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The lignan glycosides lyoniside and saracoside poison the unusual type IB topoisomerase of *Leishmania donovani* and kill the parasite both in vitro and in vivo

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ABSTRACT

Lignans are diphenyl propanoids with vast range of biological activities. The present study provides an important insight into the anti-leishmanial activities of two lignan glycosides, viz. lyoniside and saracoside. These compounds inhibit catalytic activities of topoisomerase IB (LdTopIB) of *Leishmania donovani* in non-competitive manner and stabilize the LdTopIB mediated cleavage complex formation both *in vitro* and in *Leishmania* promastigotes and subsequently inhibit the religation of cleaved strand. These two compounds not only poison LdTopIB but also can interact with the free enzyme LdTopIB. We have also shown that lyoniside and saracoside are cytotoxic to promastigotes and intracellular amastigotes. The protein–DNA complex formation leads to double strand breaks in DNA which ultimately triggers apoptosis-like cell death in the parasite. Along with their cytotoxicity towards sodium antimony gluconate (SAG) sensitive AG83 strain, their ability to kill SAG resistant GE1 strain makes these two compounds potential anti-leishmanial candidates. Not only they effectively kill *L. donovani* amastigotes inside macrophages *in vitro*, lyoniside and saracoside demonstrated strong anti-leishmanial efficacies in BALB/c mice model of leishmaniasis. Treatment with these lignan glycosides produce nitric oxide and reactive oxygen species which result in almost complete clearance of the liver and splenic parasite burden. These compounds do not inhibit human topoisomerase IB upto 200 μM concentrations and had poor cytotoxic effect on uninfected cultured murine peritoneal macrophages upto 100 μM concentrations. Taken together it can be concluded that these compounds can be developed into excellent therapeutic agent against deadly disease leishmaniasis.

1. Introduction

Kala-azar or visceral forms of leishmaniasis (VL) is one of the most fatal neglected tropical diseases (NTDs) caused by protozoan parasite *Leishmania donovani* [1]. This systemic disease presents a spectrum of clinical manifestations such as fever, weight loss, splenomegaly, hepatomegaly, lymph node enlargement, anemia, etc. [2,3]. In the global scenario, 90% of total VL cases occur in Bangladesh, Brazil, India, Nepal and Sudan [4]. Annually 100,000 cases of VL occur in India and 90% of the cases are restricted in the state of Bihar [5,6]. Until today, there is no effective vaccine against any form of human leishmaniasis [7]. Classical pentavalent antimonials such as sodium antimony gluconate (SAG) or alternative medicines like amphotericin B, miltefosine, paramomycin remained the drugs of choice though their use is compromised by their toxicity, availability, expensiveness and severe side effects [8,9]. The situation has become more complicated by emergence of parasite strains that have developed

Abbreviations: LdTOP1L, *L. donovani* topoisomerase I large subunit; LdTOP1S, *L. donovani* topoisomerase I small subunit; LdTopIB, *L. donovani* topoisomerase IB; hTopIB, human topoisomerase IB; SAG, sodium antimony gluconate; DMSO, dimethyl sulphoxide; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactopyranoside; Ni-NTA, Ni^{2+}-nitriloacetate-agarose; P11 cellulose, phosphocellulose; EtBr, ethidium bromide; M199, medium199; FBS, fetal bovine serum; K_D, dissociation constant; V_{max}, maximal velocity.

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resistance to classical antimonial drug like SAG [10]. Co-infections with HIV have made the scenario even worse [11]. Given the situations, improved chemotherapeutic agent against leishmanial infections and novel molecular targets on which to base the future treatment strategy are absolute necessities. DNA topoisomerases of *Leishmania* offer the most attractive targets for such a strategy [12].

DNA topoisomerases are ubiquitous enzymes that play a pivotal role in modulating the dynamic nature of DNA secondary and higher order structures and thus carrying out essential functions inside the cell [13,14]. Based on the number of strands they cleave, topoisomerases are classified as type I or type II and both type of enzymes are important drug targets [15,16]. Most eukaryotic type IB topoisomerases are single subunit proteins [17]. The distinctive bi-subunit topoisomerase IB of *L. donovani* (LdTopIB) in the kinetoplastid family has brought a new twist in topoisomerase research [18]. This unusual structure of LdTopIB makes it an important drug target and inhibitors for LdTopIB are of immense interest for antileishmanial therapy [12]. These topoisomerase inhibitors can be broadly classified into two classes: the class I inhibitors stabilize the formation of topoisomerase-DNA covalent complex (cleavable complex) and are coined as 'topoisomerase poisons'. Inhibitors with property to abrogate only the catalytic property of the enzyme and to interfere with the formation of covalent complex are termed as class II inhibitors (catalytic inhibitors) [19]. All of these kinds of topoisomerase inhibitions stall replication, induce cell death and thereby help preferential killing of highly replicative parasitic cells within the host [12].

Lignans are a family of natural products having two phenylpropane (C_6C_3) units in their chemical backbone. Lignans show wide range of biological activities such as cytotoxic, antiangiogenic, antiviral, antileishmanial, antifungal, hypolipidemic and antirheumatic [20]. The lignan related podophyllotoxins etoposide and teniposide are known for their anti-topoisomerase II and anticancer properties [21]. In our earlier study, we have reported isolation of a novel lignan glycoside saracoside along with a known lignan lyoniside from *Saraca indica* (Ashoka) and their potent LdTopIB inhibitory activities [22]. But no studies were done to find out their mode of action against LdTopIB and whether these compounds can be exploited as anti-leishmanial agents. Thus, further studies are needed to understand the molecular mechanism of inhibition of LdTopIB and their effect on parasite *L. donovani*.

