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Evaluation of a diospyrin derivative as antileishmanial agent and potential modulator of ornithine decarboxylase of *Leishmania donovani*

Sudipta Hazra [a], Subhalakshmi Ghosh [a], Madhushree Das Sarma [a,1], Smriti Sharma [b], Mousumi Das [c], Prakash Saudagar [c], Vijay Kumar Prajapati [b,2], Vikash Kumar Dubey [c], Shyam Sundar [b], Banasri Hazra [a,*]

[a] Department of Pharmaceutical Technology, Jadavpur University, Kolkata 700 032, India
[b] Department of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India
[c] Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati 781039, India

HIGHLIGHTS

- MTT assay of twenty derivatives of diospyrin on *Leishmania donovani* promastigotes.
- **D17**, a di-epoxide derivative with IC_{50} = 7.2 μM, was selected for this study.
- IC_{50} of **D17** was 0.18 μM in intracellular amastigotes of *L. donovani* clinical strain.
- 2 mg/kg of **D17** (i.p.) reduced 38% of parasite load in liver of infected BALB/c mice.
- ODC enzyme was identified as a probable antileishmanial target of **D17**.

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ABSTRACT

World health organization has called for academic research and development of new chemotherapeutic strategies to overcome the emerging resistance and side effects exhibited by the drugs currently used against leishmaniasis. Diospyrin, a bis-naphthoquinone isolated from *Diospyros montana* Roxb., and its semi-synthetic derivatives, were reported for inhibitory activity against protozoan parasites including *Leishmania*. Presently, we have investigated the antileishmanial effect of a di-epoxide derivative of diospyrin (**D17**), both *in vitro* and *in vivo*. Further, the safety profile of **D17** was established by testing its toxicity against normal macrophage cells ($IC_{50} \sim 20.7$ μM), and also against normal BALB/c mice *in vivo*. The compound showed enhanced activity ($IC_{50} \sim 7.2$ μM) as compared to diospyrin ($IC_{50} \sim 12.6$ μM) against *Leishmania donovani* promastigotes. Again, **D17** was tested on *L. donovani* BHU1216 isolated from a sodium stibogluconate-unresponsive patient, and exhibited selective inhibition of the intracellular amastigotes ($IC_{50} \sim 0.18$ μM). Also, treatment of infected BALB/c mice with **D17** at 2 mg/kg/day reduced the hepatic parasite load by about 38%. Subsequently, computational docking studies were undertaken on selected enzymes of trypanothione metabolism, viz. trypanothione reductase (TryR) and ornithine decarboxylase (ODC), followed by the enzyme kinetics, where **D17** demonstrated non-competitive inhibition of the *L. donovani* ODC, but could not inhibit TryR.

1. Introduction

Leishmaniasis is caused by approximately 20 kinetoplastid species from the genus *Leishmania*. It is one of the neglected infectious diseases among underprivileged communities in endemic regions of 98 tropical countries in Africa, Latin America, and Asia, and as opportunistic ailment affecting immuno-compromised people in non-endemic areas also (WHO, 2012). The disease is manifested in three major clinical forms: cutaneous (CL), mucocutaneous, and visceral leishmaniasis (VL); VL, known as Kala-azar in India, is the most severe, with symptoms of fever, hepatosplenomegaly, and anemia, leading to death if left untreated.

Pentavalent antimony (SbV) compounds, discovered nearly hundred years back, continue to be the first line of treatment against leishmaniasis. However, the disadvantages of toxic side effects, and long hospitalization required for parenteral administration, in addition to the emergence of drug resistance are challenging the clinical importance of antimony therapy (Chakravarty and Sundar, 2010). The second line of treatment, with antifungals

* Corresponding author.
E-mail addresses: banasrihazra@yahoo.co.in, hazra1@vsnl.com (B. Hazra).
[1] Present address: Department of Chemistry, A.P.C. College, New Barrackpore 700131, India.
[2] Present address: Department of Biochemistry, Central University of Rajasthan, Ajmer 305801, Rajasthan, India.

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like amphotericin B and pentamidine, also suffers from acute toxicity. Liposomal amphotericin B (AmBisome) was introduced against leishmaniasis in the USA and Europe (Meyerhoff, 1999), but this formulation could not be afforded by the majority of the VL – affected population in the world. Even the lately introduced oral formulation of miltefosine has revealed a doubling of the relapse rate, and imminent drug resistance against VL in India (Sundar et al., 2012). A clinical trial was conducted in East Africa on another experimental drug, viz. paromomycin, alone or in combination with sodium stibogluconate (SSG), revealing the importance of geographical differences to treatment responses (Hailu et al., 2010). Recently, Sundar et al. reported a randomised controlled trial involving different combinations of liposomal amphotericin B/miltefosine/paromomycin to tackle the public health problem of VL in India (Sundar et al., 2011). Thus, the on-going efforts should continue towards developing alternative strategies in order to overcome the disadvantages of the classical as well as the newly introduced therapies against leishmaniasis. One of the practical approaches to discover novel drugs is to explore structurally diverse chemical constituents of natural products, especially plants of medicinal importance, to find more potent and less toxic 'lead compounds' with better therapeutic prospect.

