PHYTOTHERAPY RESEARCH
Phytother. Res. **15**, 44–48 (2001)

In vitro and *in vivo* Leishmanicidal Activity of 2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (Lapachol)

Maria Jania Teixeira[1]*, Yacy M. de Almeida[1], Joseval R. Viana[1], Joana G. Holanda Filha[1], Tatiana P. Rodrigues[1], José Rômulo C. Prata Jr.[1], Ivo Castelo B. Coêlho[1], Vietla S. Rao[2] and Margarida M. L. Pompeu[1]

[1]Núcleo de Medicina Tropical Prof. Joaquim Eduardo de Alencar, Faculdade de Medicina, Universidade Federal do Ceará, Rua Alexandre Baraúna, 949, 60.430-160 Fortaleza, CE, Brazil
[2]Departamento de Fisiologia e Farmacologia, Faculdade de Medicina, Universidade Federal do Ceará, Rua Alexandre Baraúna, 949, 60.430-160 Fortaleza, CE, Brazil

This study aims to evaluate the *in vitro* and *in vivo* leishmanicidal activity of lapachol, a naphthoquinone found in the seeds and heartwood of certain tropical plants, and to compare its efficacy with a reference drug, sodium stibogluconate (Pentostam®). These compounds (0.0125–4.0 mg/mL) were evaluated *in vitro* against intracellular amastigotes of *Leishmania (Viannia) braziliensis* (*LVb*), then tested in an animal model (hamster) to try to reproduce the leishmanicidal activity. *In vitro*, lapachol exhibited an anti-amastigote effect, whereas *in vivo* it did not prevent the development of *LVb*-induced lesions at an oral dose of 300 mg/kg/day for 42 days. Pentostam® demonstrated a significant anti-amastigote effect *in vitro* for *LVb* and apparent clinical cure *in vivo* (60 mg/kg/day). However, it could not completely eradicate parasites from the tissues of infected animals. The observation that lapachol exerts leishmanicidal activity *in vitro* without offering significant protection against *LVb*-infected lesions in hamsters suggests that lapachol *in vivo* might possibly inhibit the microbicidal functioning of macrophages. Alternatively, it might be transformed into an inactive metabolite(s) or neutralized, losing its leishmanicidal activity. It is also possible that an optimal and sustained plasma level of the drug could not be achieved at the dose used in this study.

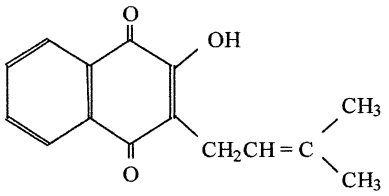
Keywords: Leishmania braziliensis; lapachol; sodium stibogluconate; hamster; treatment.

INTRODUCTION

Cutaneous leishmaniasis (CL) is an endemic disease prevalent in many parts of the world, characterized by chronic skin ulcers at the site of the sandfly bite. Antimonials, such as meglumine antimoniate and sodium stibogluconate, or pentamidine, the drugs of choice for the treatment of leishmaniasis, are drugs administered by parenteral route which have unpleasant and serious side effects such as renal, neural and cardiac toxicity, shock, pancreatitis and the risk of diabetes (Pearson and Sousa, 1995; Becker *et al.*, 1999; Ribeiro *et al.*, 1999). In addition, clinical failures and antimony-resistant parasites have been reported (Olliaro and Bryceson, 1993; Moreira *et al.*, 1998; Lira *et al.*, 1999). Therefore, drugs that are more effective, less toxic and easier to use are strongly needed.

In recent years, there has been an increase in the search for new antiparasitic drugs developed from medicinal plants (Iwu *et al.*, 1994; Phillipson *et al.*, 1995; Kirby, 1996). Lapachol, 2-hydroxy-3-(3-methyl-2-butenyl)-1, 4-naphthoquinone (Fig. 1), is an antimicrobial (Binutu *et al.*, 1996), antiinflammatory (de Almeida *et al.*, 1990) and antitumour agent (Rao *et al.*, 1968; Dinnen and Ebisuzaki, 1997) of the naphthoquinone group, isolated from some species of *Bignoniaceae*. Lapachol, as well as some of its derivatives, α and β-lapachone and quercetin, were shown to have an antiparasitic action against *Schistosoma mansoni*, *Trypanosoma cruzi* and *T. brucei* (Oswald, 1993/94). The reported biological activities of lapachol led us to investigate the leishmanicidal potential of this substance.

