀s were determined to be 13.2 mg/kg/day (95% confidence interval, 12.2 to 15.2 mg/kg/day) and 2.2 mg/kg/day (95% confidence interval, 1.1 to 4.4 mg/kg/day), respectively (reference 13 and data not shown).

The therapeutic efficacy of the association was evaluated by comparing animals treated with doses close to the maximal tolerated doses of tamoxifen and amphotericin B in this model (26 mg/kg/day and 4 mg/kg/day, respectively) with those treated with the association scheme employing half of the calculated ED₅₀ for each drug (6.5 mg/kg/day tamoxifen and 1.2 mg/kg/day amphotericin B).

Representative animals from each group are shown in Fig. 3, together with the parasite burden determination through luciferase quantification. The mean sizes of the lesions in groups treated with the combination schemes were reduced compared with the sizes of lesions in animals treated with each of the drugs alone (Fig. 4A). As for the parasite burden evaluated through luciferase quantification, a significant reduction was observed in the group treated with the combined low-dose scheme compared with that in the amphotericin B alone or untreated group (Fig. 4B). The data clearly show that the association of 1.2 mg/kg/day amphotericin B and 6.5 mg/kg/day tamoxifen produced an improved outcome compared with that for tamoxifen or amphotericin B given alone at the same doses.

For groups treated with amphotericin B and tamoxifen at the highest dose, combined therapy produced better clinical outcomes than those for the untreated or treated with each drug alone groups. The parasite burden for the combination treatment at

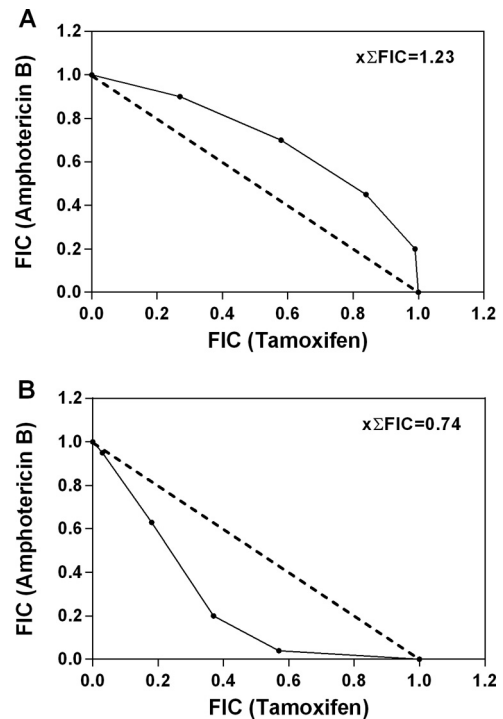


FIG 2 Isobolograms representing *in vitro* interactions between tamoxifen and amphotericin B against *L. amazonensis* promastigotes (A) and intracellular amastigotes (B). Assays were performed by a fixed-ratio method based on the EC₅₀s, with the combinations being tested at constant ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5. Results shown are from one experiment performed in triplicate, representative of at least two independent experiments. Plots were compared to a theoretical line that produced a sum of the FICs of 1 at all ratios tested (dashed line), which represents an additive effect of both compounds. The mean sum of the FICs (xΣFIC) for all interactions tested is shown at the upper right corner.

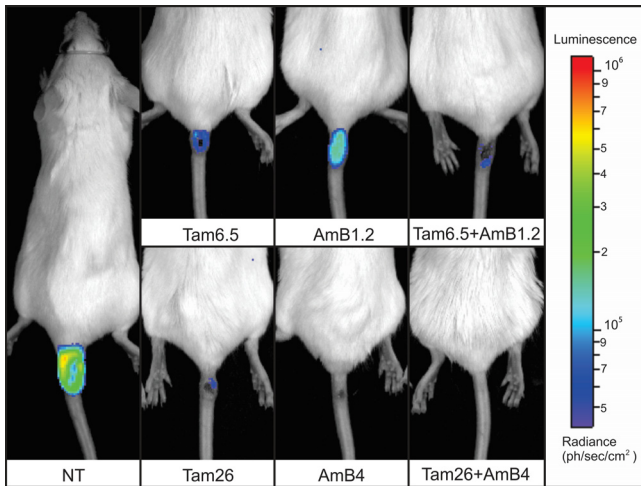


FIG 3 *In vivo* evaluation of parasite burden after combined therapy with tamoxifen and amphotericin B. Treatment was initiated 5 weeks postinfection. Images were collected after the end of treatment at 9 weeks postinfection. Images are of representative animals treated with tamoxifen and amphotericin B (alone or in association). The bar on the right shows a pseudo-color scale representing light intensities. NT, untreated; Tam6.5, Tam26, treated with 6.5 or 26 mg/kg/day tamoxifen alone; AmB1.2, AmB4, treated with 1.2 or 4 mg/kg/day amphotericin B alone; Tam+AmB, treated with combinations of the two drugs in the doses indicated.

high doses was not significantly different from that for the groups treated with single drugs at the same doses (Fig. 4).

DISCUSSION

Combination therapy has been advocated for the treatment of several infectious diseases such as malaria, tuberculosis, AIDS, and, more recently, leishmaniasis. The combination approach may increase effectiveness, especially in immunocompromised patients (19), and may reduce the length of treatment and administered doses. Consequently, the undesirable effects and costs are decreased. Combination therapy is also important in delaying the selection of resistant parasites (20).