Leishmania donovani promastigotes, the causative organism of VL, could be significantly inhibited by diospyrin, a bisnaphthoquinonoid compound isolated from *Diospyros montana* Roxb. in our laboratory (Hazra et al., 1987). Consequently, structural modification of diospyrin was undertaken to enhance its effect against *L. donovani* as well as *Leishmania major* parasites, the latter being responsible for causing CL (Yardley et al. 1996; Hazra et al., 2002). Subsequently, it was found that diospyrin and its derivative could induce apoptosis-like death in *L. donovani* promastigotes through depolarization of mitochondrial membrane potential (Mukherjee et al., 2009). Further, studies on mechanism of antileishmanial activity showed diospyrin to be a specific inhibitor of type I DNA topoisomerase, an imperative therapeutic target for rational design of antiprotozoal drugs (Ray et al., 1998). Therefore, it was envisaged to introduce novel functional groups of biological significance into this 'lead molecule' in order to optimise the potency of the prospective plant – derived antileishmanial agent.

Presently, we have carried out a comparative evaluation of disopyrin vis-à-vis some of its selected derivatives against *L. donovani* promastigotes. The most potent compound was then tested for its efficacy against intracellular amastigotes *in vitro*, followed by *in vivo* assessment using BALB/c mice infected with a clinical strain of *L. donovani* isolated from SSG-unresponsive patient. Further, a molecular docking study on the inhibitory effect was undertaken against two components of antioxidant defense system unique to the Trypanosomatidae parasites, viz. trypanothione reductase (TryR) and ornithine decarboxylase (ODC). The results obtained from the computational docking *in silico* were validated through kinetic studies on the effect of diospyrin and its derivatives on the target enzymes, viz. TryR and ODC. This is the first study undertaken on diospyrin and its semi-synthetic derivatives, particularly the di-epoxide analogue, for their response to these enzyme targets for Leishmania.

2. Materials and methods

2.1. Compounds

Diospyrin (**D1**) was isolated from *D. montana* Roxb. bark, and converted into its alkylether, acetyl, epoxide, amino- and amino-glycoside derivatives by following methods previously standardised in our laboratory (Das Sarma et al., 2007a; 2007b; 2008). Solutions (10 mM stocks) were prepared aseptically by dissolving the compounds in dimethyl sulfoxide (DMSO) and diluted with the culture medium to get the desired concentrations. The final concentration of DMSO in the culture was adjusted to 0.1% (v/v).

2.2. Parasite culture and maintenance

L. donovani promastigotes (strain MHOM/IN/1983/AG83) were obtained from the Indian Institute of Chemical Biology, Kolkata, India. Promastigotes were cultured in Schneider's insect medium (Sigma–Aldrich Co., St. Louis, USA), supplemented with streptomycin (150 μg/ml), penicillin G (100 μg/ml), gentamicin (150 μg/ml) and 10% heat-inactivated fetal bovine serum (FBS; HyClone, USA) at pH 7.2, and kept under aseptic conditions in a BOD chamber at 22 ± 2 °C (Hazra et al., 2012). Another strain of *L. donovani* which was isolated from antimony unresponsive VL patient was included in this study. This clinical strain was cultured and characterized as *L. donovani* (BHU1216) using Hsp70 gene amplification as described elsewhere (Srivastava et al., 2010). BHU1216 was also maintained *in vivo* through serial intra-cardiac passage in BALB/c mice to maintain the virulence of the strain. Written informed consent was obtained from the patient for collection of human clinical sample according to the guidelines of Ethical Committee of the Institute of Medical Sciences, Banaras Hindu University, Varanasi.

2.3. Animals

Inbred female BALB/c mice of 5–6 weeks old, weighing 20–22 g, were obtained from the Indian Institute of Chemical Biology, Kolkata, India. They were fed with water and mouse feed *ad libitum*. Animal experiments were conducted in compliance with the Institutional Animal Ethics Committee, Jadavpur University, Kolkata.

2.4. In vitro antileishmanial activity against L. donovani AG83 promastigotes

Antiparasitic activity of diospyrin and analogues against *L. donovani* (AG83) promastigotes was determined by a quantitative colourimetric assay using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sisco Research Laboratory, India]. Amphotericin B (Sigma–Aldrich Co., USA) was used as the positive control. Promastigotes (5×10^5 cells/ml; 300 μl) were treated with andwithout tested drugs at a concentration range of 0.1–25 μM, and incubated at 22 ± 2 °C. After 72 h, cells were harvested and re-suspended in PBS (500 μl) containing MTT (0.3 mg/ml). Purple formazan crystals were dissolved in DMSO and the optical density (OD) was measured at 570 nm in an ELISA reader (BIO-RAD; model 680, USA). The number of viable cells was directly proportional to the amount of formazan produced through the reduction of yellow MTT by the dehydrogenase enzymes present in the inner mitochondrial membrane of the living cells (Mosmann, 1983). The IC_{50} values (concentration of drug which inhibited at least 50% cell growth) for each compound were determined from respective dose-response curves. Each assay was performed in triplicate, and the results were expressed as the mean of three independent experiments.

2.5. Activity against L. donovani BHU1216 intracellular amastigotes

J774A.1 macrophages (200 μl; 2.5×10^5 cells/ml) were suspended in RPMI-1640 medium and seeded in 8-well Lab-Tek tissue culture slides (NUNC, USA) for 2 h at 37 °C in humidified 5% CO_2 atmosphere in a CO_2 incubator (Sanyo CO_2-Incubator, USA).