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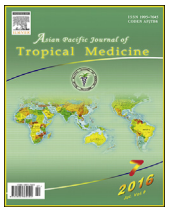

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Evaluation of hypoxia inducible factor targeting pharmacological drugs as antileishmanial agents

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ABSTRACT

Objective: To evaluate whether hypoxia inducible factor (HIF-1α) targeting pharmacological drugs, echinomycin, resveratrol and $CdCl_2$ which inhibit HIF-1α stimulation, and mimosine, which enhances the stability of HIF-1α present antileishmanial properties.
Methods: The leishmanicidal effect of drugs was evaluated in mouse macrophages and Balb/c mouse model for cutaneous leishmaniosis.
Results: Resveratrol and $CdCl_2$ reduced the parasite load [IC_{50}, (27.3 ± 2.25) μM and (24.8 ± 0.95) μM, respectively]. The IC_{50} value of echinomycin was (22.7 ± 7.36) nM and mimosine did not alter the parasite load in primary macrophages. The macrophage viability IC_{50} values for resveratrol, echinomycin and $CdCl_2$ and mimosine were >40 μM, >100 nM, >200 μM and>2000 μM, respectively. *In vivo* no differences between cutaneous lesions from control, resveratrol- and echinomycin-treated Balb/c mice were detected.
Conclusions: Resveratrol, echinomycin and $CdCl_2$ reduce parasite survival *in vitro*. The HIF-1α targeting pharmacological drugs require further study to more fully determine their anti-*Leishmania* potential and their role in therapeutic strategies.

1. Introduction

Leishmanioses are diseases caused by intracellular *Leishmania* parasites of macrophages [1] and they are endemic in more than 90 countries [2] *Leishmania amazonensis* (*L. amazonensis)* is transmitted mainly in the Amazon region and causes localized and diffuse cutaneous lesions and mucosal infection [3]. Leishmanioses are neglected diseases, there is no vaccine, current therapies fail to eradicate parasites from infected tissues and present side effects, while resistance to classical chemotherapy has become a clinical threat [2,4].

Recently our group and others have shown that mice with cutaneous leishmaniosis present hypoxic areas in damaged and infected tissues [5–7] and that *Leishmania*-infected macrophages from lesions and infected macrophage cultures accumulate hypoxia inducible factor (HIF-1α) [8–11]. HIF is a heterodimeric transcription factor consisting of HIF-1α and HIF-1β [12]. Under normoxia, HIF-1α is hydroxylated on proline residues and degraded by the ubiquitin proteasome pathway while under hypoxia, hydroxylation is inhibited and heterodimerization, nuclear translocation and transcription of HIF-dependent genes such as erythropoietin, vascular endothelial growth factor and transferrin occur [12–14]. HIF-1α overexpression is observed in a wide array of tumor cells that reprogram the metabolism for the induction of glycolytic enzymes [14]. Thus HIF-1α is originally identified as a master regulator of the adaptive response to diminished oxygen supply and accumulates in ischemic tissues and various types of cancer and their metastases; HIF-1α overexpression may trigger cell invasion and is associated with treatment failure [15,16]. The current understanding that HIF-1α can be expressed during infection with bacteria, such as *Chlamydia* [17] viruses, such as Epstein Barr [18] and protozoa, such as *Leishmania* and *Theileria* [8,9,19] via oxygen-dependent and oxygen-independent pathways reveals its additional role as a transcriptional regulator of inflammation and infection [20].

Experimental therapeutics involving the pharmacological modulation of HIF-1α has became a promising novel strategy;

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small-molecule inhibitors of the HIF-1α pathway identified through cell-based screening [15,16,21] and tests for various carcinogenesis and ischemic disease models have been reported in recent years [22–26].

Since sustained efforts are required to enrich new anti-leishmanial drug discovery, we aimed to evaluate whether echinomycin, a compound that inhibits the DNA binding activity of HIF-1α [27], resveratrol which inhibits HIF-1α though multiples mechanisms, including HIF protein degradation via the proteasome pathway [28] cadmium ($CdCl_2$), which is a heavy metal that triggers proteasome-dependent degradation of HIF-1α [29], and mimosine, a hydroxylase inhibitor agonist that stabilizes HIF-1α [30] present antileishmanial properties.

