Available online at ScienceDirect www.sciencedirect.com
Elsevier Masson France EM|consulte www.em-consulte.com
Biomedicine & Pharmacotherapy 63 (2009) 643–649

Original article

Inhibitory activity of limonene against *Leishmania* parasites *in vitro* and *in vivo*

Denise C. Arruda, Danilo C. Miguel, Jenicer K.U. Yokoyama-Yasunaka, Alejandro M. Katzin, Silvia R.B. Uliana*

Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, ICB2 - Cidade Universitaria, São Paulo, SP 05508-900, Brazil

Received 16 October 2008; accepted 24 February 2009
Available online 10 March 2009

Abstract

Limonene is a monoterpene that has antitumoral, antibiotic and antiprotozoal activity. In this study we demonstrate the activity of limonene against *Leishmania* species *in vitro* and *in vivo*. Limonene killed *Leishmania amazonensis* promastigotes and amastigotes with 50% inhibitory concentrations of 252.0 ± 49.0 and 147.0 ± 46.0 μM, respectively. Limonene was also effective against *Leishmania major*, *Leishmania braziliensis* and *Leishmania chagasi* promastigotes. The treatment of *L. amazonensis*-infected macrophages with 300 μM limonene resulted in 78% reduction in infection rates. *L. amazonensis*-infected mice treated topically or intrarectally with limonene had significant reduction of lesion sizes. A significant decrease in the parasite load was shown in the lesions treated topically with limonene by histopathological examination. The intrarectal treatment was highly effective in decreasing the parasite burden, healing established lesions and suppressing the dissemination of ulcers. Limonene presents low toxicity in humans and has been shown to be effective as an agent for enhancing the percutaneous permeation of drugs. Our results suggest that limonene should be tested in different experimental models of infection by *Leishmania*.

Keywords: *Leishmaniasis*; Chemotherapy; Terpene

1. Introduction

Parasites of the *Leishmania* genus are the etiological agents of leishmaniasis, a widely distributed protozoal disease with a prevalence of 12–14 million people and a population at risk of 350 million people in 88 different countries [1].

First choice treatment of leishmaniasis still relies on the parenteral administration of highly toxic antimonial compounds. Therapy with pentavalent antimonials is commonly associated with high rates of non-compliance and parasite resistance to these drugs is rising alarmingly [2]. Therapeutic alternatives have been sought for a long time and some progresses have been achieved with new formulations of amphotericin B in liposomes and, more recently, with the use of miltefosine as an oral-administered drug for the treatment of kalazar [3] and testing of paromomycin as a topical agent [4]. However, the need for alternative drugs is still very clear.

Reports on the antileishmanial activity of a variety of plant extracts containing terpenoid compounds have been published [5–8]. We have recently shown that nerolidol – a sesquiterpene present in essential oils of several citrus plants – is active against promastigotes and amastigotes of *Leishmania in vitro* and, applied topically, is partially effective in the treatment of experimental leishmaniasis [9].

Limonene is a 10-Carbon cyclohexanoid monoterpene found in a variety of plants, particularly in oils of lemon, orange, dill and bergamot. Limonene's insecticide and antimicrobial properties are representative of plants' natural defense mechanisms. Both quiral forms R-(+)- and S-(−)-limonene are used in the cosmetic industry as flavoring agents. This terpene has also been shown to be effective as an

* Corresponding author. Tel.: +55 11 30917334; fax: +55 11 30917417.
E-mail address: srbulian@icb.usp.br (S.R.B. Uliana).

0753-3322/$ - see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biopha.2009.02.004

agent for enhancing the percutaneous permeation of drugs *in vitro* and *in vivo* [10,11].

Several studies reported that limonene has an antiproliferative effect in a variety of cell types, such as melanoma, gastric, and prostate cancer cells [12–14]. The therapeutic properties of limonene in animal models of tumorigenesis were used as the basis for Phase I clinical trials in breast and colon cancer patients. The drug was considered well tolerated and resulted in partial clinical response [15,16].

Rodrigues Goulart and coworkers determined that limonene was active *in vitro* against *Plasmodium falciparum*. The antiplasmodial effect was associated with inhibition of dolichol and ubiquinone biosynthesis and reduction in protein isoprenylation [17].

In this paper we describe the antileishmanial activity of limonene against *Leishmania amazonensis* promastigotes and amastigotes and its efficacy in the treatment of experimentally induced cutaneous leishmaniasis.

