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Research Brief

Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone

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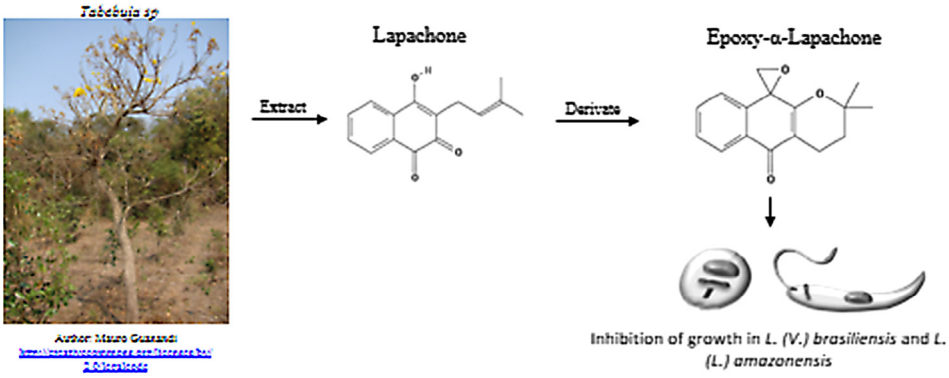
HIGHLIGHTS

- Epoxy-α-lapachone has activity against *L. (V.) braziliensis* and *L. (L.) amazonensis*.
- It is able to decrease promastigotes growth in culture.
- It also drastically affected the survival of amastigotes inside human macrophages.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, we analyze the leishmanicidal effects of epoxy-α-lapachone on *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*. Promasigotes and amastigotes (inhabiting human macrophages) from both species were assayed to verify the compound's activity over the distinct morphological stages. The incubation with epoxy-α-lapachone led to a significant decrease in the numbers of promastigotes from both species in the cultures, in a dose-and time-dependent fashion. The survival of amastigotes inhabiting human macrophages was also drastically affected by the compound, as shown by the variations in the endocytic index. Our results indicate that the epoxy-α-lapachone has an antiparasitic effect over *Leishmania* in both morphological stages and may potentially affect a range of species in two distinct subgenera of this parasite.

Leishmaniasis is a very relevant parasitic disease, especially in the tropical and subtropical regions of the world, with increasing importance as its geographical spreading is a reported fact (Hotez et al., 2007). Currently, the first-line treatment for leishamniasis involves antimonial, which are expensive and potentially toxic, requiring also a long therapy timeframe (Berman et al., 1997; Deps et al., 2000). Additionally, the emergence of cases of parasites

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presenting resistance to this treatment has been reported (Sundar et al., 2014).

The drugs used in cases where treatment with antimonials is ineffective or must be avoided, known as second choice drugs, e.g. Pentamidin, Amphotericin B and Miltefosine, are also toxic and present difficulties to their administration (Jha, 2006; Saldanha et al., 1999). These drugs present differences in their efficiency against distinct *Leishmania* species (Santos et al., 2008). Therefore, leishmaniasis still lacks adequate therapies and the development of new drugs is a point of great and, even, urgent interest.

The derivates of naphthoquinones, organic compounds originally described in the heartwood of *Bignoniaceae* and *Verbanaceae* trees are known by their significant anti-microbial properties (Ferreira et al., 2006; Jorqueira et al., 2006). β-lapachone (1,2-naphthoquinone) and its isomer α-lapachone (1,4-naphthoquinone) were shown to have trypanocidal activity but with a significant cytotoxicity on mammalian cells.

As they have been described as effective against *Trypanosoma cruzi*, derivatives of naphthoquinones were also analyzed as potential leishmanicidal drugs: It was reported that lapachol, isolapachol and dihydrolapachol, as well as soluble derivatives and acetate, are able to kill *in vitro* metacyclic promastigotes of *Leishmania (Leishmania) amazonensis* and *Leishmania (Leishmania) braziliensis* (Lima et al., 2004). Other derivatives, such as 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone and 2,3-dibromo-1,4-naphthoquinone, were also proven to kill promastigotes and intracellular amastigotes of *Leishmania (L.) donovani* (Lezama-Dávila et al., 2012).

However, the toxicity of the lapachone derivatives could limit their potential to be used in the treatment of leishmaniasis and other parasite infections. Thus, studies have applied chemical modifications in these compounds to address this issue and a variation at the quinonoid center of α-lapachone generated the epoxy-α-lapachone (2,2-dimethyl-3,4-dihydrospiro[benzo[g]chromene-10,20-oxiran]-5(2H)-one), a derivative potentially less toxic for mammalian cells (Ferreira et al., 2006).