2. Material and methods

2.1. Reagents

Echinomycin, $C_{51}H_{64}N_{12}O_{12}S_2$, was purchased from Alexis Biochemicals (San Diego, CA, USA), L-mimosine, $C_8H_{10}N_2O_4$, was purchased from Enzo Life Sciences (Lausen, Switzerland), resveratrol, $C_{14}H_{12}O_3$, and cadmium chloride, $CdCl_2$, were purchased from Sigma–Aldrich (St. Louis, MO, USA), and meglumine antimoniate (glucantime) was purchased from Sanofi-Aventis (São Paulo, Brazil). Each of these compounds was dissolved in phosphate-buffered saline (PBS) or RPMI medium, resveratrol was dissolved in RPMI medium using small amounts (<0.01%) of dimethyl sulfoxide (DMSO) as required. Unless otherwise stated, all other reagents were obtained from Sigma–Aldrich.

2.2. Cell culture and parasites

Peritoneal mouse macrophages were obtained from normal BALB/c mice by peritoneal lavage, as previously described [31]. The cells were cultured in RPMI medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (Cultilab, Campinas, SP, Brazil) at 37 °C in 5% CO_2, 5% O_2 and balanced N_2. *L. amazonensis* (MHOM/BR/73/M2269) amastigotes were isolated from active skin lesions of BALB/c mice [32].

2.3. Assessment of the effect of drugs on L. amazonensis infected macrophages

Macrophages (5×10^5 cells/well) cultured in 24-well cell culture plates containing 13 mm diameter glass coverslips were exposed to *L. amazonensis* at a parasite/macrophage ratio of 3:1 for 2 h. Following exposure, the cultures were washed to remove extracellular parasites and then incubated in the presence of the drugs for 48 h. To evaluate the parasite load (number of amastigotes per macrophage), cells on coverslips were stained with Giemsa. The intracellular amastigotes, which are located exclusively in parasitophorous vacuoles, and 200 cells were examined microscopically at 1 000 magnification [31]. All tests were performed in triplicate. The reduction in parasite load induced by the compounds was calculated as a percentage of the control (assuming 100% parasite load of untreated macrophages). The IC_{50} describes the drug concentration that inhibits 50% of parasite load and was calculated using a curve fitting program (GraphPad Prism 6 software). Cellular viability was assessed by counting the adherent cells in 20 random fields of infected and uninfected macrophage cultures [33]. The IC_{50} describes the drug concentration that inhibits 50% macrophage viability and, was calculated using a curve fitting program (GraphPad Prism 6 software).

2.4. Assessment of the effect of drugs on L. amazonensis infected mice

The Ethics Committee for Animal Research of the Institute of Biology of the State University of Campinas approved the experimental protocols. Six-week-old female BALB/c mice were subcutaneously inoculated in the right hind footpad with 10^5 amastigotes. For each group of mice, 3 per group were administered the same vehicles (PBS and DMSO) without the compounds, resveratrol 15 mg/kg/day, echinomycin 0.13 mg/kg/day or glucantime 100 mg/kg/day [33–36] injected intraperitoneally for 20 d, 26 d after parasite inoculation. The course of infection was monitored by measuring the increase in footpad thickness with a dial caliper, compared with the contra lateral uninfected footpad [33]. This study was approved by the Ethics Committee of Universidade Estadual de Campinas (process numbers: 1742-1 and 2715-1).

2.5. Immunoblot analyses

The macrophages were scraped from the culture flasks and rinsed twice with PBS. Lysis buffer (62.5 mM Tris–HCl, pH 6.8, 69 mM SDS, 10% glycerol, 2% 2-mercaptoethanol, 34 mM ethylenediaminetetraacetic acid, 2 μg/mL pepstatin and 1 mM phenylmethylsulfonyl fluoride) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added to the cell pellets. Proteins were denatured at 95 °C for 3 min, electrophoresed on a 10% SDS-PAGE (poly-acrylamide) gel system (Thermo EC, USA) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). After blotting, membranes were probed with rabbit polyclonal anti-HIF-1α antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Sigma–Aldrich) and secondary antibody peroxidase-conjugated goat anti-rabbit IgG (Amersham, Poole, UK and Sigma–Aldrich); development was performed with 3,3-diaminobenzidine. Immunoreaction images were scanned and the densitometric value of each band was determined using Image Master Total Lab version 1 software (Amersham Pharmacia Biotech).