2. Materials and methods

2.1. Parasites

Leishmania promastigotes were grown in M199 supplemented with 10% fetal bovine serum (FBS) as described [18]. The strains used were: *L. (Leishmania) amazonensis* MHOM/BR/1973/M2269, *L. (Leishmania) chagasi* MHOM/BR/1974/M2682, *L. (Viannia) braziliensis* MHOM/BR/1975/M2903 and *L. (Leishmania) major* (MHOM/IL/1981/Friedlin). Amastigotes of *L. amazonensis* were obtained from experimentally infected BALB/c mice as described [19].

2.2. Drugs

(R)-(+)-limonene, was purchased from Sigma–Aldrich (St. Louis, MO, USA). Limonene was diluted in methanol. Topical formulations containing 10% (wt/wt) limonene were prepared in lanovaseline (LV) (70% lanoline, 30% vaseline, wt/wt). Ointments were freshly prepared every 20 days and kept at 4 °C. Intrarectal doses of 100 mg of limonene/kg/day were delivered by instilling 20 μl of 10% limonene in 20% ethanol/0.01 M phosphate buffer (pH 7.4). Stock solutions of amphotericin B (Sigma–Aldrich) were prepared in DMSO (5 mM final concentration) and stored at −20 °C.

2.3. Antileishmanial in vitro assays

Activity against parasites was tested *in vitro* by cultivating promastigotes (1×10^6) or amastigotes (5×10^6) in the presence of increasing concentrations of drug in 24-well culture dishes (Corning Life Sciences, NY, USA) for 2, 24 and 48 h. Cell viability was assessed by measuring the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma–Aldrich, St. Louis, MO, USA) as described previously [9]. Assays were performed in triplicate and results are expressed as the mean percent reduction of parasite numbers compared to untreated control wells calculated for at least three independent experiments. The 50% inhibitory concentration (IC_{50}) was determined from sigmoidal regression of the concentration–response curves using Scientific Graphing and Analysis Software ORIGIN 7.5.

Efficacy of limonene against intracellular amastigotes was assessed by counting the number of infected cells in J774.A1 macrophage monolayers. Macrophages were plated in round glass coverslips inside the wells of a 24-well culture dish at a concentration of 5×10^5 cells per coverslip in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 50 mg/ml gentamicin. After 2 h of incubation at 37 °C in an atmosphere of 5% CO_2, *L. amazonensis* stationary phase promastigotes were added to the wells (2.5×10^6 per well) and the cultures were incubated at 33 °C in a 5% CO_2 atmosphere. After 3 h, free promastigotes were removed by extensive washing with RPMI without FBS and infected cultures were treated with the different drug concentrations for 48 h. The monolayers were washed, fixed and stained with the Instant Prov kit (Newprov, PR, Brazil). The percentage of infected macrophages was assessed by light microscopy observation by counting 100 cells in triplicate coverslips.

Cytotoxicity was evaluated by cultivating 1×10^6 LLC-MK2 or HEK-293 epithelial kidney cells in 24-well plates for 24 h in the presence of increasing concentrations of limonene. Cell viability was assessed by the MTT assay as described above and results are expressed as percent reduction in cell viability compared to untreated control cultures. The 50% cytotoxic concentration (CC_{50}) was determined as described above for IC_{50} values.

2.4. In vivo assays

Groups of 5–7 female C57BL/6 mice were infected subcutaneously at the basis of the tail with 10^6 amastigotes of *L. amazonensis*. After 5–6 weeks, swelling at the inoculation site developed. Infected animals were randomized according to lesions sizes and treatment was initiated.

Lesion size was recorded once a week by measuring the thickness of the tail in two dimensions (D and d) at right angles to each other with a caliper. The size of the lesion (S) was estimated by calculating the mean diameter of the tail using the formula $S = (D + d)/2$. All animal experiments were approved by the Ethical Committee. Data on the lesion progression were analyzed for statistical significance by using the two-tailed Student's t-test for unpaired samples. A result was considered significant at $P < 0.05$.

2.5. Histopathology

Fragments of lesions were removed and fixed in neutral buffered formalin for subsequent paraffin embedding. Sections (5 μm thick) were stained with Hematoxylin–Eosin (HE).

2.6. Limiting dilution

The tissue at the lesion site was removed and separated from the bone, weighed and homogenized in 1 ml culture

medium. Parasites from tissue were quantified as described [20].