The present study demonstrates for the first time that the lignan glycoside saracoside and lyoniside poison LdTopIB and exhibit potent anti-leishmanial activity towards promastigote and more deadly amastigote forms (in cultured mouse peritoneal macrophages) of *L. donovani*. Both these compounds are cytotoxic not only to antimony sensitive *L. donovani* strain AG83 but also antimony resistant GE1 strain. Our study confirms that these lignan glycosides stabilize the LdTopIB mediated cleavage complex formation both *in vitro* and in parasitic cells. These compounds also can interact reversibly with the free enzyme (LdTopIB). As lyoniside and saracoside stabilize *in vivo* topoisomerase mediated protein-DNA complex, double strand breaks in DNA are formed which ultimately triggers apoptosis-like cell death in parasitic cells and lead to genomic DNA fragmentation. Not only they effectively kill *L. donovani* amastigotes inside macrophages *in vitro*, lyoniside and saracoside demonstrated strong anti-leishmanial efficacies in *in vivo* BALB/c mice model of leishmaniasis. Treatment with these lignan glycosides (intraperitoneal administration) produce nitric oxide and reactive oxygen species which results in almost complete clearance of the liver and splenic parasite burden in BALB/c mice. Our results show that the two lignans lyoniside and saracoside can be exploited as potential antileishmanial agents.

2. Materials and methods

2.1. Chemicals

MTT was purchased from Invitrogen Life Technologies (Carlsbad, California, USA). DMSO and Camptothecin were purchased from Sigma chemicals (St. Louis, MO, USA). All drugs were dissolved in 100% DMSO at a concentration of 20 mM and stored at −20 °C. Recombinant human topoisomerase I was purchased from Topogen Inc. (Florida, USA).

2.2. Isolation of lignans (lyoniside and saracoside) from Saraca indica

Lyoniside and Saracoside used in the study were isolated from stem bark of *S. indica* as described previously [22]. The air dried powdered stem bark of *S. indica* (3 kg) was subjected to percolation with light petroleum ether (60–120 °C) [RFCL Limited (RANKEM), India], ethyl acetate (Finar Limited, India) and methanol (Qualigens Fine Chemicals, India) respectively at room temperature. The methanol extract (32 g) obtained after evaporation of solvent *in vacuo* was chromatographed over silica gel (100–200 mesh) [Merck Millipore, Mumbai, India]. Gradient elution was carried out with chloroform (Finar Limited, India), followed by various mixtures of chloroform-methanol (2%, 5%, 10%, 12%, 15%, 20% and 30%). A total of 180 fractions (each 200 ml) were collected and those giving similar spots on TLC were combined. Fractions 120–140, eluted with 15% methanol in chloroform, were repeatedly chromatographed on a Sephadex LH20 column (Sigma chemicals, St. Louis, MO, USA) eluted with water and methanol (5%, 10%, 20% methanol in water), resulted in the isolation of saracoside (8 mg) and lyoniside (10 mg). The homogeneity of the compounds was determined by TLC, RPHPLC, ESIMS spectral studies. The structures of the lignans were established by detailed spectral analysis [^{1}H and ^{13}C NMR, 2D NMR (HMQC, HMBC, COSY, NOESY), ESIMS, CD] studies [22].

2.3. Bioethics

Balb/c mice, originally obtained from Jackson Laboratories, Bar Harbor, ME and reared in the Institute animal facilities, were used for experimental purposes with prior approval of the animal ethics committee. The studies and animal handling were approved by IICB Animal Ethical Committee (Registration no. 147/1999, CPCSEA), registered with Committee for the purpose of Control and Supervision on Experiments on Animals (CPCSEA), Govt. of India.

2.4. Purification and reconstitution of recombinant proteins of topoisomerase I activity

Escherichia coli BL21 (DE3) pLysS cells harbouring pET16bLdTOP1L and pET16bLdTOP1S, described previously [18], were separately induced at OD_{600} = 0.6 with 0.5 mM IPTG (isopropyl β-D-thiogalactoside) at 22 °C for 12 h. Cells harvested from 1 l of culture were separately lysed by lysozyme/sonication, and the proteins were purified through Ni-NTA (Ni^{2+}-nitriloacetate-agarose column (Qiagen, Hilden, Germany) followed by a phosphocellulose column (P11 cellulose; Whatman, Maidstone Kent, UK) as described previously [18]. Finally, the purified proteins LdTOP1L and LdTOP1S were stored at −70 °C. The concentrations of each protein were quantified by using Bradford Reagent (PIERCE, Thermo Fisher Scientific Inc., Rockford, USA).

Purified LdTOP1L was mixed with purified LdTOP1S at a molar ratio of 1:1 at a total protein concentration of 0.5 mg/ml in reconstitution buffer [50 mM potassium phosphate, pH 7.5, 0.5 mM DTT, 1 mM EDTA, 0.1 mM PMSF and 10% (v/v) glycerol].