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Epoxy-α-Lapachone Has *In Vitro* and *In Vivo* Anti-*Leishmania* (*Leishmania*) *amazonensis* Effects and Inhibits Serine Proteinase Activity in This Parasite

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***Leishmania* (*Leishmania*) *amazonensis* is a protozoan that causes infections with a broad spectrum of clinical manifestations. The currently available chemotherapeutic treatments present many problems, such as several adverse side effects and the development of resistant strains. Natural compounds have been investigated as potential antileishmanial agents, and the effects of epoxy-α-lapachone on *L.* (*L.*) *amazonensis* were analyzed in the present study. This compound was able to cause measurable effects on promastigote and amastigote forms of the parasite, affecting plasma membrane organization and leading to death after 3 h of exposure. This compound also had an effect in experimentally infected BALB/c mice, causing reductions in paw lesions 6 weeks after treatment with 0.44 mM epoxy-α-lapachone (mean lesion area, 24.9 ± 2.0 mm^2), compared to untreated animals (mean lesion area, 30.8 ± 2.6 mm^2) or animals treated with Glucantime (mean lesion area, 28.3 ± 1.5 mm^2). In addition, the effects of this compound on the serine proteinase activities of the parasite were evaluated. Serine proteinase-enriched fractions were extracted from both promastigotes and amastigotes and were shown to act on specific serine proteinase substrates and to be sensitive to classic serine proteinase inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and antipain). These fractions were also affected by epoxy-α-lapachone. Furthermore, *in silico* simulations indicated that epoxy-α-lapachone can bind to oligopeptidase B (OPB) of *L.* (*L.*) *amazonensis*, a serine proteinase, in a manner similar to that of antipain, interacting with an S1 binding site. This evidence suggests that OPB may be a potential target for epoxy-α-lapachone and, as such, may be related to the compound's effects on the parasite.**

An array of *Leishmania* species are able to infect humans, as well as other mammalian hosts, and cause diseases that are known under the common name of leishmaniasis. Leishmaniasis has high incidence and prevalence in tropical and subtropical regions of the world, affecting mostly populations in poor or emerging countries, and is included among the 17 neglected tropical diseases defined by the World Health Organization (http://www.who.int/neglected_diseases/diseases/en).

These parasites can affect cells in the skin, mucosa, and cartilage, causing cutaneous leishmaniasis (CL). Some species may infect internal tissues and organs, such as the liver, spleen, and bone marrow, causing visceral leishmaniasis (VL) (1). Mucosal leishmaniasis (ML) is a metastatic outcome of a CL infection, resulting in the dissemination of parasites to the oropharynx mucosa.

In Brazil, *Leishmania* (*Leishmania*) *amazonensis* is a species described to cause a wide spectrum of clinical manifestations (2), accounting for unusual clinical presentations (3). Great genetic diversity among strains isolated from patients (4) has been reported, as well as a trend toward increasing geographical distribution.

The currently available treatments for these infections are restricted to two option groups, namely, (i) the antimonials, which are the first-choice drugs (5, 6), and (ii) pentamidine and amphotericin B, the second-choice drugs (7). Both groups of drugs have many limitations regarding their use, such as (i) high cost, (ii) difficulty of administration, (iii) toxicity, and (iv) the development of resistance by parasite strains. Undoubtedly, these limitations represent obstacles for successful therapy (8), emphasizing the need to develop new drugs for the treatment of leishmaniasis.

Several natural compounds have been reported to have antileishmanial effects, but none has transitioned into an effective drug for treatment of leishmaniasis. In this context, some natural products obtained from plant extracts or their derivatives, such as quinones, alkaloids, terpenes, and phenolic derivatives, have been

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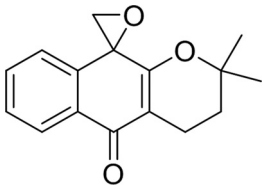

FIG 1 Structure of 2,2-dimethyl-3,4-dihydrospiro[benzo[*g*]chromene-10,20-oxiran]-5(2*H*)-one, also known as epoxy-α-lapachone (CID 12000280; molecular formula, $C_{16}H_{16}O_3$; molecular weight, 256.29644 g/mol) (https://pubchem.ncbi.nlm.nih.gov/compound/12000280#section=Top).

proposed for leishmaniasis chemotherapy (9). Recently, we reported evidence that quinone derivatives exhibited promising properties against protozoan parasites, such as *Trypanosoma cruzi* (10–14), *Leishmania* (*Viannia*) *braziliensis*, and *Leishmania* (*Leishmania*) *amazonensis* (15). These compounds can be isolated from *Bignoniaceae* or *Verbenaceae* trees, and their antimicrobial properties have been well established (16).

Among the naphthoquinone derivatives, epoxy-α-lapachone (Fig. 1) is a good candidate to serve as the basis for antileishmanial treatments, as it has been shown to have low cytotoxicity for mammalian cells (10, 11) while being effective against *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* (15); it was able to kill promastigotes of both species *in vitro* and affected amastigotes infecting human macrophages. We previously reported that epoxy-α-lapachone can inhibit serine and cysteine proteinase activities in *Trypanosoma cruzi* (17), but we have not yet assessed this possibility in *Leishmania* spp.