When used in assays with *T. cruzi*, the compound epoxy-α-lapachone presented a trypanocidal activity equivalent to β-lapachone but, as expected, with lower cytotoxic effect against mammalian cells, such as human macrophages (Bourguignon et al., 2009; Ferreira et al., 2006; Jorqueira et al., 2006).

In the present study, the *in vitro* effects of epoxy-α-lapachone compound against two species of *Leishmania* – *L. (V.) braziliensis* and *L. (L.) amazonensis* – and their two morphological stages, is described for the first time.

Leishmania (Viannia) braziliensis (strain MCAN/BR/1998/R619) was obtained from Instituto de Pesquisa Clínica Evandro Chagas (IPEC-Fiocruz) and *Leishmania (Leishmania) amazonensis* (strain MHOM/BR/73/LTB0016) was obtained from Coleção de *Leishmania* do Instituto Oswaldo Cruz (CLIOC/IOC – Fiocruz). The conditions of promastigotes cultures are described elsewhere (Cysne-Finkelstein et al., 1998).

The epoxy-α-lapachone was obtained by epoxidation of α-lapachone (Ferreira et al., 2006). And this quinone was previously extracted from the heartwood of *Tabebuia* sp. (Pinto et al., 1980). The samples were solubilized in dimethyl sulfoxide (DMSO – Sigma-Aldrich, St. Louis, MO, USA); stock solution of 5 mM were used in the performed assays.

The compound direct effect over promastigotes *in vitro* was assessed as follows: parasites (5×10^5/mL) in Schneider's medium (containing 10% fetal calf serum) were incubated at 28 °C in 24-well plates for 24 or 48 h in the presence or absence of epoxy-α-lapachone (25, 50 or 75 μM), in 1% DMSO. A control with 1% DMSO was added as this compound was used as a diluent for the epoxy-α-lapachone. Samples were analyzed each day by counting in a Neubauer chamber. Promastigotes viability was determined by observing cell motility and staining with 0.1% trypan blue (Sigma-Aldrich) in a dye exclusion test.

Table 1
Cytotoxicity effects of epoxy-α-lapachone in mammalian macrophage cells.

Epoxy-α-lapachone concentration (μM)	Incubation time (% of viable macrophages)	
	24 h	48 h
25	100 ± 8.3	99 ± 6.2
50	100 ± 8.5	98 ± 8.1
75	96 ± 6.4	90 ± 8.8

The values represent the average and standard deviation (±) of three independent experiments, as measured by CBBR-250 absorbance. In these assays, the control (not treated – 100% viable) macrophages (10^5 cells) showed an optical density of 0.8 at 570 nm.

For the assays with human macrophages, peripheral blood mononuclear cells (PBMC) from healthy donors were separated by Ficoll-Hypaque (Sigma-Aldrich) gradient centrifugation and the monocyte-derived macrophages (MØ) were isolated by plastic adherence assays (Bourguignon et al., 2009). The use of human samples in the present study was approved by the Committee of Ethics in Human Research of UFF (process CEP CMM/HUAP – 162/06).

To further use in tests for assessing *in vitro* leishmanicidal activity of epoxy-α-lapachone on infected cells, the MØ were incubated with infective promastigotes (ratio of cell/parasites 1:10) for 3 h at 37 °C in 5% CO_2, following washing (three times) with RPMI medium (Sigma-Aldrich) to remove unbound parasites.

The effects of the compound over MØ and their infecting parasites were assessed as follows: infected or non-infected MØ were incubated on Lab-Tek Chamber Slides (Sigma-Aldrich), under the same conditions above, in the presence or absence of 75 μM epoxy-α-lapachone (or 1% DMSO) for different times (24, 48 or 72 h). At each time point, the macrophages were fixed with methanol, stained with May-Grünwald-Giemsa (Sigma-Aldrich) and observed in an optical microscope (Nikon Eclipse E200).

The rate of the infected human macrophages was determined by analyzing at least 100 randomly selected cells at 1000× magnification. Infection data are expressed as endocytic index, was calculated by the percentage of parasites multiplied by the percentage of infected cells.