The antileishmanial activity was evaluated both *in*



2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone

Figure 1. Chemical structure of lapachol.

* Correspondence to: Dr M. J. Teixeira, Rua Alexandre Baraúna, 949, 60.430-160 Fortaleza, CE, Brazil.
E-mail: pato@ufc.br
Contract/grant sponsor: CNPq.
Contract/grant sponsor: Escola de Saúde Pública do Ceará (ESP-CE).

Received 16 September 1999
Revised 13 December 1999
Accepted 10 February 2000

vitro by testing on *Leishmania (Viannia) braziliensis* (*LVb*) amastigotes and *in vivo* on cutaneous *LVb*-infected lesions in hamsters, comparing its efficacy with a reference antimonial drug, sodium stibogluconate, Pentostam®.

MATERIALS AND METHODS

Drugs tested. Pure lapachol was supplied by the Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE) as yellow coloured crystals. Vials of sodium stibogluconate (Pentostam®, MEHECO National Medicines & Health Products, China) were provided by the Fundação Nacional de Saúde do Ceará (FNS-Ce), Brazil.

Parasites. The *L. (V.) braziliensis* stock (MHOM/BR/94/H-3227) used was originally isolated from skin lesions of a patient with CL, and typed using enzyme electrophoresis and monoclonal antibodies. The parasites were maintained in Schneider's drosophila medium (Sigma Chemical Co., St. Louis, USA) with antibiotics [100 IU/mL penicillin G-potassium, 100 μg/mL streptomycin sulphate (Sigma)] in agar-blood bottles at 26 °C. The parasites were serially passaged in hamsters or cryopreserved. When needed they were freshly isolated on Schneider's medium supplemented with antibiotics, 2 mM L-glutamine (Gibco BRL, Grand Island, USA), 10% of sterile fetal bovine serum (FBS) and 2% of sterile normal human urine. Stationary phase promastigotes were used for infection. Before use, promastigotes were harvested from culture, washed in sterile saline and resuspended to get the desired concentration.

In vitro assays

Macrophage cultures. Swiss mice peritoneal macrophages were stimulated by injection of starch (1 mL of 3%, i.p.) and after 3 days, collected with 10 mL of RPMI-1640 medium (Sigma) containing heparin (10 IU/mL). The macrophages were then centrifuged (1200 rpm, 4 °C), washed in sterile saline and resuspended in RPMI medium with 10% FBS to obtain a concentration of 10^6 cell/mL. Aliquots of 200 μL were deposited in each well of 96-well culture plates. The cells were allowed to adhere for 3 h at 37 °C in 5% CO_2. Nonadherent cells were removed by washing with warm culture medium and supernatant replaced for fresh RPMI + 10% FBS + antibiotics, followed by additional incubation for 24 h before evaluation of the drugs.

Cytotoxicity to macrophages. Cytotoxicity was evaluated by exposing the non-parasitized peritoneal macrophages to different concentrations (0.0125–4.0 mg/mL) of lapachol and Pentostam®. After incubating for 12 h at 37 °C in 5% CO_2, the percentage of viable cells was determined by staining with 0.4% trypan blue (dye exclusion procedure for viable cell counting). Concentrations of drugs that produced less than 20% cell death were considered not toxic.

Anti-amastigote activity. Swiss mice peritoneal macrophages were plated in 96-well plates at 10^6 cells/mL per well in RPMI medium supplemented with antibiotics and 10% FBS. Macrophages were infected with stationary-phase *LVb* promastigotes (3×10^6 parasites/mL per well) for up 12 h at 37 °C in 5% CO_2. Extracellular parasites were removed by washing with warm culture medium and the compounds to be tested were added in different concentrations. The drugs were diluted in 2% dimethylsulphoxide (DMSO) and in RPMI medium at final concentrations of 0.0125 to 4.0 mg/mL. Cultures were followed by additional incubation for 24 h. The supernatant was replaced for Schneider's medium supplemented with antibiotics, 10% FBS and 2% of sterile human urine, and the plates were incubated at 26 °C up to 72 h in order to allow surviving *LVb* amastigotes to transform into promastigotes. Then, 1 μCi per well of [^{3}H]thymidine (Amersham International, Amersham, UK) was added and the cells were incubated for another 24 h and harvested. [^{3}H]thymidine incorporation was measured in a β-counter (Pharmacia, Finland). Assays were performed in six replicates and the inhibition of growth was expressed as the percent decrease of radioactive incorporation in treated cells compared with untreated control. The formula for calculation of antileishmanial activity was:

$$\frac{\text{cpm of control cells (parasitized macrophages)} - \text{cpm of treated cells (parasitized macrophages + drugs)}}{\text{cpm of control cells (parasitized macrophages)}} \times 100$$

It was considered a good antileishmanial activity when values equal or greater than 70% were obtained.