2.6. Immunofluorescence analyses

Cells attached to the slide-chambers were fixed for 10 min with 4% paraformaldehyde and washed 3 times in PBS. The cells were permeabilized with 1% Tween 20 and then washed twice in PBS. Nonspecific binding sites were blocked with 3% BSA (Amresco, Solon, OH, USA) for 30 min. The macrophages were then incubated with mouse anti-*L. amazonensis* serum or anti-HIF-1α antibody (Santa Cruz Biotechnology) overnight at 4 °C in a wet room. The cells were washed 4 times in PBS + 0.1% Tween 20 and incubated with FITC-conjugated goat anti-mouse secondary antibody or FITC-conjugated goat anti-rabbit secondary antibody for 1 h in a wet room at room temperature. The cells were washed four times in PBS + 0.1%

Tween 20 and mounted with DAPI-containing DABCO mounting media. The cells were visualized under a Nikon Eclipse 50*i* fluorescence microscope (Nikon, Melville, NY, USA). All images were captured and analyzed with a digital camera (Nikon DXM1200-F) and imaging software (ACT-1, Nikon).

2.7. Statistical analyses

All the experiments were repeated at least three times for *in vitro* assays and twice for *in vivo* assays. Statistical significance between the control and experimental groups were determined by the Student *t* test and the resulting data are expressed as the mean ± SD.

3. Results

3.1. Expression of HIF-1α in Leishmania-infected macrophages

Leishmania is an intracellular parasite that interferes with HIF-1α expression *in vitro and in vivo* [9]. The immunofluorescence analyses confirmed that under the chosen experimental conditions intracellular amastigotes were established inside macrophages with (8.9 ± 3.2) parasites per infected cell (Figure 1A–B). The intensity and pattern of HIF-1α immunostaining were similar between macrophage cultures in normoxia (21% O_2) and hypoxia (2% O_2) (Figure 1C–D), confirming that *Leishmania* activates HIF-1α in macrophages [8,9]. HIF-1α was expressed in *L. amazonensis* infected macrophages, as shown in the western blots, and was reduced following treatment with resveratrol, echinomycin and $CdCl_2$ (Figure 1E).

3.2. Effect of HIF-1α targeting pharmacological drugs on viability of macrophages

The dose range of the pharmacological drugs used in the antileishmanial assays were chosen based on macrophage viability data for each compound; the IC_{50} values obtained for resveratrol, echinomycin, $CdCl_2$, and mimosine were >40 μM, >100 nM, >200 μM, and>2000 μM, respectively (Table 1); these results corroborate previous findings [9,34,37–39].

3.3. Effect of HIF-1α targeting pharmacological drugs on Leishmania within macrophages

Next, the compounds were tested in antileishmanial assays. The IC_{50} values of each drug are listed in Table 1. Resveratrol, $CdCl_2$ and echinomycin reduced the parasite load after 48 h of treatment [IC_{50} (27.30 ± 2.25) μM, (24.80 ± 0.95) μM and (22.70 ± 7.36) nM, respectively]. Mimosine did not inhibit significantly the parasite load in *L. amazonensis* infected macrophages under the conditions tested (IC_{50} > 80 μM) (Table 1). Similar results were obtained

Table 1
In vitro antileishmanial and cytotoxicity of HIF-1α targeting drugs.

Drugs	IC_{50}	
	Intracellular amastigotes[a]	Macrophages[b]
Resveratrol	27.3 ± 2.25 μM	>40 μM
$CdCl_2$	24.8 ± 0.95 μM	>200 μM
Echinomycin	22.7 ± 7.36 nM	>100 nM
Mimosine	>80 μM	>2000 μM

[a] Drug concentration that inhibit 50% of the parasite load (number of amastigotes per macrophage) at 48 h incubation time. [b] Drug concentration that inhibit 50% of the macrophage viability at 48 h incubation time.