It is known that proteinases are pivotal virulence factors for *Leishmania* spp. (18). Serine proteinases, such as oligopeptidase B (OPB; clan SC, family S9), have been reported to correlate with the infection of murine macrophages by parasites and the survival of those located within infected cells (19). Therefore, in the present study, we aimed to assess the potential inhibitory effect of epoxy-α-lapachone on *L.* (*L.*) *amazonensis* serine proteinase activity, as it may be part of the antileishmanial mechanism of this compound, and we applied a molecular modeling approach to investigate how this inhibitor binds to target enzymes such as OPB. In parallel, we investigated the effects of epoxy-α-lapachone on both parasite forms and on the outcome of experimental murine infection with *L.* (*L.*) *amazonensis*.

MATERIALS AND METHODS

Chemicals and culture reagents. Coomassie brilliant blue R-250, detergents (sodium dodecyl sulfate [SDS] and Triton X-100), proteinase inhibitors (phenylmethylsulfonyl fluoride [PMSF], aprotinin, and antipain), HiTrap Benzamidine FF, Tris, glycerol, dimethyl sulfoxide (DMSO), penicillin, streptomycin, Schneider's *Drosophila* medium, and fluorogenic peptide substrates (*Z*-Phe-Arg-7-amido-4-methylcoumarin [*Z*-FR-AMC], Ala-Phe-Lys-7-amido-4-methylcoumarin [AFK-AMC], and *Z*-Gly-Gly-Arg-4-methoxy-β-naphthylamide [*Z*-GGR-MβNA]) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Amicon Centriprep YM-10 filter devices were purchased from Millipore (Billerica, MA). Fetal calf serum (FCS) was purchased from Cultilab S/A (Brazil). Brain heart infusion (BHI) medium was purchased from Oxoid Australia (West Heidelberg, Australia). The micro-bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Appleton, WI). TO-PRO-3 (Invitrogen, Waltham, MA) and tetramethylrhodamine ethyl ester perchlorate (TMRE) were purchased from Molecular Probes (Eugene, OR). Meglumine antimonate (Glucantime) was purchased from Sanofi-Aventis Farmacêutica (Suzano, Brazil). The epoxy-α-lapachone compound was synthesized by the Department of Organic Chemistry of the Instituto de Química, Universidade Federal Fluminense.

Parasite cultures. *L.* (*Leishmania*) *amazonensis* (strain MHOM/BR/73/LTB0016) was obtained from the *Leishmania* collection of the Instituto Oswaldo Cruz (Fiocruz). *In vitro* promastigote cultures were maintained at 28°C in Schneider's medium (pH 7.2) containing 1 mM L-glutamine, 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin, with frequent subpassages to maintain the parasites in the logarithmic growth phase.

Axenic amastigote transformation. Axenic amastigotes were obtained as described previously (20–22). Briefly, promastigotes of both parasite species, in the logarithmic growth phase (5×10^5 cells/ml), were seeded in axenic medium (Schneider's medium [pH 7.2] containing 10 mM HEPES buffer, 1 mM L-glutamine, 60 IU/ml penicillin, and 60 μg/ml streptomycin) and incubated for 24 h at 26°C. The promastigotes were then reseeded in new axenic medium with the pH adjusted to 5.5 and were cultivated under the same conditions. Following 4 days of incubation, the parasites were reseeded in new axenic medium at pH 5.5 and incubated at a higher temperature (32°C). To assess the degree of successful differentiation, the morphology of the cells in the cultures was analyzed by optical microscopy.

Effects of epoxy-α-lapachone on promastigotes and amastigotes. The parasites were seeded at a density of 1.0×10^7 parasites/ml in Schneider's medium and were incubated under different conditions (1 h or 3 h at 28°C or 32°C) in the absence or presence of epoxy-α-lapachone (0.175 μM) or DMSO, which was used as a diluent for the compound and as a control. Parasite viability was then assessed by flow cytometry using specific fluorescent markers. TO-PRO-3, a membrane-impermeable DNA marker, was used (10 μM) to assess parasites' membrane integrity. The TMRE probe was used (50 nM) to verify variations in ionic pumping metabolism and transmembrane potential (plasma membrane and organelles). Parasites (1.0×10^6 cells/well) were incubated for 20 min with the markers, and the samples were immediately analyzed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was carried out using Summit version 4.3 software.

Experimental murine infections and treatment of animals with epoxy-α-lapachone. Experimental infections were conducted with 6- to 8-week-old BALB/c mice weighing approximately 22 g. The animals were obtained from the animal breeding center of Fiocruz, and all experimental procedures were performed as approved by the Committee for the Ethical Use of Animals of Fiocruz (P-40/13-2). The mice were inoculated in the footpad of the left hind limb with 1.0×10^6 promastigotes of *L.* (*L.*) *amazonensis* in 10 mM phosphate-buffered saline (PBS). The parasites were in the stationary growth phase after 5 days of culture in Schneider's medium.

The experimental treatments were performed with either Glucantime (as a comparative control for treatment efficacy) or epoxy-α-lapachone at different concentrations (0.44, 0.09, and 0.02 mM). The drugs (0.5 ml/animal) were administered subcutaneously in the dorsal region of each mouse. Treatments were carried out for 1 week with daily injections, starting 1 week after challenge infection, when the paw lesions had already become noticeable. Two negative-control groups were included, in which sterile PBS or DMSO was administered during treatment. The lesions were evaluated on a weekly basis, by measuring lesion areas (in mm^2) with a caliper.