In vivo studies

Experimental animals. Three to four months old female and male golden hamsters (*Mesocricetus auratus*) were obtained from Núcleo de Medicina Tropical Prof. Joaquim Eduardo de Alenca/UFC. Animals were housed in groups of eight and had water and food *ad libitum* throughout the study. All experimental procedures were approved by the Animal Care and Utilization Committee of the Universidade Federal do Ceará, Brazil.

Infection and treatment

Twenty-two hamsters were infected in the right hind footpad with 20 μL containing 10^6 stationary-phase *LVb* promastigotes. The treatments were initiated 15 days after inoculation when infection was well established and lesions were obvious. The animals were randomly divided into four groups of eight and the drugs were administered for 42 consecutive days: (1) Lapachol group. The drug was dissolved in distilled water containing 0.1% of Tween 80 and 0.5% of ethanol and administered at 300 mg/kg/day (1/16 DL_{50}) by oral route (intragastric intubation); (2) Pentostam® group. The antimonial was administered at 60 mg/kg/day by i.m. route; (3) Lapachol + Pentostam® group. Lapachol was administered at 300 mg/kg/day by oral route and Pentostam® was administered at 60 mg/kg/day by i.m. route; (4) Control group. The untreated group received, orally, drugless vehicle (Tween 80 + ethanol + H_2O).

Lesion growth. Lesion sizes were measured with a dial gauge caliper (Mitutoyo, 0.01 mm sensitivity) and

Table 1. *In vitro* activities of lapachol and Pentostam® in Swiss mice peritoneal macrophage culture

Drug	mg/mL	Cytotoxicity to macrophages (%) (mean ± SEM)	Anti-amastigote activity (%) (mean ± SEM)
Lapachol	0.4	36.5 ± 3.04	–
	0.2	32.8 ± 2.85	96.8 ± 2.20
	0.1	24.9 ± 0.07	90.2 ± 2.90
	0.05	13.8 ± 0.07	88.8 ± 3.25
	0.025	4.4 ± 0.74	80.7 ± 0.65
	0.0125	–	76.4 ± 1.65
Pentostam®	4.0	59.1 ± 0.85	–
	2.0	50.2 ± 0.14	95.5 ± 0.81
	1.0	32.4 ± 0.60	92.1 ± 3.30
	0.50	18.7 ± 2.12	74.3 ± 13.3
	0.25	5.7 ± 0.36	64.0 ± 12.0
	0.125	–	59.8 ± 8.55

expressed as the difference in thickness (mm) between the infected footpad and contralateral uninfected footpad. Measurements were initiated 1 week after the inoculation of promastigotes and continued for a period of 48 days.

Effect of treatment. The animals were killed after cessation of treatment to assess parasitological loads in the draining lymph nodes, using a limiting dilution assay (Titus *et al.*, 1985), modified by Silva *et al.* (1994). Briefly, draining lymph nodes were aseptically excised, weighed and homogenized with a tissue glass grinder in 2 mL of Schneider's medium. After removal of debris by sedimentation for 5 min, the homogenates were serially diluted in Schneider's medium supplemented with antibiotics, 10% FBS and 2% of sterile human urine in 96-well plates containing blood agar slants. Twelve replicates of each dilution (100 μL/well) were incubated at 26 °C for 3 weeks and wells containing motile parasites were identified using an inverted microscope.

Statistical analysis. The number of parasites present in the lymph nodes was determined by minimum χ^2 analysis applied to a Poisson distribution (Taswell, 1984). Statistical significance ($p < 0.05$) in all data was analysed by Student's *t*-test.