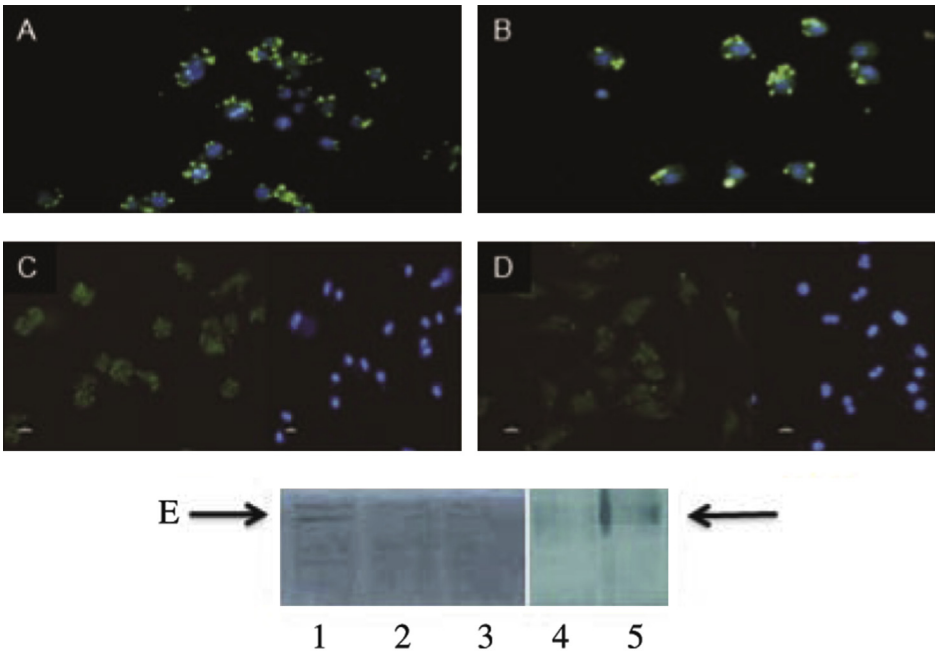

Figure 1. HIF-1α expression in *L. amazonensis* infected macrophages.
Fluorescent images of infected macrophages under normoxia labeled with anti-*L. amazonensis* serum (green) and nuclei labeled with DAPI (blue) (A); infected macrophages under hypoxia labeled with anti-*L. amazonensis* serum (green) and nuclei labeled with DAPI (blue) (B); or labeled with anti-HIF-1α antibody (DAPI image, right side) (C); or labeled with anti-HIF-1α antibody (DAPI image, right side) (D). Western blots (E) of extracts from infected macrophages nontreated (1) or treated with resveratrol 50 μM; (2) echinomycin 10 nM; (3) $CdCl_2$ 25 μM; (4) or mimosine 50 mM for 24 h.

using macrophage cell lines and primary human macrophages; the exception was *L. amazonensis* infected J774 macrophage cultures, which reduced the parasite load after mimosine treatment: IC_{50} (56.00 ± 1.51) μM. The IC_{50} value of a reference anti-*Leishmania* drug, amphotericin B was (0.040 ± 0.002) μM but a complete reduction of infection was not obtained [33].

3.4. Effect of resveratrol and echinomycin on *Leishmania*-infected mice

Since resveratrol and echinomycin have been tested in various animal models of cancer and other diseases [22,27,28,34], both compounds were evaluated in mice. Observation of individual footpad sizes of *L. amazonensis* infected Balb/c mice over time detected no significant differences between PBS/DMSO, resveratrol and echinomycin treatments; lesion in individual mouse progressively increased in size (Figure 2). In this experiment, three of the five (60%) echinomycin-treated mice died prematurely. No mortality was observed in PBS/DMSO- and resveratrol-treated mice; similar results were obtained in another independent experiment. Glucantime, which is used in the clinical treatment of leishmaniosis, prevented lesions (Figure 2) and ulcerations developed slowly, although a complete cure was not obtained.