Parasite protein extracts. Protein extracts were obtained as described previously (23). Briefly, parasites (2.0×10^9), either promastigotes in the logarithmic growth phase or amastigotes, were washed three times by centrifugation (3,000 × *g* for 10 min at 4°C) in PBS (pH 7.2) and then were subjected to 4 cycles of vortex-mixing for 30 min in the presence of lysis buffer (100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 0.6% Triton X-100). The soluble protein fraction was obtained by centrifugation of the samples (25,000 × *g* for 30 min at 4°C) and then was stored at −20°C until further use. The protein concentrations of the extract samples were determined using the micro-BCA protein assay kit.

Serine proteinase-enriched fractions from parasites. Soluble protein fraction samples of promastigotes or amastigotes (adjusted to 35 or 40 mg/ml, respectively, in 10 mM Tris-HCl [pH 7.5]) were bound in a Hi-Trap Benzamidine FF column that had been previously equilibrated with binding buffer (0.05 M Tris-HCl, 0.5 M NaCl [pH 7.4]). The column was washed with the same buffer to flush out unbound proteins; the bound proteins were retrieved using elution buffer (0.05 M glycine [pH 3.0]) and preserved in 1 M Tris-HCl (pH 9.0). The eluted proteins (here called the serine proteinase-enriched fraction) were concentrated for 30 min and dialyzed against a buffer (10 mM Tris-HCl [pH 7.5]) for further use in proteinase assays.

Zymographic assays. The serine proteinase-enriched fraction (5 μg of total protein) was subjected to electrophoresis under reductive conditions using 12% acrylamide gels copolymerized with 0.1% gelatin (24). Following electrophoresis, the gels were washed for 1 h at 4°C in 0.1 M Tris-HCl (pH 7.5) (washing buffer) containing 2.5% Triton X-100 and then were incubated for 6 h at 37°C in washing buffer without supplements. The gel was then stained with Coomassie brilliant blue R-250.

Assessments of proteinase activity and inhibitory efficacy. The proteinase activities (in solution) of the serine proteinase-enriched fraction (0.5 μg of total proteins) and trypsin, which was used as a positive activity control, were characterized in activation buffer (10 mM Tris-HCl [pH 7.5]), at a final volume of 60 μl, using specific fluorescent peptide substrates for serine proteinase (*Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA at 0.1 mM). Samples were incubated for 60 min at 37°C, and the variance in the relative fluorescence units (RFU), corresponding to enzymatic cleavage of the substrates, was monitored with a Molecular Devices SpectraMax spectrophotometer (Gemini XPS) (7). Concomitantly, the efficacy of various serine proteinase inhibitors was assessed under the same conditions. Inhibition assays were performed with 1 mM PMSF, 0.3 μM aprotinin, 5 μM antipain (all used as controls), and 1 mM epoxy-α-lapachone.

The substrate enzymatic cleavage rate was defined using the formula $v = \Delta s/\Delta t$, where v represents velocity (reaction rate), Δs represents substrate concentration variation, and Δt represents the total reaction time (20). Self-degradation of the fluorescent peptide substrate was controlled throughout the assay, to avoid incorrect readings. The enzymatic activity is expressed as $(\times 10^{-3})$ mmol · min^{-1} · mg $protein^{-1}$.

Determination of IC_{50} values for serine proteinase inhibitors. The 50% inhibitory concentration (IC_{50}) values for all tested inhibitors were obtained as described previously (25). Briefly, the tests were performed by combining a fixed *Z*-FR-AMC substrate concentration (0.1 mM) with 10 distinct concentrations (from 1.5×10^{-4} mM to 5 mM) of each inhibitor, using the same methodology as described above. These results were applied to a linear interpolation of the concentrations for each inhibitor versus the corresponding percentage of enzymatic inhibition and were analyzed using the following equation: IC_{50} = [(50% − lower inhibition %)/(higher inhibition % − lower inhibition %) × (higher concentration − lower concentration) + lower concentration] For the linear interpolation analysis, the mean values for triplicate determinations in each assay were used.

Statistical analysis. To compare results, Student's test was applied; data matrices were considered statistically different when the *P* value was less than 0.05. Statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA).

Molecular docking of oligopeptidase B. In order to investigate the binding mode of epoxy-α-lapachone, this compound was docked into *L.* (*L.*) *amazonensis* oligopeptidase B (OPBa) using the DockThor program (27). First, the three-dimensional structures of ligand molecules were built and minimized with the Avogadro 1.1 program. The crystal structure of *Leishmania* (*Leishmania*) *major* oligopeptidase B (OPBm) complexed with antipain was obtained from the Protein Data Bank (PDB accession number 2XE4), and the OPBa model was constructed using the Modeller 9.14 program (28), which was used with the OPBm template. The model construct with the lowest value for discrete optimized protein energy (DOPE) was selected and evaluated with ProCheck (29), Errat (30), and Prosa (31) software. The molecular docking was established in a cubic grid box of 8 by 8 by 8 $Å^3$, and the parameters are referred to as defaults in DockThor. Structures with positional root mean square deviation (RMSD) of up to 2 Å were clustered together, and the results with the most favorable free energy of binding were selected as the resultant complex structures. We also performed redocking of antipain to the crystal structure of *L.* (*L.*) *major* OPB, with a success rate (RMSD of ≤2.0 Å for the interface backbone atoms) of 53%.