Additionally, cytotoxic effects of both epoxy-α-lapachone and DMSO on the human MØ were assessed. The cells were incubated with the compounds, stained with 0.2% CBBR-250 and the cells viability after incubation was measured using the correlation between the cell number and the CBBR-250 absorbance (Ferreira et al., 2006; Jorqueira et al., 2006). In these experiments, the DMSO did not affect the cells, as 100% of the MØ remained viable in culture even after 48 h incubation with 1% DMSO, (data not show). Similarly, the compound epoxy-α-lapachone, in both tested incubation time frames (24 and 48 h), showed no effects over MØ viability in the two lower concentrations (Table 1).

Our results show that co-incubation of epoxy-α-lapachone with promastigotes is able to significantly decrease the number of viable promastigotes in test cultures compared with control cultures. In addition, the compound effect on parasites was shown to be dose- and time-dependent (Fig. 1).

The tested species were both susceptible to the compound effects, but with some differences regarding their susceptibility rate. After a 24 h incubation with epoxy-α-lapachone, decreases of 44% (25 μM), 97.1% (50 μM) and 98.5% (75 μM) in the numbers of viable promastigotes of *L. (V.) braziliensis* was observed. For *L. (L.) amazonensis* parasites, similar conditions induced decreases of 61% (25 μM), 72% (50 μM) and 80% (75 μM). To both parasites species we have found an IC_{50} 37.0 ± 0.4 μM.

A more extensive incubation period (48 h) potentialized the effects of the compound, especially at lower concentrations: *L. (V.) braziliensis*

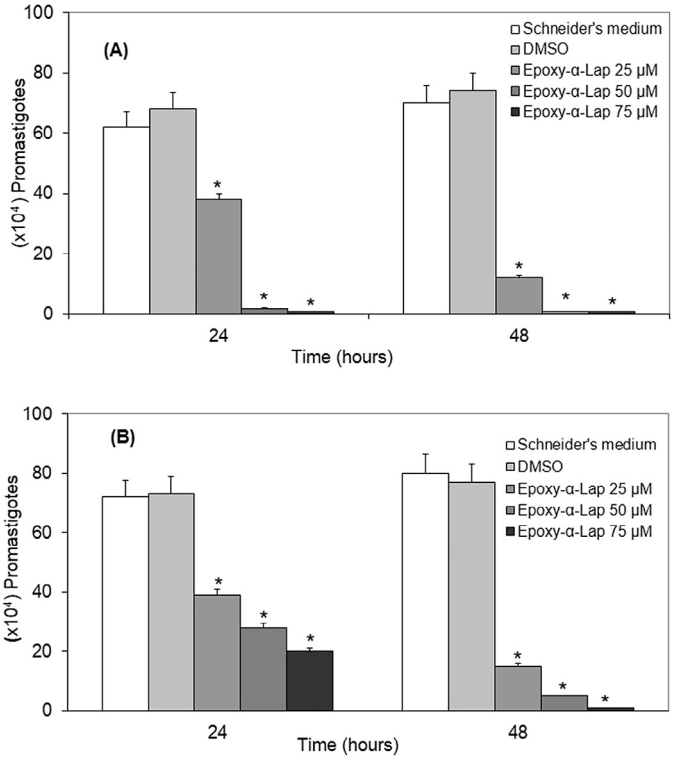

Fig. 1. Dose-response curve of epoxy-α-lapachone effects on the survival of promastigotes of *Leishmania* species in culture. Promastigotes from *L. (V.) braziliensis* (A) and *L. (L.) amazonensis* (B) were cultivated in Schneider's medium, during 24 or 48 h, in the presence (at the concentrations of 25, 50 or 75 μM) or absence of epoxy-α-lapachone. Cultures cultivated in the presence of 1% DMSO were also used as controls. Data are expressed as the number of viable promastigotes ($\times 10^4$) in the cultures. The graphics present mean and standard deviation of five independent experiments. *Indicates statistically significant difference from the controls (Student's T, $p < 0.05$).

promastigotes numbers reduced by 83.8% (25 μM), 98.6% (50 μM) and 98.9% (75 μM), while *L. (L.) amazonensis* promastigotes numbers reduced by 80.5% (25 μM), 93.4% (50 μM) and 98.7% (75 μM).

In addition, we could observe that the compound was also able to kill intracellular parasites in a time-dependent fashion (Fig. 2). After 72 h of culture, almost no parasite of either tested species survived, as indicated by the comparison of endocytic index of samples coincubated or not with epoxy-α-lapachone: *L. (L.) braziliensis* (21.0 ± 2 and 491.1 ± 40, respectively) and *L. (L.) amazonensis* (6.0 ± 0.8 and 290.0 ± 30, respectively).

