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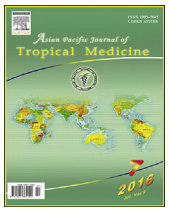

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Evaluation of hypoxia inducible factor targeting pharmacological drugs as antileishmanial agents

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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%