RESULTS

Epoxy-α-lapachone has leishmanicidal activity with promastigotes and amastigotes of *Leishmania* (*L.*) *amazonensis*. We used flow cytometry to demonstrate that epoxy-α-lapachone can affect both promastigotes and amastigotes. First, the drug causes metabolic dysfunction in ionic pumping, which can be mainly due to mitochondrial damage, and this was evidenced by a reduction in TMRE labeling. Then, it leads to parasite death through the loss of membrane integrity, as evidenced by an increase in TO-PRO-3-positive cells. Our results also indicated that the drug is capable of quickly crossing the plasma membrane (Fig. 2).

As indicated in Fig. 2A, 87.9% of control promastigotes had normal ionic pump activity, with a mean fluorescence intensity (MFI) value of 96.9. After 1 hour of incubation with 0.175 μM epoxy-α-lapachone, there was an abrupt decrease in metabolic activity, as evidenced by a reduction in TMRE staining (MFI of 49.5). Exposure for 3 h led to a further decrease in MFI to 40.6. The loss of membrane integrity was confirmed by an increase in promastigotes stained with TO-PRO-3 (control, 4.0%; 1 h of exposure, 39.9%; 3 h of exposure, 78.9%) (Fig. 2A, upper right quadrant of each graph). Amastigote forms also showed a reduction in the difference in transmembrane potential, with MFI values from 35.0 in control cells to 35.4 at 1 h and 29.4 at 3 h. Regarding membrane integrity, we observed that only 1.7% of cells were TO-PRO-3 positive in the control and there was an increase to 44.5% after 1 h of incubation. We observed no increase in this result after 3 h of incubation (Fig. 2B).

Mouse lesions caused by *Leishmania* (*L.*) *amazonensis* infection decrease after treatment with epoxy-α-lapachone. The role of epoxy-α-lapachone in the progression of lesions during the course of an experimental infection was analyzed in BALB/c mice injected with different concentrations of the compound after 1 week of infectious challenge. The results indicated that the treated animals exhibited reductions in paw lesion areas, compared to animals from the control group (Fig. 3). Although no dose-response correlation was observed in assays with Glucantime and epoxy-α-lapachone, the effects of the compounds were statically significant, compared with negative-control results (Fig. 3A and B).

Serine proteinase activity in *Leishmania* (*L.*) *amazonensis* is inhibited by epoxy-α-lapachone. The potential of epoxy-α-lapachone to act as an inhibitor of *L.* (*L.*) *amazonensis* serine proteinases from promastigotes and amastigotes was assessed in this study. To this end, assays were performed with serine proteinase-enriched fractions obtained by affinity chromatography, which were analyzed by using gelatin-SDS-PAGE and fluorogenic peptide substrates. These fractions yielded approximately 0.1 and 0.05 mg of protein, corresponding to 0.28 and 0.13% of the total applied protein, respectively, for promastigotes and amastigotes. SDS-PAGE analysis revealed a major proteinase band with an estimated molecular mass of 68 kDa, which was stained with both