3. Results

3.1. Activity of limonene against Leishmania in vitro

Incubation of *L. amazonensis* promastigotes in the presence of increasing concentrations of limonene resulted in a dose-dependent decrease in the viability of parasites. Drug activity in 24-h assays, expressed as the concentrations that killed 50% of the parasites (IC_{50}) was 252.0 ± 49.0 μM. The volumes of methanol used to deliver the highest drug concentrations used in the tests were not responsible for parasite toxicity. Limonene had a leishmanicidal effect as demonstrated by microscopic examination of treated parasites, which revealed cells with disrupted structure, and by the absence of growth in cultures treated with concentrations above the IC_{90} and recultured in media without drug (data not shown). The IC_{50} of amphotericin B against *L. amazonensis* promastigotes determined in parallel assays was 0.15 ± 0.06 μM, compatible with previously reported data [21].

Amastigotes of *L. amazonensis* purified from lesions and grown *in vitro* for 24 h were also killed by limonene with an IC_{50} of 147.0 ± 46.0 μM.

The susceptibility of *L. major* promastigotes to this terpene was also tested and proved to be similar to that of *L. amazonensis* with IC_{50} of 354.0 ± 33.0 μM. Limonene was also active against *L. braziliensis* and *L. chagasi* promastigotes with IC_{50} of 185.0 ± 19.0 and 201.0 ± 17.0 μM, respectively.

Limonene was also able to inhibit the growth of intracellular amastigotes. The treatment of *L. amazonensis*-infected macrophages for 48 h with 200 and 300 μM limonene resulted in significant reductions of intracellular parasitism, by 52.0 ± 1.7 and 78.0 ± 4.2%, respectively.

3.2. In vitro cytotoxicity activity of limonene

The toxic effect of limonene was tested using epithelial-derived cell lines. The results of *in vitro* experiments showed that toxicity of limonene to mammalian cells was lower than that obtained for the parasites. The CC_{50} for human (HEK-293) and *Rhesus* monkey epithelial kidney cells (LLC-MK2) were higher than 1012 μM.

3.3. In vivo efficacy of limonene in L. amazonensis-infected C57BL/6 mice

Limonene was administered to C57BL/6 mice infected with *L. amazonensis* by topical or intrarectal routes. The treatment with limonene by the intrarectal route was initiated 5–6 weeks after infection and performed for 2 weeks. No toxic effects were observed in treated mice. A significant reduction on the average lesion size was achieved in 80% of the treated animals after administration of this terpene (Fig. 1A and B). The therapeutic response was sustained and 18 weeks after the onset of treatment responsive mice remained healthy while control animals presented clear evidence of disease dissemination to snout, footpad, and neck (Fig. 1B).

Parasite burden was quantified in intrarectally-treated mice 15 weeks after the interruption of the treatment by limiting dilution assay (Fig. 2). A reduction of more than 99.9% in the parasite load was noted in 80% of the treated mice.

Topical treatment was administered to *L. amazonensis*-infected mice with preparations of limonene in a LV ointment. Treatment was initiated 5–6 weeks after infection and control-infected groups treated with the basis alone were included in all experiments. Treatment with 10% limonene in LV resulted in a significant reduction on the average lesion size. Fig. 3 shows the follow up of individual limonene-treated mice, in a representative experiment, up to 19 weeks after the start of treatment. Complete healing at the inoculation site was detected in 67–86% of the treated mice. No side effects were detected in *L. amazonensis*-infected and control uninfected mice treated with ointments containing 10% limonene in LV. Thirteen weeks after the interruption of treatment, tails from mice treated with limonene were prepared for tissue analysis and showed parasites to be absent or very scarce (Fig. 4d–f).

In comparison to control untreated animals, the lesions from mice receiving LV alone had reduced sizes while the ointment was being applied but soon reverted to sizes comparable to the untreated controls when therapy was interrupted (data not shown). Moreover, lesions on LV treated mice did not heal and the histopathological examination of the inoculation site in control untreated mice or in tissue taken from the mock treated lesions revealed the classical picture of a cutaneous leishmaniasis lesion with a large number of parasitized macrophages (Fig. 4a–c).

Amastigotes recovered from treated mice were transformed *in vitro* and grown as promastigotes. The sensitivity of these cultures to limonene was the same as the parental line used for infection (data not shown), indicating that the remaining parasites were not resistant to limonene.

4. Discussion

In this study we have evaluated the activity of limonene against *Leishmania*. Our interest in this terpene was raised by previous reports on its effect against tumor cells. Limonene has been shown to suppress the growth of neoplastic cells *in vitro* and *in vivo* [22,12,13]. Furthermore, the safety and chemopreventive or chemotherapeutic activity of limonene's systemic administration has been shown in several *in vivo* models and for several types of cancer [22,23]. Long-term analysis of limonene's toxicity in mice and rats has not found evidences of severe side effects in animals receiving from 250 to 1000 mg/kg/day limonene for 2 years [16]. Limonene application was tested in Phase I clinical trials, resulting in a very satisfactory tolerance [15,23]. In addition, D-limonene does not pose a mutagenic, carcinogenic, or nephrotoxic risk to humans and low toxicity was observed after single or repeated doses for up to 1 year [24].