RESULTS AND DISCUSSION

Lapachol displayed marked *in vitro* anti-amastigote activity (76–89%) against *LVb* at concentrations of 0.0125 to 0.05 mg/mL and no toxicity to macrophages at concentrations <0.1 mg/mL. In contrast, the antimonial Pentostam® showed an inhibitory activity (60–74%) against *LVb* amastigotes at concentrations ≤0.5 mg/mL, but was found to be toxic for macrophages at concentrations >1.0 mg/mL. Thus, *in vitro*, lapachol exhibited a more powerful anti-amastigote effect and a lower cytotoxicity to macrophages than Pentostam® (Table 1).

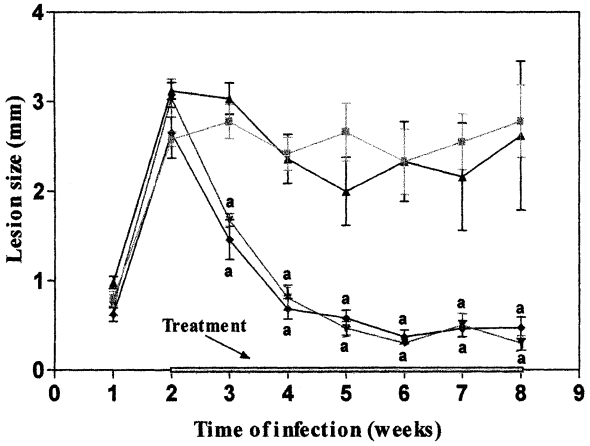


Figure 2. Effect of treatment with lapachol (30 mg/kg/day, p.o) and Pentostam® (60 mg/kg/day, i.m) or their combination for 42 days on the time course of lesion development in *LVb*-infected hamsters. Each point represents the mean ± SEM of eight animals. [a] $p < 0.001$ vs untreated control. ■ Untreated; ▲ Lapachol; ◆ Pentostam®; ▼ Lapachol + Pentostam®.

Despite showing an important *in vitro* anti-amastigote activity, lapachol could not protect hamsters against *LVb* lesion development in the *in vivo* model. Hamsters treated with lapachol (300 mg/kg/day) did not present significantly smaller footpad lesions compared with the untreated control, which represents only 6% of reduction of the lesion size (Fig. 2, Table 2).

The results in the *in vivo* model also showed that, whereas untreated control presented progressive lesions, Pentostam® treatment effectively brought footpad sizes down to normal within 2 weeks of treatment. The efficacy of lapachol + Pentostam® treatment was similar to Pentostam® alone.

To better evaluate if the drugs effectively reduced the parasites burden after treatment, viable parasites were quantitated in draining lymph nodes of treated and untreated hamsters using a limiting dilution assay, as previously described (Silva *et al.*, 1994).

Table 2. Treatment effects with lapachol (300 mg/kg/day, p.o) and Pentostam® (60 mg/kg/day, i.m.) or their combination for 42 days in *LVb*-infected hamsters

Group	*n*	Lesion size (mm) (mean ± SEM)	Mean parasite load in lymph nodes
Untreated	8	2.78 ± 0.43	5.8×10^7
Lapachol	8	2.62 ± 1.13 (6.0)	1.4×10^7
Pentostam®	8	0.47 ± 0.12[a] (83.2)	1.5×10^{4b}
Lapachol + Pentostam®	8	0.30 ± 0.08[a] (98.9)	2.4×10^{3a}

Figures in parenthesis indicate percentage reduction of lesion size compared with untreated group of animals. *n* = number of animals in each group.
[a] $p < 0.001$ vs untreated control.
[b] $p < 0.016$ vs untreated control.

Lymph nodes from hamsters treated with lapachol + Pentostam® as well as those from hamsters treated with Pentostam® alone had a significantly lower parasite load ($p < 0.001$; $p < 0.016$, respectively) compared with the untreated control. Nevertheless, no significant difference was observed between the untreated and the lapachol treated groups, which indicates the inefficiency of lapachol in suppressing parasite growth in the *in vivo* model (Fig. 2 and Table 2).

It has been reported that naphthoquinones extracted from several members of the plant family *Bignoniaceae* possess activity both *in vitro* and *in vivo* against various strains of *Leishmania* (Iwu *et al.*, 1994). Lapachol, diospyrin, plumbagin and β-lapachone are the most active members of this group (Fournet *et al.*, 1992; Guiraud *et al.*, 1994; Ray *et al.*, 1998).