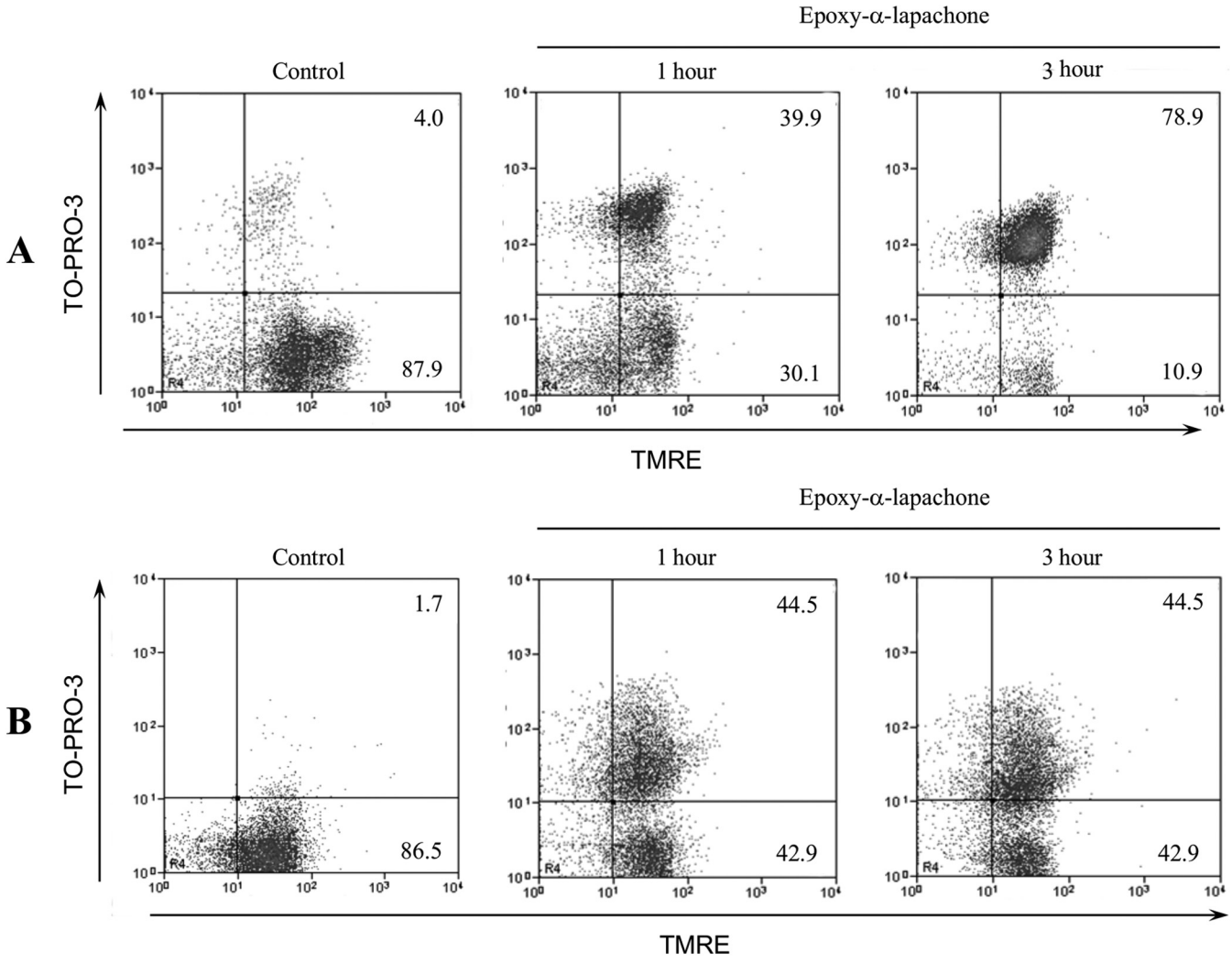

FIG 2 Flow cytometry assays demonstrating that the epoxy-α-lapachone compound can affect promastigotes and amastigotes. Dot-plot analyses of untreated (control) and epoxy-α-lapachone (0.175 μM)-treated (1 h and 3 h) promastigote (A) and amastigote (B) forms are shown. Before the acquisition of data (10^6 events), the parasites were stained with TMRE and TO-PRO-3 in Schneider's medium. The data are representative of three experiments, and values within the graphs are percentages.

silver (Fig. 4) and Coomassie brilliant blue (data not shown), presenting similar results by both methods.

Serine proteinase activity in fractions from both parasite forms was subsequently detected in assays of enzymatic activity in solution. The protein fractions from promastigotes and amastigotes were both able to hydrolyze *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA but at different velocity rates, with the amastigote fraction exhibiting higher velocity rates for substrate hydrolysis than the promastigote fraction, as follows: for the amastigote fraction, *Z*-FR-AMC, $(16 \pm 0.03) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; AFK-AMC, $(20 \pm 0.7) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; and *Z*-GGR-MβNA, $(18 \pm 0.4) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; and for the promastigote fraction, *Z*-FR-AMC, $(8 \pm 0.8) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; AFK-AMC, $(3 \pm 0.6) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; and *Z*-GGR-MβNA, $(3 \pm 0.3) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}. These fractions exhibited distinct profiles of inhibition by classic serine proteinase inhibitors (Fig. 4).

In the course of this study, we were able to verify that the epoxy-α-lapachone inhibits serine proteinase activity in protein fractions from both *L.* (*L.*) *amazonensis* promastigotes and amastigotes. This inhibitory effect was observed for parasite enzymatic activity with all tested substrates, i.e., 85%, 80%, and 93% inhibition of promastigote enzymes and 80%, 77%, and 91% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively. With the three assayed substrates, this was a better profile than those of PMSF (11%, 14%, and 2% inhibition of promastigote enzymes and 15%, 14%, and 3% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively), aprotinin (2%, 0%, and 7% inhibition of promastigote enzymes and 11%, 11%, and 4% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively), and antipain (85%, 65%, and 67% inhibition of promastigote enzymes and 66%, 92%, and 99% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively) (Fig. 4).

Additionally, the IC_{50} values for all of the tested inhibitors were

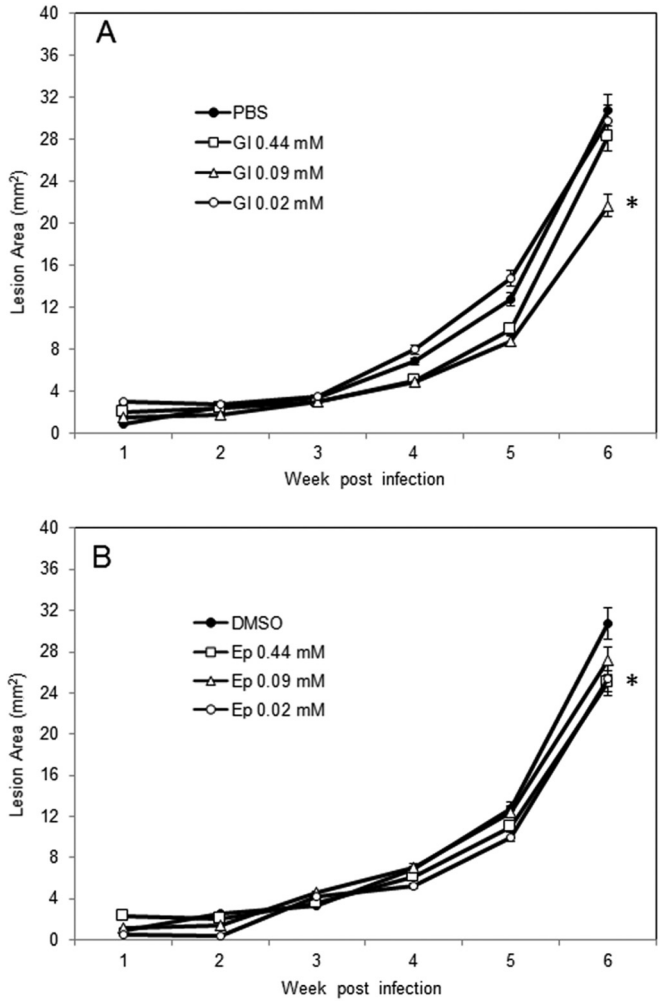

FIG 3 Experimental treatment of infections in mice caused by *Leishmania* (*L.*) *amazonensis*. BALB/c mice were inoculated subcutaneously, in the left footpad, with 1.0×10^6 promastigotes at the logarithmic phase of growth. After 1 week of infection, the mice were treated weekly with meglumine antimoniate (GI) (A) or epoxy-α-lapachone (Ep) (B) at concentrations of 0.44 mM, 0.09 mM, and 0.02 mM administered subcutaneously, with five animals per group. Controls were treated with PBS (A) or 0.44 mM DMSO (B) alone. The lesion sizes were measured, and the results represent the means ± standard deviations from three independent experiments. *, $P < 0.05$.