Antileishmanial properties of terpenoids have been previously reported. Triterpenoid saponins isolated from *Maesa*

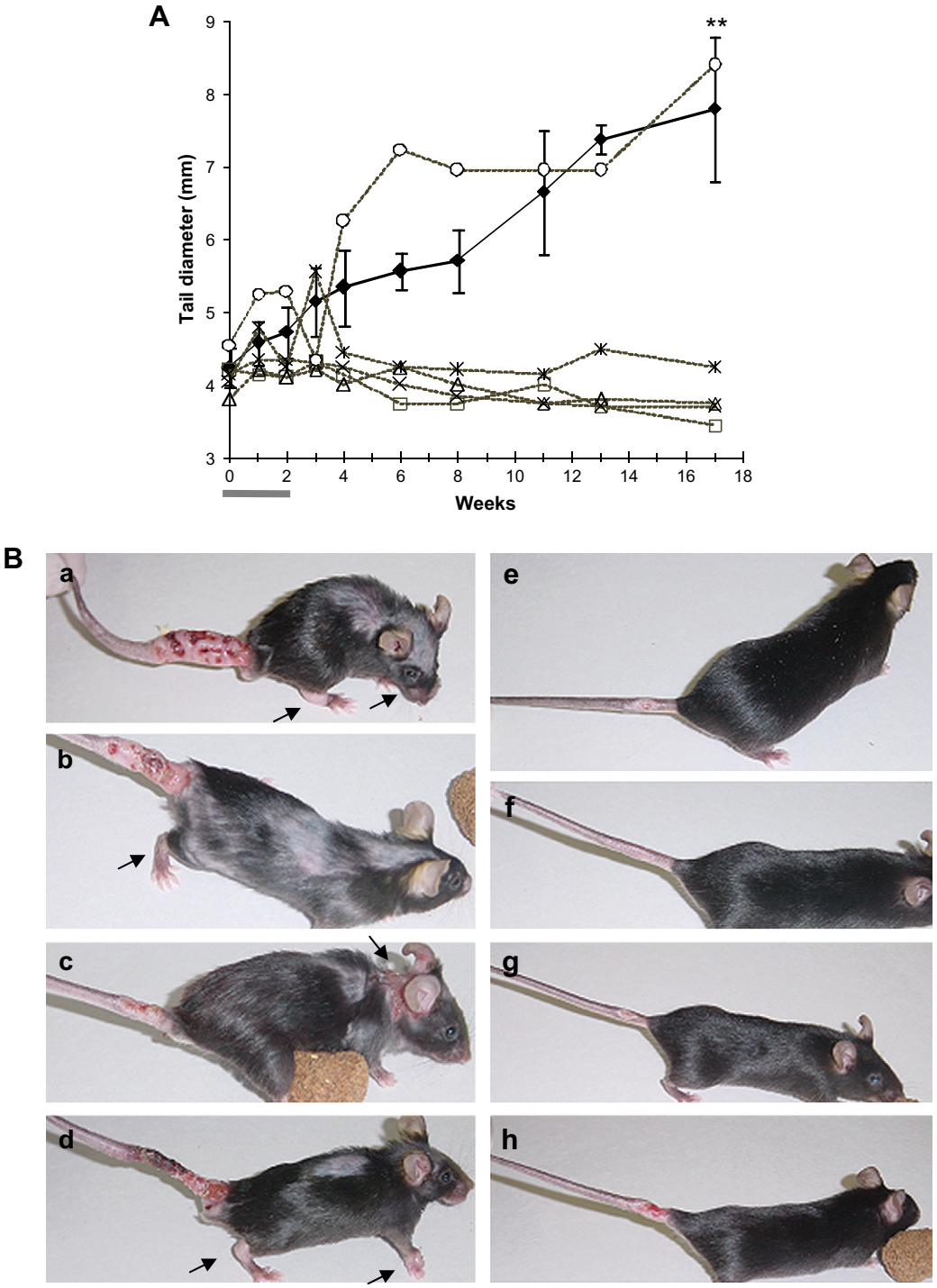

Fig. 1. Evaluation of disease development in mice treated with limonene by the intrarectal route. (A) Intrarectal treatment with 100 mg/kg/day limonene for 2 weeks (indicated by the horizontal bar) starting 40 days after infection (week 0). The results shown are the individual measurements for each treated animal (grey symbols, dashed lines) and the mean and standard deviation for a group of five control animals (black diamonds, solid line) (**P: 0.03). Results are representative of two independent experiments. (B) Pictures were taken 15 weeks after the interruption of treatment. Control untreated mice (a–d) and limonene-treated animals (e–h). Disseminated ulcers in control untreated animals are indicated by arrows.

