INTRODUCTION

Leishmaniasis caused by the intracellular protozoan parasite of mononuclear phagocytes *Leishmania* is endemic in 88 countries [1]. *Leishmania amazonensis*, a species transmitted mainly in the Amazon region, has been associated with localized cutaneous lesions, diffuse cutaneous disease, and mucosal infection [1,2]. The disease is neglected by the pharmaceutical industry, even though no vaccine exists, and significant side effects and signs of increasing resistance continue to occur with the use of the few effective drugs available [3,4].

More recently, the pharmaceutical proprieties of tellurane compounds were investigated [5,6]. Several tellurides show antioxidative and immunomodulating proprieties and antitumor activities [5-12]. Clinical trials with a tellurane compound are presently underway [13,14]. The synthetic organotellurane compound RT-01 (Fig. 1) has been previously shown to inhibit cathepsin B, a cysteine protease involved in tumor invasion, and presents a cytotoxic effect on cancer cells [7]. In an attempt to find new leishmanial drugs, RT-01 was tested both in vitro and in vivo against *L. amazonensis*. The results reported here suggest that RT-01 is effective against the flagellate and nonflagellate parasitic forms and in *L. amazonensis*-infected BALB/c mice.

MATERIALS AND METHODS

Organotellurane compound (RT-01)

The organotellurane RT-01 used to evaluate the effects in vitro against *L. amazonensis* and in vivo in *L. amazonensis*-infected mice was prepared by the reaction of tellurium tetrachloride with propargyl alcohol in benzene followed by complexation with triethylbenzylammonium chloride, as described previously [7,15] (Fig. 1). The compound was dissolved in DMSO and diluted in phosphate buffer pH 7.4, medium or saline. The final concentration of DMSO in vitro and in vivo experiments was 0.1%.
Parasite

Promastigotes of *L. amazonensis* (MHOM/BR/73/M2269) were cultured at 26 ℃ in RPMI 1640 medium (Sigma, St. Louis, Missouri, USA) supplemented with 25 μg/ml gentamicin, 2 mM L-glutamine, and 10% fetal calf serum (FCS) (Cultilab, Campinas, São Paulo, Brazil). Amastigotes were isolated from active skin lesions from BALB/c mice and used immediately after isolation [16].

Assessment of RT-01 effects on *L. amazonensis* promastigotes and amastigotes

Promastigotes growing in 25 cm² plastic flasks at 26 ℃ (5 × 10⁶ promastigotes in total volume of 5 ml) were treated with different doses of RT-01 (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, and 5 μg/ml) and their numbers were determined using a Neubauer hemocytometer at 400 × magnification [17]. Extracellular amastigotes cultured in 25 cm² plastic flasks at 26 ℃ (5 × 10⁶ amastigotes in total of 5 ml) were treated with different doses of RT-01 (0.1, 0.2, 0.4, 0.5, 0.6, 0.8, 1, and 2 μg/ml) and their viability was determined by hemocytometer counts after staining with erythrosine-B [18]. The transformation of extracellular amastigotes into promastigotes was estimated microscopically. The stage of transformation was classified as previously described [19].