determined with each protein fraction (and trypsin, used as a positive control) using the *Z*-FR-AMC substrate. As shown in Table 1, the IC_{50} of epoxy-α-lapachone, although higher than those of aprotinin and antipain for both protein fractions, was lower than the IC_{50} value of PMSF, suggesting that it effectively impairs serine proteinase activities.

***In silico* simulations of epoxy-α-lapachone.** Due to the absence of crystallographic data for *L.* (*L.*) *amazonensis* OPB in data banks, it was necessary to build a three-dimensional model of this enzyme to proceed with molecular docking tests. OPBa showed a high degree of identity (90%) with OPBm. The model of OPBa with a lower DOPE value revealed an RMSD of 0.19 Å when aligned with OPBm. The stereochemical evaluation exhibited 90.2% and 85.5% of residues with most favored regions in a Ramachandran plot and G-factor values of −0.25 and −0.19 for OPBm and OPBa, respectively. In addition, analysis of nonbonded interactions showed Errat scores of 93.5% and 87.0% and *Z*-scores of −11.56 and −11.26 for OPBm and OPBa, respectively.

An analysis of the results of the redocking of antipain into OPBa showed an RMSD of 2.0 Å when aligned with the original cocrystallized conformation, demonstrating that the methodology was theoretically reliable for showing ligand-bound conformations (Fig. 5A). In order to theoretically analyze the binding mode of epoxy-α-lapachone, we docked these molecules into OPBa and compared them with antipain. The comparison of epoxy-α-lapachone docking with that of antipain revealed several differences in binding to the amino acid residues (data not shown). The OPBa-epoxy-α-lapachone complex conserved the main binding in S1 by hydrogen bonds and the hydrophobic interactions that are observed in antipain (Fig. 5B). The data showed that the main hydrogen bonds occurred with the residues Ser577, Ala578, and Try496 and the hydrophobic interactions with the residues Phe698, Arg576, Ile501, and Leu617 (Fig. 5C), between OPB and the epoxy-α-lapachone. The interaction energy value of epoxy-α-lapachone (−22.08 kcal/mol) was comparable to the energy value of antipain redocking (−26.95 kcal/mol).

DISCUSSION

American tegumentary leishmaniasis has spread across North and South America and remains without an efficient treatment (32). The current treatment, based on pentavalent antimony, is associated with severe side effects, such as pain, gastrointestinal disorders, headache, anorexia, and cardiac, hepatic, and pancreatic toxicity (33). For this reason, the development of new chemotherapeutic agents, potentially including plant-derived compounds such as the naphthoquinones, is required (10, 11). We therefore aimed to assess the potential leishmanicidal activity of epoxy-α-lapachone in the treatment of experimental murine infections and to identify the targets in the parasite affected by this compound. This compound was selected for further analysis because of its previously reported low cytotoxicity in mammalian cells (10, 15), which highlighted its usefulness to serve as a basis for the development of novel antileishmanial drugs.

With our assays, we collected evidence that epoxy-α-lapachone is in fact a potent leishmanicidal agent; it readily affected *L.* (*L.*) *amazonensis* promastigotes and axenic amastigotes *in vitro* after a short coincubation period, as well as inducing decreases in infection-related paw lesions in experimentally infected mice. The results presented show that both epoxy-α-lapachone and Glucantime had effects in the reduction of paw lesions in the treated BALB/c mice. These results, in association with the previous data on the low toxicity of epoxy-α-lapachone for mammalian cells (15, 17), are strong indicators of the potential use of this compound in the treatment of leishmaniasis. Possibly the effects of epoxy-α-lapachone in the control of lesions in mice are due to multifactorial actions on parasite physiology. The chemical structures of naphthoquinone derivatives, such as the compound in this study, contribute to the formation of reactive oxygen and accelerate intracellular hypoxic conditions, causing severe damage to the parasite cells (34, 35).