balansae reduced liver parasite burden in *Leishmania infantum*-infected BALB/c mice [6]. Thymol, a monoterpene phenol derivative of cymene, presented partial *in vivo* leishmanicidal activity decreasing by 46% the parasite burden in *Leishmania panamensis* infected golden hamsters [25].

L. amazonensis-infected mice initially develop swelling at the inoculation site that progress into an ulcerated lesion, eventually leading to the loss of the tail and to lesion dissemination. Intrarectal or topical treatment with limonene inhibited the progression of lesions and suppressed metastasis

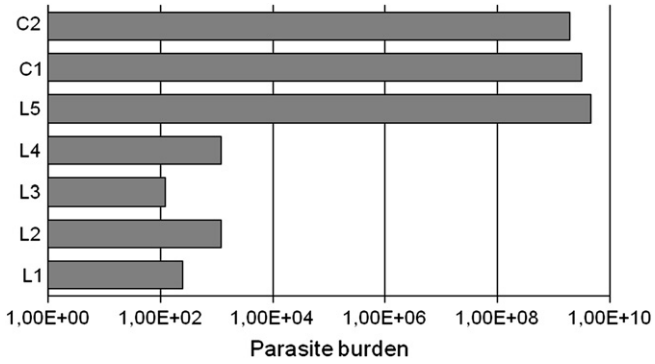

Fig. 2. Parasite burden at the inoculation site in two control untreated (C1 and C2) and from five limonene-treated mice (L1–L5) taken 15 weeks after the interruption of treatment. Parasites were quantified by limiting dilution.

development. Limonene also led to a reduction of more than 99.9% in the parasite load observed by limiting dilution and histopathological examination.

Reasons why response to treatment was not uniform in treated groups, varying from 67 to 86%, are not clear. Genetic variation in C57Bl/6 littermates has been detected due to copy number variations [26]. On the other hand, behavioural differences in topically treated mice could result in reduced absorption of the drug, with early removal by friction or licking. Similarly, gut intolerance in some mice could explain early expulsion of the instilled drug. In an attempt to control these variables, both intraperitoneal and intravenous routes were also employed to administer limonene. In both cases, treatment was ineffective (data not shown). Since limonene is rapidly metabolized in the liver to perillyl alcohol [27], these results suggest that activity is lost after first-passage metabolism. Taken together, our results suggest that limonene could be considered a template compound to be chemically modified to increase activity and reduce metabolic quick removal.

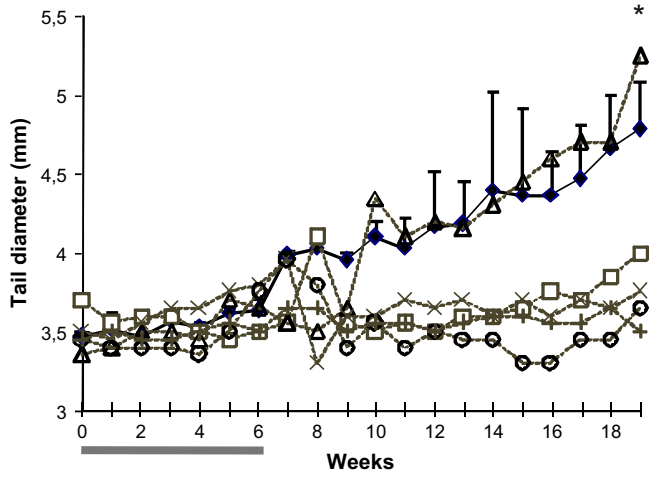

Fig. 3. Topical treatment of *L. amazonensis*-infected mice with limonene. Treatment of C57BL/6 mice infected at the basis of the tail and treated with a cream containing 10% (wt/wt) limonene in LV basis for 6 weeks (indicated by the horizontal bar) starting 40 days after infection. The results shown are the individual measurements for each treated animal (grey symbols, dashed lines) and the mean and standard deviation for a group of six control animals (black diamonds, solid line) (*P: 0.01). Results are representative of three independent experiments.