Treatment and evaluation of RT-01 efficacy in *L. amazonensis*-infected mice

The Ethical Committee for Animal Research of the Institute of Biology of the State University of Campinas (UNICAMP) approved the experimental protocols. Six-week-old female BALB/c mice were subcutaneously infected in the right hind footpad with 10⁵ amastigotes in a volume of 20 μl. The mice were housed 7 to a cage and received sterile food and water in the Laboratory Animal Center of the Department of Parasitology, UNICAMP. RT-01 was dissolved in DMSO, diluted with saline and administered by intraperitoneal injection 30 and 15 days before infection, and 1, 15, and 30 days post-infection (PI). For each mice group (7 per group) were administered a total of 5 doses of RT-01: 180, 360, or 720 μg/kg/day. Alternatively, other groups of mice (7 per group) received the drug 3 times by intranasal injection at intervals of 10 days PI. RT-01 doses were 180, 360, or 720 μg/kg/day. Control groups of mice (7 per group) were tested with the same vehicles (DMSO and saline) without RT-01 or Glucantime (N-methyl glucamine antimonite; Rhodia, Santo Amaro, São Paulo, Brazil) at 100 mg/kg/day injected intraperitoneally for 20 days after *L. amazonensis* infection used as the standard antileishmanial agent [20]. The course of infection was monitored by measuring the increase in footpad thickness with a dial caliper, compared with the contra lateral uninfected footpad [20]. To estimate the parasite burden in the lesions, the mice were sacrificed at the designated period, the entire infected footpads were weighed and amastigotes were recovered from the lesion, as previously described [20]. The mice were also regularly examined to detect cutaneous ulcers, secondary lesions and secondary infection by bacteria. To perform histopathological evaluation, mouse foot tissues were fixed by immersion in 4% paraformaldehyde in 0.1 M PBS/0.1 M sucrose for 6 hr and processed for standard paraffin embedding. Tissue sections were stained with hematoxylin and eosin and examined for pathological changes using an optical microscope (Eclipse E800-Nikon, Tokyo, Japan) [20].

Statistical analyses

All experiments were repeated at least 3 times. Three independent experiments involving 7 mice per group were performed to analyze the drug efficacy in vivo. Statistical significance between control and experimental groups were determined by the Student’s *t*-test and the resulting data are expressed as mean ± standard error of mean (SEM).

RESULTS

Experiments were undertaken to study the possible effects of RT-01 on promastigotes and amastigotes of *L. amazonensis*. RT-01 caused a concentration-dependent loss of promastigote viability, and its toxicity effect (IC50, 24 hr) was found at approximately 2 μg/ml concentration (Fig. 2A). Interestingly, RT-01-treated promastigotes that remained viable lost their capacity to proliferate (data not shown). Amastigotes derived from lesions were also sensitive to RT-01. Treatment of axenic amastigotes with the compound affected their viability (data not shown) and
their ability to transform into promastigotes (Fig. 2B).

Experiments were realized to determine whether RT-01 could modulate the course of infection in a cutaneous leishmaniasis model. Fig. 3 demonstrates that cutaneous lesions progressively increased in size in BALB/c mice infected with *L. amazonensis*. The mice failed to resolve the infection and were unable to control parasite burden in footpad lesions (Fig. 3A, B, D). Mice treated with 360 μg/kg/day RT-01 by intraperitoneal administration showed reduced footpad thickness and approximately 3-fold fewer parasites in the footpad lesion compared to the untreated mice (Fig. 3C, D). Mice treated with lower doses of RT-01 by intraperitoneal injection exhibited lesion progression and parasite burden similar to that of untreated mice (Fig. 3A, D). Treatment with 720 μg/kg/day RT-01 by intralesional administration delayed the onset of lesion development and reduced parasite tissue burden; mice presented 11-fold fewer parasites in the footpad lesion compared to the untreated mice (Fig. 4A, C, D). We compared the effect of the standard antileishmanial drug, Glucantime.
cantine, to that of RT-01 treatment. The results showed a significant decrease in the size of the lesions and parasite burden after treatment with Glucantime injected intraperitoneally, an effective route of administration of this antimonial drug for treating mice (Fig. 3A) [20]. A complete reduction of lesion size was not obtained, similar to the pattern observed in animals treated with RT-01 (Figs. 3A, 4A).