In this work we demonstrate that lapachol exhibits marked leishmanicidal activity *in vitro*. The mechanism by which lapachol induces lysis of intracellular amastigotes *in vitro* is not clear. However, a characteristic of the naphthoquinones (especially lapachol and β-lapachone) is that they interfere with the oxygen metabolism of the tumour cell, blocking cell respiration and generating free oxygen radicals (Oswald, 1993/94). Besides free oxygen radicals, there is evidence that nitric oxide (NO) production, which follows the induction of nitric oxide synthetase by IFN-γ, plays an important role for the death of intracellular amastigotes in murine macrophages (Liew *et al.*, 1990, McSorley *et al.*, 1996). NO is considered the most important metabolite involved in *Leishmania* killing in mice (Assreuy *et al.*, 1994). Recently, Kumagai *et al.* (1998) have suggested that quinone compounds like lapachol could possibly inhibit NO-dependent physiological and/or pathophysiological actions *in vivo*, and since lapachol showed leishmanicidal activity *in vitro* but no therapeutic effect in the *in vivo* experimental leishmaniasis, we can suggest that: (a) lapachol *in vitro* could be acting directly on the parasite and not necessarily through the activation of macrophages, and (b) lapachol *in vivo* might be transformed into non-active metabolite(s) or be neutralized, losing its leishmanicidal activity. With regard to β-lapachone and the other naphthoquinone derivatives, Lopes *et al.* (1978) have demonstrated that these drugs can be inactivated either by reduction or by interacting with serum proteins. The structure–activity relationships of lapachol and various analognes have been reviewed by Discoll *et al.* (1974). They found that removal of the hydroxyl group or the isopentenyl group reduced antitumour activity 50%, while removal of the aromatic ring gave an inactive product. Replacement of one or both methyl groups on the side chain by hydrogen abolished activity. Reduction, oxidation or isomerization of the side chain also abolished activity, as did the addition of water to the double bond or the replacement of the hydroxyl by an amino or a methylamino group. Finally, it is also possible that an optimal and sustained plasma level of the drug could not be achieved at the dose (300 mg/kg/day) used in this study.

To our knowledge, this is the first report on lapachol that shows direct inhibition of *L. (V.) braziliensis* amastigotes. It would be rewarding to continue to evaluate other lapachol derivatives since differently substituted quinones might represent new chemotypes with less toxicity and more efficacy.

It has been estimated that 60% of people living in developing countries depend on traditional medicine for their primary health care. Therefore, new therapies derived from medicinal herbs offer a promising and practical avenue to the development of new drugs for the most needed in *Leishmania*-endemic countries.

Acknowledgements

We thank Professor Francisco Abreu Matos, Professor Said Fonseca and Dr Antônio Wilson Vasconcelos for collaboration. We wish to thank Herivaldo L. Costa and Adriana P. do Nascimento for technical advice. We are also grateful to CNPq and Escola de Saúde Pública do Ceará (ESP-CE) for financial support.

REFERENCES

Assreuy J, Cunha FQ, Epperlein M *et al.* 1994. Production of nitric oxide and superoxide by activated macrophages and killing of *Leishmania major*. *Eur J Immunol* **24**: 672–676.

Becker I, Volkow P, Velasco-Castrejon O *et al.* 1999. The efficacy of pentamidine combined with allopurinol and immunotherapy for the treatment of patients with diffuse cutaneous leishmaniasis. *Parasitol Res* **85**: 165–170.

Binutu OA, Adesogan KE, Okogun JI. 1996. Antibacterial and antifungal compounds from *Kigelia pinnata*. *Planta Med* **62**: 352–353.

De Almeida ER, da Silva Filho AA, dos Santos ER, Lopes CA. 1990. Anti-inflammatory action of lapachol. *J Ethnopharmacol* **29**: 239–241.

Dinnen RD, Ebisuzaki K. 1997. The search for novel anticancer agents: a differentiation-based assay and analysis of a folklore product. *Anticancer Res* **17**: 1027–1033.

Driscoll JS, Hazard GF Jr, Wood HB Jr, Goldin A. 1974. Structure–antitumor activity relationships among quinone derivatives. *Cancer Chemother Rep Part 2* **4**: 1–362.

Fournet A, Barrios AA, Munoz V, Hocquemiller R, Cave A. 1992. Effect of natural naphthoquinones in BALB/c mice infected with *Leishmania amazonensis* and *L. venezuelensis*. *Trop Med Parasitol* **43**: 219–222.