In neoplastic cells, limonene's mechanism of action was initially associated with inhibition of protein prenylation, particularly of small G-proteins such as Ras. Early data suggested that limonene was in fact an inhibitor of the enzymes protein farnesyl transferase and geranylgeranyl transferase [28]. There are also evidences suggesting that limonene may act by decreasing the steady state levels of Ras, by transcription or translation inhibition or stimulation of specific pathways of protein degradation [29]. Other possible mechanisms underlying the tumor-suppressive activity of limonene include induction of apoptosis and reduction of hydroxy-3-methylglutaryl coenzyme A reductase activity [23]. In *P. falciparum* limonene was shown to have wider effects on the isoprenoid biosynthesis pathway, inhibiting the synthesis of dolichol, ubiquinone and reducing levels of small prenylated proteins [17].

There are also reports on the effect of limonene modulating the immune response in mice with increased delayed-type hypersensitivity reactions, phagocytosis and microbicidal activity, higher total antibody production and bone marrow cellularity [30,31]. The discrepancy between limonene's weak antileishmanial activity *in vitro*, with IC_{50} values in the high micromolar range, and its effectiveness in a highly susceptible experimental model *in vivo* might be related to these immune enhancing properties. *In vitro* studies indicated that D-limonene increased NO production in peritoneal macrophages obtained from tumor-bearing mice [30]. We did not detect increased accumulation of nitrate on supernatants of *L. amazonensis*-infected macrophages treated with limonene, suggesting that increased production of NO is not operating as the killing mechanism in this case. We also investigated the pattern of humoral immune responses on infected mice treated or not with this terpene and did not detect increased anti-*Leishmania* antibody titres or a shift on *Leishmania*-specific antibody subtypes produced (data not shown). At present, we cannot exclude other effects of the drug on the immune response of infected mice.

In summary, we showed that topical treatment of *L. amazonensis*-infected mice with limonene induced healing in 67–86% of the treated animals. Intrarectal administration of limonene was also effective in the resolution of cutaneous lesions.

Topical treatment of cutaneous leishmaniasis is a very desirable goal. Patients with this disease often live in areas with poor access to medical care and where injections are difficult to obtain and use. Our best results were obtained by the intrarectal instillation of limonene. Different vehicles or formulations for the topical administration of limonene can potentially increase absorption and effective concentration in the tissue, while chemical modifications of limonene deserve to be investigated. Therefore, this terpene may have great potential in the development of new antileishmanial chemotherapeutic agents.