Histopathology sections are shown in Figs. 4E, F. The footpad lesions of untreated mice showed a large infiltration of infected vacuolar macrophages that replaced the normal skin tissue (Fig. 4E). In contrast, lesions from RT-01-treated mice displayed normal skin structures; the epidermis, dermal connective tissue and sebaceous glands were histologically well-preserved (Fig. 4F). No expressive accumulation of inflammatory cells occurred and a marked reduction in the number of macrophages and in parasitism intensity of the lesioned footpad of RT-01-treated mice was observed compared to the lesions of untreated mice (Fig. 4F). It should be noted that RT-01 elicited no apparent toxicity; the mice neither lost weight nor did they show any alteration in gait or food consumption. In addition, long-term survival was observed in groups of mice infected and uninfected, treated 3 to 5 times with different doses of RT-01 (180 to 800 μg/kg/day).

**DISCUSSION**

The investigation of biological and therapeutic applications of tellurane compounds has substantially increased over the past 2 decades [5,6]. Organotellurane compounds present protease inhibition activity, which is specific for cysteine proteases, like papain and cathepsin B [7,8]. Various tellurane compounds have been described as presenting tumoricidal, antiviral, anti-protozoan parasite *Babesia rodhaini* activities, and immunomodulatory properties [7-14,21]. In addition to their effects, tellurium containing-compounds are characterized by low toxicity [8,13,14]. RT-01, the compound studied in this work, inhibits the extracellular form of cathepsin B and showed a marked effect on human leukemia HL60 cells [7]. To the best of our knowledge, the effects of tellurane compounds on *Leishmania* have never been reported in the literature.

In the present study, direct antileishmanial activity of RT-01 on *L. amazonensis* was shown. RT-01 caused a concentration-dependent loss of promastigote viability and promastigote multiplication was also hindered. The antileishmanial effects were extended to amastigotes derived from lesions. The way in which RT-01 altered the viability of *L. amazonensis* in vitro was not addressed in this study. Based on the fact that tellurane compounds inhibit proteolytic enzymes, such as cysteine proteases [7], we suggest the inhibition of parasite cysteine proteases, a virulence factor required for survival and the infection process of *Leishmania* [22,23] by RT-01 as a hypothesis that should be investigated further.

The current study also showed that while treatment with RT-
01 did not induce complete cure, it influenced the development of clinical disease in *L. amazonensis* infection of an extremely susceptible mouse strain [20,24]. The data obtained indicate that RT-01 treatment by intraleSIONal route generated more efficient control of parasite infection in mice than by intraperitoneal route. The best results were obtained following intraleSIONal administration with 720 μg/kg/day, and the mice showed significant delay in the development of cutaneous lesions and a decrease in the number of the parasites obtained from the lesions that eventually developed. These results were expected, considering that a localized disease like cutaneous leishmaniasis may be affected more directly by therapeutic agents, when applied topically [25]. The recruitment of inflammatory cells induced by RT-01 treatment in association with *Leishmania* infection was not observed, but the possibility of proinflammatory effects induced by RT-01 cannot be excluded. In fact, another tellurane compound that presents a protective ability against *B. rodhaini* increased the number of peripheral blood neutrophils of infected mice [21,26-30].

It should be noted that RT-01 treatment produces a similar effect to that of pentavalent antimonial, Glucantime, in the lesions of mice. The antimonial compounds, the first choice treatment to all forms of leishmaniasis has the disadvantage of both toxicity (pancreatitis, cardiotoxicity, hematological disorders, and skeletal muscle toxicity) and clinical resistance where they have been used for a long time [3,4]. Although a toxicity profiles of RT-01 are not yet established, it can be expected that RT-01 may present similar non- or low toxicity as other organotellurane compounds due to lack of apparent toxicity of RT-01 in mice as reported in this study, excellent safety profile on clinical trials for cancer of other organotelluranes and the common tel-lurium-thiol chemistry of these compounds [8,13,14,21].

Evaluation of the mode of action of RT-01 treatment may lead to improved protocols for inducing parasite clearance and the cure of leishmaniasis. Finally, these findings highlight the fact that the apparent potency of organotellurane compounds, together with their relatively simple structure, may represent a new avenue for the development of novel drugs to combat parasitic diseases.

**ACKNOWLEDGEMENTS**

This work was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil.

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