Flow cytometry results indicated that epoxy-α-lapachone was able to freely enter both parasite forms and eventually led to a loss of parasite plasma membrane integrity, as parasites exposed to this compound had DNA that was stainable by TO-PRO-3, a marker that is unable to cross intact plasma membranes (36). TMRE

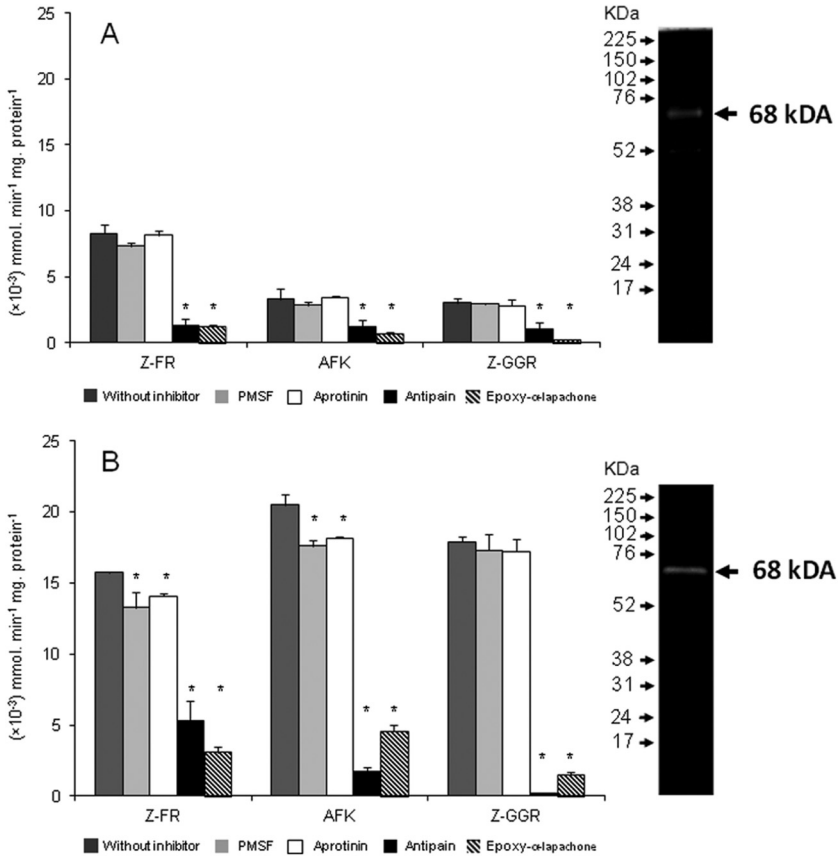

FIG 4 Proteinase activities of *Leishmania* (*L.*) *amazonensis* in solution. Fractions enriched in serine proteinase from promastigotes (A) and amastigotes (B) were obtained through benzamidine-Sepharose affinity chromatography. The enzymatic activities of fractions (10 μg) were measured with 100 μM levels of the substrates *Z*-FR-AMC, *Z*-GGR-MβNA, and AFK-AMC, in the absence (control) or presence of inhibitors (1 mM PMSF, 1 mg of aprotinin, or 1 mM antipain) or 1 mM epoxy-α-lapachone. The reaction mixtures were incubated for 60 min at 37°C in 10 mM Tris-HCl buffer (pH 7.5). The enzymatic activity of the fractions is expressed as $(\times 10^{-3})$ mmol · min^{-1} · mg $protein^{-1}$. Inset, zymographic profile of enriched serine proteinase fractions (5 μg). The molecular mass markers are indicated, and results are expressed as the means ± standard deviations from three independent experiments. *, $P < 0.05$.

staining demonstrated that this compound also possibly induced alterations in the membrane potential of parasite mitochondria, revealing yet another physiological effect of epoxy-α-lapachone on the parasites.

This effect was evidenced by fluorescent labeling indicating membrane potential ($\Delta\psi_m$) changes, mainly in mitochondria, using TMRE labeling (37). An experiment in which the organelle was reconstructed in three dimensions showed that the physical continuity of intact functional mitochondria can be determined by fluorescence from TMRE (38). Here, we propose that epoxy-α-lapachone is able to act in any metabolic pathway by compromising the $\Delta\psi_m$ of intracellular (primarily mitochondrial) organelles, as revealed by reductions in TMRE staining. The collapse of the mitochondrial transmembrane potential is related to the opening of mitochondrial permeability pores, leading to the release of cytochrome *c* into the cytosol, which then leads to other events in the apoptotic cascade (39).

Data from this study confirmed that epoxy-α-lapachone inhibited a 68-kDa proteinase from *L.* (*L.*) *amazonensis*, which was subsequently characterized as a serine proteinase, as it was isolated by benzamidine-based affinity chromatography and its hydrolytic activity was inhibited in the presence of classic serine proteinase inhibitors but was not affected by other proteinase inhibitors (data not shown). Additional data suggested that, similar to trypsin, the folding of the serine proteinase from *L.* (*L*) *amazonensis* is

TABLE 1 IC_{50} values for inhibition of serine proteinase activity

Enzyme or fraction[a]	IC_{50} (mM)			
	Epoxy-α-lapachone	Antipain	PMSF	Aprotinin
Trypsin	0.9 ± 0.05	$(4.0 \pm 1.7) \times 10^{-3}$	1.4 ± 0.2	$(33 \pm 2.8) \times 10^{-3}$
Fraction from promastigotes	0.9 ± 0.1	$(4.1 \pm 0.2) \times 10^{-3}$	2.8 ± 0.8	$(0.9 \pm 0.04) \times 10^{-3}$
Fraction from amastigotes	1.2 ± 0.06	$(1.8 \pm 0.5) \times 10^{-3}$	9.5 ± 2.4	$(4.8 \pm 0.08) \times 10^{-3}$

[a] Serine proteinase fractions from *L.* (*L.*) *amazonensis* promastigotes and amastigotes were tested. Enzymatic assays were performed with 0.1 mM *Z*-FR-AMC substrate in 10 mM Tris-HCl (pH 7.5) and at least five concentrations of epoxy-α-lapachone, antipain, PMSF, and aprotinin. The data are expressed as means ± standard deviations.