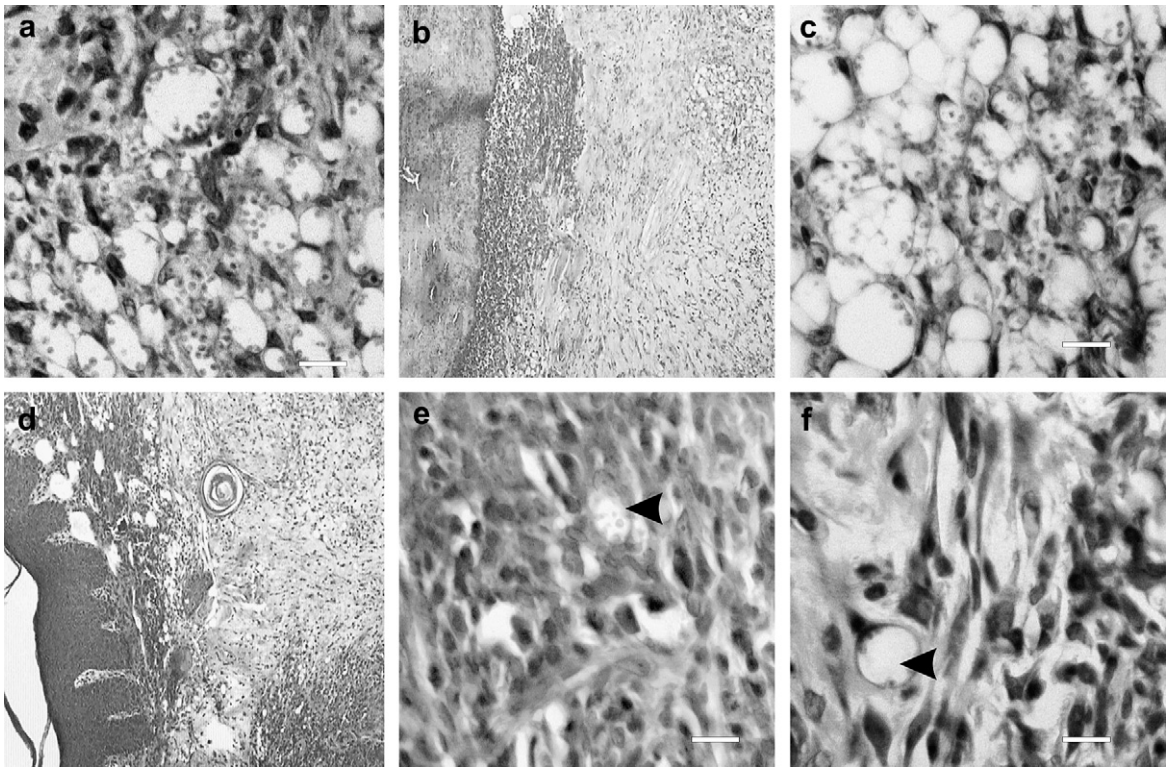

Fig. 4. Topical treatment of *L. amazonensis*-infected C57BL/6 mice with limonene. Fragments were taken from lesions or from the inoculation site 13 weeks after the end of treatment and submitted to histopathological analysis. Samples from control (a), placebo (LV) (b, c) and limonene-treated (d–f) mice are shown. Figures are representative of at least two animals analyzed in each group. The outcome of footpad lesion of control (a) and placebo-treated mice (b, c) is marked by numerous parasitized macrophages in the dermis. In limonene-treated mice a fibrotic scar is observed at the inoculation site (d), which is surrounded by a mononuclear inflammatory reaction with sparse (arrowheads) or absent infected macrophages (e, f and data not shown). HE staining; (a, c, e, f) ×400; (b, d) ×100. Bars represent 20 μm.

Acknowledgments

We thank Dr. Tania Bijovsky de Katzin, Emilia Kimura and Márcia Ribeiro Pinto for valuable suggestions and Dr. Maria Irma Seixas Duarte, Roosecelis Araújo Brasil and Bernardo Paulo Albe for help with the histopathology. D.C.A. (2008/51256-7) and D.C.M. (2005/59881-0) held studentships from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). This work was supported by FAPESP and CNPq and was carried out according to the rules and regulations of animal experimentation in Brazil.

References

[1] Desjeux P. Leishmaniasis. Nat Rev Microbiol 2004;2:692.
[2] Sundar S. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Health 2001;6:849–54.
[3] Croft SL, Engel J. Miltefosine – discovery of the antileishmanial activity of phospholipid derivatives. Trans R Soc Trop Med Hyg 2006;100:4–8.
[4] El-On J, Bazarsky E, Sneir R. *Leishmania major*: *in vitro* and *in vivo* antileishmanial activity of paromomycin ointment (Leshcutan) combined with the immunomodulator Imiquimod. Exp Parasitol 2007;116:156–62.
[5] do Socorro SM, Rosa RR, Mendonça-Filho HR, Bizzo I, de Almeida Rodrigues RM, Soares T, et al. Antileishmanial activity of a linalool-rich essential oil from *Croton cajucara*. Antimicrob Agents Chemother 2003;47:1895–901.
[6] Germonprez N, Maes L, Van Puyvelde L, Van Tri M, Tuan DA, De Kimpe N. *In vitro* and *in vivo* anti-leishmanial activity of triterpenoid saponins isolated from *Maesa balansae* and some chemical derivatives. J Med Chem 2005;48:32–7.
[7] Torres-Santos EC, Lopes D, Oliveira RR, Carauta JP, Falcao CA, Kaplan MA, et al. Antileishmanial activity of isolated triterpenoids from *Pourouma guianensis*. Phytomedicine 2004;11:114–20.
[8] Tiuman TS, Ueda-Nakamura T, Garcia Cortez DA, Dias Filho BP, Morgado-Diaz JA, de Souza W, et al. Antileishmanial activity of parthenolide, a sesquiterpene lactone isolated from *Tanacetum parthenium*. Antimicrob Agents Chemother 2005;49:176–82.
[9] Arruda DC, D'Alexandri FL, Katzin AM, Uliana SRB. Antileishmanial activity of the terpene nerolidol. Antimicrob Agents Chemother 2005;49:1679–87.
[10] El-Kattan AF, Asbill CS, Kim N, Michniak BB. The effects of terpene enhancers on the percutaneous permeation of drugs with different lipophilicities. Int J Pharm 2001;215:229–40.
[11] Krishnaiah YS, Al-Saidan SM. Limonene enhances the *in vitro* and *in vivo* permeation of trimetazidine across a membrane-controlled transdermal therapeutic system. Curr Drug Deliv 2008;5:70–6.
[12] Raphael TJ, Kuttan G. Effect of naturally occurring monoterpenes carvone, limonene and perillic acid in the inhibition of experimental lung metastasis induced by B16F-10 melanoma cells. J Exp Clin Cancer Res 2003;22:419–24.
[13] Lu XG, Zhan LB, Feng BA, Qu MY, Yu LH, Xie JH. Inhibition of growth and metastasis of human gastric cancer implanted in nude mice by D-limonene. World J Gastroenterol 2004;10:2140–4.
[14] Chen J, Lu M, Jing Y, Dong J. The synthesis of L-carvone and limonene derivatives with increased antiproliferative effect and activation of ERK pathway in prostate cancer cells. Bioorg Med Chem 2006;14:6539–47.