Tamoxifen is a SERM that has been in use as a therapeutic and prophylactic agent in breast cancer therapy for decades. Tamoxifen has also been studied as an alternative treatment in other conditions such as retroperitoneal fibrosis (21) and bipolar disease (22, 23). It is a low-cost orally administered drug with a good safety profile, especially in short-term therapeutic schemes, such as the ones proposed in the treatment of bipolar disorder. We previously described the effectiveness of tamoxifen given for 15 to 20 days in the treatment of established cutaneous and visceral leishmaniasis in animal models (11, 12). The work described herein was performed to investigate whether tamoxifen would be a good partner in combination therapy. We chose to test tamoxifen in combination with amphotericin B, one of the most widely used drugs in leishmaniasis treatment nowadays. Amphotericin B is effective, especially for visceral leishmaniasis, but toxicity is considerable (24). The selection of amphotericin B-resistant strains was considered unlikely until recently when some reports suggested that it was possible (9, 10).

In vitro interactions between tamoxifen and amphotericin B, studied through fixed-ratio dilutions and isobologram analysis, were shown to be indifferent or additive. Parameters for the anal-

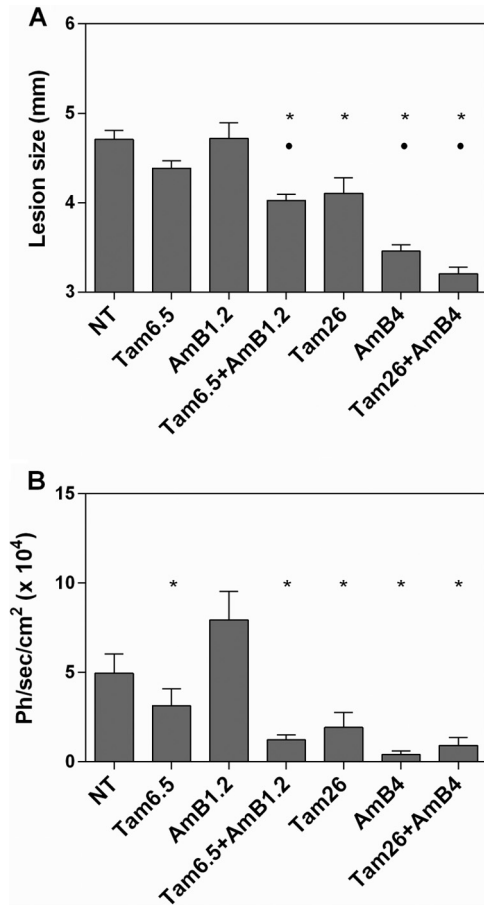


FIG 4 Combined therapy with tamoxifen and amphotericin B. Evaluation of lesion size (A) and parasite burden (B) in mice ($n = 6$) treated with tamoxifen (6.5 or 26 mg/kg/day) and/or amphotericin B (1.2 or 4 mg/kg/day) at the end of treatment. NT, untreated; Tam6.5, Tam26, treated with 6.5 or 26 mg/kg/day tamoxifen alone; AmB1.2, AmB4, treated with 1.2 or 4 mg/kg/day amphotericin B alone; Tam+AmB, treated with combinations of the two drugs in the doses indicated; Ph/sec/cm², photons per second per square centimeter. *, $P < 0.05$ versus the untreated group and versus amphotericin B 1.2 mg/kg/day; •, $P < 0.05$ versus tamoxifen 6.5 mg/kg/day.

ysis and classification of drug interaction profiles *in vivo* are less clear-cut than those for *in vitro* studies. *In vivo* synergism has been suggested to occur when the effect of the drug combination is significantly more pronounced than the sum of the effects of each agent alone (25). In an attempt to quantify *in vivo* the effects of combining tamoxifen and amphotericin B, we calculated the percentages of reduction in lesion size and parasite burden for each treated group compared with those for the untreated controls. While the lesion size was reduced by 25% in the 6.5 mg/kg/day tamoxifen group and by 0% in the 1.2 mg/kg/day amphotericin B group, there was a 55% reduction in the group assigned to low-dose combined therapy. When parasite burdens were considered, there was a 36% reduction in the 6.5 mg/kg/day tamoxifen group and an increase in the 1.2 mg/kg/day amphotericin B group, while a 75% reduction was noted in the group treated with the low-dose combined scheme. Therefore, in both cases, the effect of combined therapy was superior to the sum of effects of the individual drugs, suggesting that tamoxifen and amphotericin B have additive and possibly synergistic behavior *in vivo*.

A potential lack of correlation between *in vitro* (additive) and *in vivo* (additive/synergistic) interaction profiles is not unexpected since several factors come into play *in vivo* that cannot have an effect *in vitro*, such as pharmacokinetics, metabolism, distribution, and a possible role of the immune system in controlling the infection.

In mice, the elimination half-life of micellar amphotericin B is 89 min (26), while the rate of elimination from serum following a single dose of tamoxifen in mice is 11.9 h (27). Tamoxifen is rapidly metabolized into *N*-desmethyltamoxifen and 4-hydroxytamoxifen, both of which are active and have plasma half-lives of 9.6 and 6.0 h, respectively. The different rates of elimination may provide an extended effect for the combination scheme.

Another factor that needs to be taken into account is the mechanism of action. It is generally accepted that amphotericin B interacts with membrane sterols and with higher affinity with ergosterol, which is abundant in parasite membranes (28). That results in membrane disorganization, possibly through pore formation (28, 29). Amphotericin B also leads to lipid peroxidation and generation of free radicals (30–32).

Tamoxifen's mechanism of action against *Leishmania* is still unclear, but in mammalian cells this highly lipophilic molecule interacts with the membrane lipids (33). Therefore, altered membrane physiology might extend the lesions caused by amphotericin B. On the other hand, tamoxifen has been shown to prevent lipid peroxidation (34) and to behave as an antioxidant (35, 36), which might reduce the adverse effects of amphotericin B.

In conclusion, the data presented herein indicate clearly that tamoxifen does not hinder amphotericin B activity and that lower doses of the two drugs combined result in good clinical and parasitological responses.

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