Guiraud P, Steiman R, Campos-Takaki GM, Seigle-Murandi F, Simeon de Buochberg M. 1994. Comparison of antibacterial and antifungal activities of lapachol and β-lapachone. *Planta Med* **60**: 373–374.

Iwu MM, Jackson JE, Schuster BG. 1994. Medicinal plants in the fight against leishmaniasis. *Parasitol Today* **10**: 65–68.

Kirby GC. 1996. Medicinal plants and the control of parasites. Medicinal plants and the control of protozoal disease, with particular reference to malaria. *Trans R Soc Trop Med Hyg* **90**: 605–609.

Kumagai Y, Nakajima H, Midorikawa K, Homma-Takeda S, Shimojo N. 1998. Inhibition of nitric oxide formation by neuronal nitric oxide synthase by quinones: nitric oxide synthase as a quinone reductase. *Chem Res Toxicol* **11**: 608–613.

Liew FY, Li Y, Millott S. 1990. Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. *J Immunol* **15**: 4306–4310.

Lira R, Sundar S, Makharia A *et al.* 1999. Evidence that the high incidence of treatment failures in Indian Kala-azar is due to the emergence of antimony-resistant strains of *Leishmania donovani*. *J Infect Dis* **180**: 564–567.

Lopes JN, Cruz FS, Docampo R *et al.* 1978. *In vitro* and *in vivo* evaluation of the toxicity of 1,4-naphthoquinone and 1,2-

naphthoquinone derivatives against *Trypanosoma cruzi*. *Ann Trop Med Parasitol* **72**: 523–531.
McSorley S, Proudfoot L, O'Donnell CA, Liew FY. 1996. Immunology of murine leishmaniasis. *Clin Dermatol* **14**: 451–464.
Moreira ES, Anacleto C, Petrillo-Peixoto ML. 1998. Effect of glucantime on field and patient isolates of New World *Leishmania:* use of growth parameters of promastigotes to assess antimony susceptibility. *Parasitol Res* **84**: 720–726.
Olliaro PL, Bryceson ADM. 1993. Practical progress and new drugs for changing patterns of leishmaniasis. *Parasitol Today* **9**: 323–328.
Oswald EH. 1993/94. Lapacho. *Br J Phytother* **3**: 112–117.
Pearson RD, Sousa AQ. 1995. *Leishmania* species: visceral (kala-azar), cutaneous, and mucosal leishmaniasis. In *Principles and Practice of Infections Diseases*, Mandell GL, Bennett J, Dolin R (eds). Churchill Livingston: New York; 2066–2077.
Phillipson JD, Wright CW, Kirby GC, Warhurst DC. 1995. Phytochemistry of some plants used in traditional medicine for the treatment of protozoal disease. *Proc Phytochem Soc Eur* **37**: 95–135.
Rao KV, McBride TJ, Oleson JJ. 1968. Recognition and evaluation of lapachol as an antitumor agent. *Cancer Res* **28**: 1952–1954.
Ray S, Hazra B, Mittra B, Das A, Majumder HK. 1998. Diospyrin, a bisnaphthoquinone: a novel inhibitor of type I DNA topoisomerase of *Leishmania donovani*. *Mol Pharmacol* **54**: 994–999.
Ribeiro AL, Drummond JB, Volpini AC, Andrade AC, Passos VM. 1999. Electrocardiographic changes during low-dose, short-term therapy of cutaneous leishmaniasis with the pentavalent antimonial meglumine. *Braz J Med Biol Res* **32**: 297–301.
Silva EM, Bertho AL, Mendonça SCF. 1994. Effect of *in vivo* depletion of CD4+ T cells on experimental infection of susceptible BALB/c mice with *Leishmania amazonensis*. *Acta Trop* **56**: 111–120.
Taswell C. 1984. Limiting dilution assays for the determination of immunocompetent cell frequencies III. Validity tests for the single-hit Poisson model. *J Immunol Meth* **72**: 29–40.
Titus RG, Ceredig R, Cerottini JC, Louis JA. 1985. Therapeutic effect of anti-L3T4 monoclonal antibody GK 1.5 on cutaneous leishmaniasis in genetically susceptible BALB/c mice. *J Immunol* **135**: 2108–2114.