This result suggests that the compound is capable of crossing the plasma membrane of the macrophages and affect the amastigote, supporting its potential for acting as an antiparasitic agent in future treatments against leishmaniasis.

The precise mechanism of action of epoxy-α-lapachone over the parasites is not yet known, but previous reports in the literature (Bourguignon et al., 2009, 2011; Taddei, 1999) suggest it may be through inhibition of parasites proteinases activity. Preliminary zymographic assays by our group (data not shown) seem to confirm this hypothesis, but further studies are still required.

Taken together our data show a relevant effect of the compound epoxy-α-lapachone over the survival of promastigotes and amastigotes *in vitro*, including the potential to reach and affect parasites inhabiting host cells. Additionally, they suggest that this leishmanicidal activity affects a range of *Leishmania* species, as, during our assays, two species belonging to two different subgenera were equally affected.

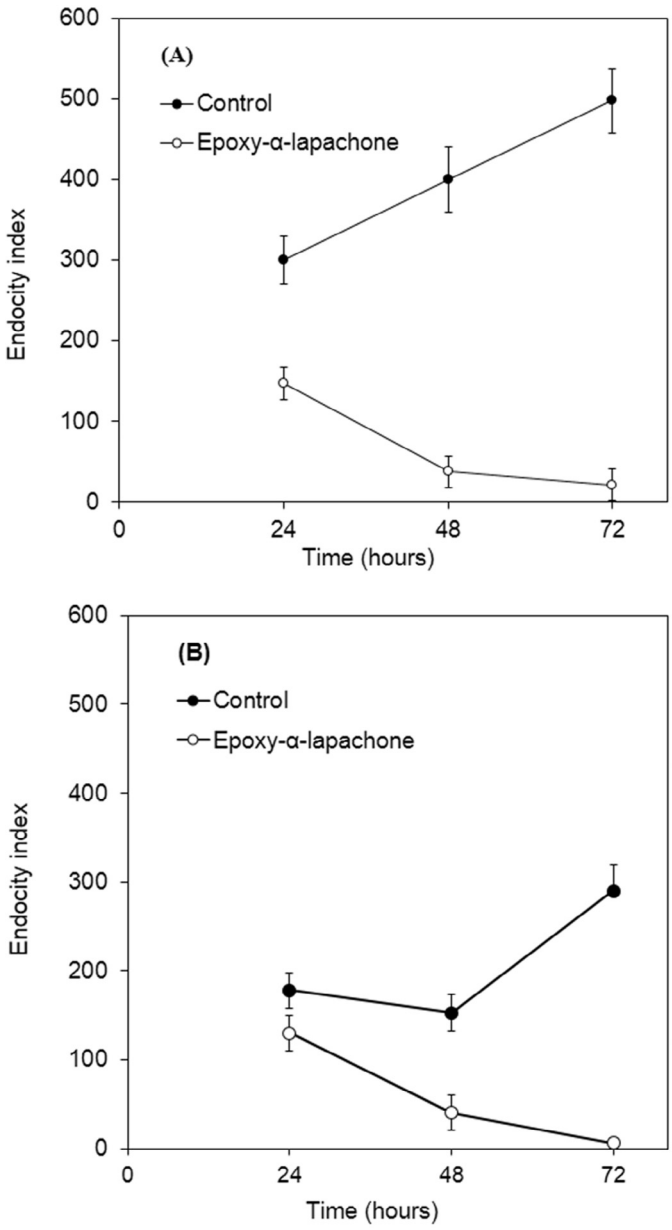

Fig. 2. Effects of the epoxy-α-lapachone on the endocytic index of the amastigotes in human macrophages. Epoxy-α-lapachone (75 μM: ○) or DMSO (1%: ●), used as control, were co-incubated in cultures of amastigotes-infected human macrophages, with either *L. (V.) braziliensis* (A) or *L. (L.) amazonensis* (B) parasites, for 24, 48 or 72 h. The results are expressed as the mean and standard deviation of three independent experiments, performed with macrophages from different healthy human donors. All time points analyzed presented statically significant differences from their respective controls (Student's *t*, $p < 0.05$).

Thus, the evidences presented herein present for the first time the potential of using epoxy-α-lapachone as basis for the development of novel or complementary treatments against human leishmaniasis.