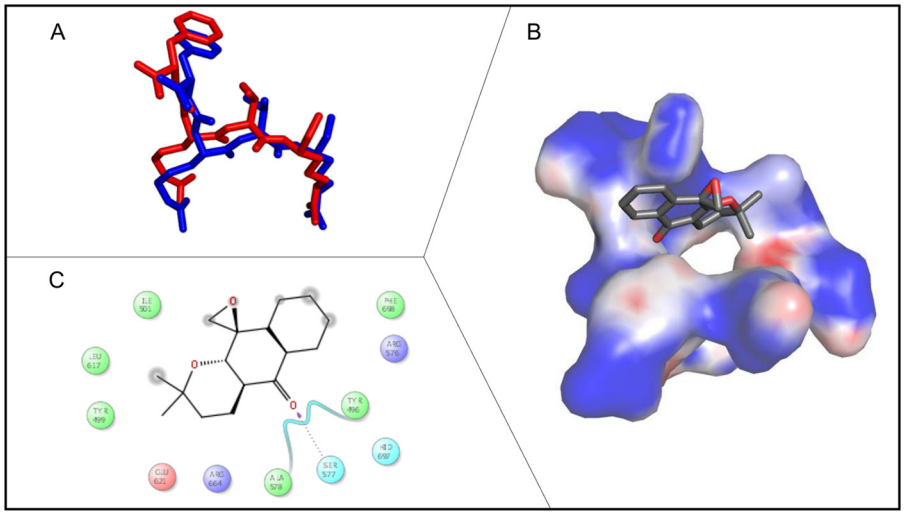

FIG 5 Docking complexes of compounds with *L.* (*L.*) *amazonensis* oligopeptidase B. (A) Structural alignment of the redocking complexes of antipain (blue) and antipain cocrystallized with oligopeptidase B (red). (B) Binding of epoxy-α-lapachone (sticks) in the active site (surface). (C) Details of amino acid residues at approximately 5 Å, showing interactions with epoxy-α-lapachone. Black, carbon atoms; red, oxygen atoms.

resistant to mild denaturing conditions, indicating that the structural stability of the isolated enzyme was maintained during the enzyme activity assays.

Other serine proteinases of various molecular sizes (i.e., 115 kDa [40], 68 kDa [41, 42], and 56 kDa [43]) were previously identified in *L.* (*L.*) *amazonensis* and may also be affected by the compound. Additionally, a serine proteinase named OPB has been described for other *Leishmania* species and has been found to play roles in many essential events for the parasites in their mammalian hosts (19, 44). These data indicate that many other potential serine proteinase targets that may be affected by epoxy-α-lapachone are present in the parasites, and they emphasize the importance of these molecules in parasite survival.

Notably, the affinity chromatography approaches applied here were able to demonstrate that, in *L.* (*L*) *amazonensis*, the amastigotes contain more serine proteinase than the promastigotes. Both parasite forms hydrolyze a selective group of substrates related to the fibrinolytic serine proteinases, i.e., *Z*-FR-AMC (kallikrein) (45), AFK-AMC (plasmin, urokinase, and thrombin) (46), and *Z*-GGR-AMC (urokinase) (47). These enzymes preferably cleave Arg and Lys residues in the P1 position and Gly and Ser (urokinase) and Pro, Ala, Gly, and Leu (thrombin) in the P2 position (48). Additionally, enzyme activity is greater at the parasite stage related to the infection of mammalian cells, which reinforces the hypothesis that serine proteinases are essential for *Leishmania* survival, are feasible targets for the development of new inhibitors such as epoxy-α-lapachone, as proposed here, and can be targeted in combined treatments for effective antileishmanial therapy, as recently suggested (49).

Generally, desired IC_{50} values for potential inhibitors are in the nanomolar or low micromolar ranges. Values for epoxy-α-lapachone were within this range, which importantly demonstrates that, in molecular docking, this compound was able to bind to the active center of a serine proteinase with inhibitory capabilities. Therefore, the mechanism of action of epoxy-α-lapachone on a *Leishmania* serine proteinase was assessed here by molecular docking.

Due to the absence of crystallography coordinates for the serine proteinase structure of *L. (L.) amazonensis*, molecular docking studies for these enzymes in *Leishmania* are constrained, because target-based ligand selection methods depend on the availability of target structural information (50). We experimentally and theoretically tested the ability of epoxy-α-lapachone to inhibit this enzyme.

Our theoretical evaluation of the docking complexes of epoxy-α-lapachone with *L.* (*L.*) *amazonensis* OPB and comparison of those complexes with a classic inhibitor (antipain) revealed that epoxy-α-lapachone underwent hydrophobic binding with residue Leu617 in the S3 pocket and formed hydrogen bonds with Ala578, Ser577, and Try496 in the S1 pocket of *L.* (*L.*) *major* OPB, with distances of 2.7 to 3.9 Å. These interactions may contribute to the stabilization and maintenance of epoxy-α-lapachone at the active site. In addition, epoxy-α-lapachone has an electrophilic moiety susceptible to nucleophilic attack by the activated catalytic serine at a distance that suggested this reaction. In summary, this study presents additional evidence that epoxy-α-lapachone can affect *L.* (*L.*) *amazonensis* parasites in mice during experimental infections and this compound can act as a serine proteinase inhibitor, making it a promising candidate to serve as a basis for the development of novel drugs for controlling leishmanial infections.

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All authors approved the final version of the manuscript.

We confirm that there are no conflicts of interest associated with this article.

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