[15] Vigushin DM, Poon GK, Boddy A, English J, Halbert GW, Pagonis C, et al. Phase I and pharmacokinetic study of D-limonene in patients with advanced cancer. Cancer Research Campaign Phase I/II Clinical Trials Committee. Cancer Chemother Pharmacol 1998;42:111–7.
[16] National Toxicology Program. NTP toxicology and carcinogenesis studies of D-limonene (CAS No. 5989-27-5) in F344/N rats and B6C3F1 mice (gavage studies). Natl Toxicol Program Tech Rep 1990; 347:1–165. Available at: <http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr347.pdf> [accessed 10.03.09].
[17] Rodrigues Goulart H, Kimura EA, Peres VJ, Couto AS, Aquino Duarte FA, Katzin AM. Terpenes arrest parasite development and inhibit biosynthesis of isoprenoids in *Plasmodium falciparum*. Antimicrob Agents Chemother 2004;48:2502–9.
[18] Kapler GM, Coburn CM, Beverley SM. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. Mol Cell Biol 1990;10: 1084–94.
[19] Uliana SRB, Goyal N, Freymüller E, Smith DF. *Leishmania*: overexpression and comparative structural analysis of the stage-regulated meta 1 gene. Exp Parasitol 1999;92:183–91.
[20] Titus RG, Marchand M, Boon T, Louis JA. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunol 1985;7:545–55.
[21] Tanaka AK, Valero VB, Takahashi HK, Straus AH. Inhibition of *Leishmania (Leishmania) amazonensis* growth and infectivity by aureobasidin A. J Antimicrob Chemother 2007;59:487–92.
[22] Crowell PL. Prevention and therapy of cancer by dietary monoterpenes. J Nutr 1999;129:775–8.
[23] Mo H, Elson CE. Studies of the isoprenoid-mediated inhibition of mevalonate synthesis applied to cancer chemotherapy and chemoprevention. Exp Biol Med 2004;229:567–85.
[24] Sun J. D-limonene: safety and clinical applications. Altern Med Rev 2007;12:259–64.
[25] Robledo S, Osorio E, Muñoz D, Jaramillo LM, Restrepo A, Arango G, et al. *In vitro* and *in vivo* cytotoxicities and antileishmanial activities of thymol and hemisynthetic derivatives. Antimicrob Agents Chemother 2005;49:1652–5.
[26] Watkins-Chow DE, Pavan WJ. Genomic copy number and expression variation within the C57BL/6J inbred mouse strain. Genome Res 2008; 18:60–6.
[27] Miyazawa M, Shindo M, Shimada T. Metabolism of (+)- and (−)-limonenes to respective carveols and perillyl alcohols by CYP2C9 and CYP2C19 in human liver microsomes. Drug Metab Dispos 2002;30: 602–7.
[28] Crowell PL, Chang RR, Ren ZB, Elson CE, Gould MN. Selective inhibition of isoprenylation of 21-26-kDa proteins by the anticarcinogen D-limonene and its metabolites. J Biol Chem 1991;266:17679–85.
[29] Hohl RJ, Lewis K. Differential effects of monoterpenes and lovastatin on RAS processing. J Biol Chem 1995;270:17508–12.
[30] Del Toro-Arreola S, Flores-Torales E, Torres-Lozano C, Del Toro-Arreola A, Tostado-Pelayo K, Guadalupe Ramirez-Duenas M, et al. Effect of D-limonene on immune response in BALB/c mice with lymphoma. Int Immunopharmacol 2005;5:829–38.
[31] Raphael TJ, Kuttan G. Immunomodulatory activity of naturally occurring monoterpenes carvone, limonene, and perillic acid. Immunopharmacol Immunotoxicol 2003;25:285–94.