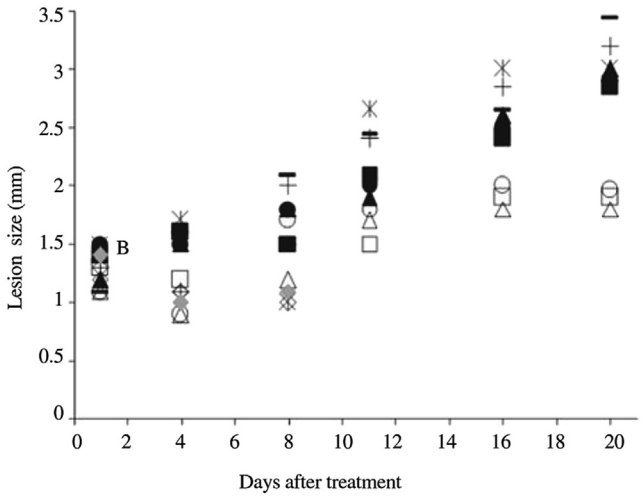

Figure 2. Effects of HIF-1α target drugs on *L. amazonensis* infection. Balb/c mice (3 per group) were treated intraperitoneally with PBS/DMSO (■, ▲, ●), glucantime (100 mg/kg/day) (□, △, ○), resveratrol (15 mg/kg/day) (*, –, +) or echinomycin 0.13 mg/kg/day (rhombuses) for 20 d, 26 d after parasite inoculation with 10^5 amastigotes in the footpad. Lesion size is expressed as the difference in size between the infected and the contra lateral uninfected footpads.

4. Discussion

The modulation of HIF-1α has been an interesting chemotherapy approach for many diseases and drugs that achieve this could be repositioned as infectious disease therapeutics [20]. Recently we showed that HIF-1α is activated during *L. amazonensis* infection [5,7–9]. The picture emerging from these studies is that HIF-1α induction and the target genes constitute part of an adaptation mechanism resulting from *Leishmania* infection and that this could permit the macrophage to attenuate damage, maintain integrity and survive the infection [9], since HIF-1α transcriptional regulation can support microbicidal and inflammatory phenotypes [20,24]. Reports on the anti-microorganism properties of HIF-1α modulating drugs are scarce.

The addition of $CdCl_2$ to *L. amazonensis* infected macrophage cultures significantly reduced parasite survival, confirming our previous data [9]. Cadmium is a heavy metal that triggers proteasome-dependent degradation of HIF-1α protein, depressing its activity as a hypoxia mimic; $CdCl_2$ was used only as an *in vitro* control since it is classified as a human carcinogen [40].

Our results also indicated that resveratrol reduced parasite survival in macrophage cultures. In fact, resveratrol anti-*Leishmania in vitro* activity has also been reported for *L. major* [41] and the authors speculated that resveratrol could exert antiproliferative activities in promastigotes and intracellular amastigotes through the inhibition of tubulin polymerisation. Since resveratrol downregulate HIF-1α expression [22] and it is a transcription factor that can reprogram the cell metabolism [14], we suggest that the antileishmanial effect of resveratrol could be linked to negative modulation of HIF-1α in host macrophages. Our data showed that resveratrol did not have a significant effect on the reduction of Balb/c mice lesion, even though it is nontoxic in rodents [42]. The animal model of infection chosen was Balb/c mice, the most used *in vivo* model for experimental studies because infection with cutaneous *Leishmania* results in very aggressive and non-healing cutaneous lesions [43]. Future investigation of resveratrol treatment in other mouse strains and different *Leishmania* species would assist in a fuller understanding of how this drug effects the progression of different leishmanioses.

Echinomycin is a cyclic depsipeptide antibiotic which has been described as inhibiting HIF-1α DNA binding and transcription activity [27,28]. It is reported to have antitumor and antibacterial activity, despite a low toxicity in *Trypanosoma brucei* and *T. rhodesiense* [44] and showed no *in vivo* antileishmanial effect (our data). In fact, administration of echinomycin induced lethality in Balb/c mice, even though previous toxicological evaluation performed in mice and dogs indicated that the clinical signs induced by the drug, such as hypoactivity, ataxia and weight loss, were reversed [34]. The reasons for this discrepancy are unclear but it is possible that *L. amazonensis* infected mice are more sensitive to the drug than uninfected mice.

Mimosine is an alkaloid that stabilizes HIF-1α and boosts the capacity of human neutrophils to kill *Staphylococcus aureus* [30]. Our data shown that mimosine did not have a significant effect on the parasite load in *L. amazonensis* infected primary macrophages, with the exception of the *L. amazonensis* infected J774 macrophage cell line. As previously shown, the compound did not inhibit the growth of *L. donovani* promastigotes, but had effect on amastigotes within a macrophage cell line [45]. The authors suggested that the anti-*Leishmania* activity of mimosine is related to its inhibitory activity in deoxyhypusine hydroxylase, an enzyme involved in cell proliferation [45]. The differences in sensitivity between *Leishmania* species and macrophage origins to mimosine and its mechanism of action could be explored for drug development.

In conclusion, analysis of our results suggests that HIF-1α modulation require further study to more fully determine the anti-*Leishmania* potential of HIF-1α modulator compounds and their role in therapeutic strategies.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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