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References

Berman, E., Wiernik, P., Vogler, R., Velez-Garcia, E., Bartolucci, A., Whaley, F.S., 1997. Long-term follow-up of three randomized trials comparing idarubicin and daunorubicin as induction therapies for patients with untreated acute myeloid leukemia. Cancer 80, 2181–2185.

Bourguignon, S.C., Castro, H.C., Santos, D.O., Alves, C.R., Ferreira, V.F., Gama, I.L., et al., 2009. *Trypanosoma cruzi*: in vitro activity of Epoxy-alpha-Lap, a derivative of alpha-lapachone, on trypomastigote and amastigote forms. Exp. Parasitol. 122, 91–96.

Bourguignon, S.C., Cavalcanti, D.F., de Souza, A.M., Castro, H.C., Rodrigues, C.R., Albuquerque, M.G., et al., 2011. *Trypanosoma cruzi*: insights into naphthoquinone effects on growth and proteinase activity. Exp. Parasitol. 127 (1), 160–166.

Cysne-Finkelstein, L., Temporal, R.M., Alves, F.A., Leon, L.L., 1998. *Leishmania amazonensis*: long-term cultivation of axenic amastigotes is associated to metacyclogenesis of promastigotes. Exp. Parasitol. 9, 58–62.

Deps, P.D., Viana, M.C., Falqueto, A., Dietze, R., 2000. Comparative assessment of the efficacy and toxicity of N-methyl-glucamine and BP88 sodium stibogluconate in the treatment of localized cutaneous leishmaniasis. Rev. Soc. Bras. Med. Trop. 3, 535–543.

Ferreira, V.F., Jorqueira, A., Souza, A.M., da Silva, M.N., de Souza, M.C., Gouvêa, R.M., et al., 2006. Trypanocidal agents with low cytotoxicity to mammalian cell line: a comparison of the theoretical and biological features of lapachone derivatives. Bioorg. Med. Chem. 14, 5459–5466.

Hotez, P.J., Molyneux, D.H., Fenwick, A., Kumaresan, J., Sachs, S.E., Sachs, J.D., et al., 2007. Control of neglected tropical diseases. N. Engl. J. Med. 357, 1018–1127.

Jha, T.K., 2006. Drug unresponsiveness & combination therapy for kala-azar. Indian J. Med. Res. 123, 389–398.

Jorqueira, A., Gouvêa, R.M., Ferreira, V.F., Silva, M.N., Souza, M.C.B.V., Zuma, A.A., et al., 2006. Oxyrane derivative of α-lapachone is potent growth inhibitor of *Trypanosoma cruzi* epimastigote forms. Parasitol. Res. 99, 429–433.

Lezama-Dávila, C.M., Isaac-Márquez, A.P., Kapadia, G., Owens, K., Oghumu, S., Beverley, S., et al., 2012. Leishmanicidal activity of two naphthoquinones against Leishmania donovani. Biol. Pharm. Bull. 35 (10), 1761–1764.

Lima, N.M.F., Correia, C.S., Leon, L.L., Machado, G.M.C., Madeira, M.F., Santana, A.E.G., et al., 2004. Antileishmanial activity of lapachol analogues. Mem. Inst. Oswaldo Cruz 99, 757–761.

Pinto, A.V., Coutada, L.C., Ferreira, V.F., 1980. Síntese de análogos de xiloidonas e lapachonas. Anais da Academia Brasileira de Ciências 52, 478–483.

Saldanha, A.C., Romero, G.A., Merchan-Hamann, E., Magalhães, A.V., Macedo, V.O., 1999. A comparative study between sodium stibogluconate BP 88R and meglumine antimoniate in the treatment of cutaneous leishmaniasis. I. The efficacy and safety. Rev. Soc. Bras. Med. Trop. 32, 383–387.

Santos, D.O., Coutinho, C.E., Madeira, M.F., Bottino, C.G., Vieira, R.T., Nascimento, S.B., et al., 2008. Leishmaniasis treatment – a challenge that remains: a review. Parasitol. Res. 103, 1–10.

Sundar, S., Singh, A., Singh, O.P., 2014. Strategies to overcome antileishmanial drugs unresponsiveness. J. Trop. Med. 4, 646932.

Taddei, M., 1999. Synthesis of oligopeptides containing an oxirane ring in the place of a peptidic bond. Methods Mol. Med. 23, 189–207.