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Novel β-carboline-quinazolinone hybrids disrupt *Leishmania donovani* redox homeostasis and show promising antileishmanial activity

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ABSTRACT

Visceral Leishmaniasis is a deadly parasitic disease caused by *Leishmania donovani*. Paucity exists in the discovery of novel chemotherapeutics against Leishmaniasis. In this study, we synthesized a natural product inspired Diversity Oriented Synthesis library of *L. donovani* Trypanothione reductase (LdTR) inhibitor β-carboline-quinazolinone hybrids, which are different in stereochemical architecture and diverse in the bioactive chemical space. It is noteworthy that chirality affects drug-to-protein binding affinity since proteins in any living system are present only in one of the chiral forms. Upon evaluation of the hybrids, one of the chiral forms i.e. Compound 1 showed profound cytotoxic effect in micromolar range as compared to its other chiral form i.e. Compound 2. *In-silico* docking studies confirmed high binding efficiency of Compound 1 with the catalytic pocket of LdTR. Treatment of *L. donovani* parasites with Compound 1 inhibits LdTR activity, induces imbalance in redox homeostasis by enhancing ROS, disrupts the mitochondrial membrane potential, modifies actin polymerization and alters the surface topology and architecture. All these cellular modifications eventually led to apoptosis-like death of promastigotes. Furthermore, we synthesized the analogues of Compound 1 and found that these compounds show profound antileishmanial activity in the nanomolar range both in promastigotes and intracellular amastigotes. The enhanced inhibitory potential of these compounds was further supported by *in-silico* analysis of protein-ligand interactions which revealed high binding efficiency towards the catalytic pocket of LdTR. Taken together, this study reports the serendipitous discovery of β-carboline-quinazolinone hybrids with enhanced antileishmanial activity along with the in-depth structure-activity relationships and mechanism of action of these analogues.

1. Introduction

Leishmaniasis is a neglected tropical disease (second largest parasitic infection), endemic in several parts of the tropics, subtropics, and southern Europe [1]. This disease is caused by protozoan parasites of genus *Leishmania* and transmitted through the bite of female phlebotomine sand-fly. Leishmaniasis is manifested in several different forms among which the deadly ones are cutaneous and visceral forms. Visceral Leishmaniasis (VL) caused by *Leishmania donovani* is also commonly known as Kala-azar. Almost 3 million people are within the risk zone of infection and ~200,000–400,000 new cases are reported each year [1]. The typical symptoms include fever, weight loss and enlargement of visceral organs such as spleen and liver. The life-cycle of *L. donovani* consists of two distinct forms: a promastigote flagellar form found in the gut of the sandfly, and an amastigote form which develops intracellularly in macrophages of human host [2].

For many years, treatment for VL relied on pentavalent antimonials using compounds like sodium stibogluconate and meglumine antimoniate but these antimonials were found to be toxic with life threatening adverse effects, including cardiac arrhythmia and acute pancreatitis. Later, antimonials were successfully replaced by Amphotericin B, which was then followed by emergence of miltefosine as the first oral drug therapy for VL [3]. But, roadblocks like slow release of drugs, adverse side effects and development of drug resistance persisted.

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Current preventive strategies for VL rely on deltamethrin-coated collars for disease control in reservoirs (canines and rodents) and usage of insecticides and impregnated materials for prevention of transmission through vectors [4,5]. However, research on transmission blocking drugs for visceral leishmaniasis is still under infancy.

For effective drug discovery and development, enzymes involved in cellular metabolism of VL are considered as one of the promising targets. Previous studies have identified Trypanothione reductase (TR) as the key enzyme responsible for the maintenance of cellular redox homeostasis in *Leishmania* [6–8]. This enzyme plays role in protecting the parasite from oxidative stress by catalyzing the NADPH-dependent reduction of trypanothione disulfide [TS2] to the dithiol-trypanothione [bis(glutathionyl)spermidine, T(SH)2]. This reaction is similar to the reduction of glutathione disulfide by glutathione reductase (GR) in mammals. Although *Leishmania donovani* Trypanothione reductase (LdTR) is found to be structurally and mechanistically similar to the mammalian GR, it specifically differs in its disulfide-binding site making. Precisely, absence of LdTR in the mammalian system and its vital role in the antioxidant defense of the parasite makes LdTR a lucrative target for rational drug designing [9,10].

For targeting LdTR, we designed a Diversity Oriented Synthesis (DOS) library, which is a forward, directional and unbiased approach for designing molecules. The rationale behind DOS is to efficiently synthesize a large number of molecules spanning diverse regions of the bioactive chemical space, which is an inherent property seen in natural products [11,12]. The resulting molecules are subjected to phenotypic screening to assess their relevant biological activity. Based on their 3-dimensional structure, stereogenicity, selectivity towards targets (DNA, RNA and proteins), and lack of toxicity, natural product-inspired DOS library scaffolds are found to be effective in generating compounds with better bioactivities. Recently, natural alkaloids like β-carboline and quinazolinone [13] are reported to possess LdTR inhibition activity.

Based on this information we designed advanced scaffolds of β-carboline and quinazolinone hybrids having diastereomeric properties. Upon evaluation of these hybrids, we found that out of two chiral forms (Compound 1 and 2), Compound 1 showed profound anti-leishmanial activity in micromolar range.

Through in-depth analysis involving LdTR inhibition assays, *in-silico* protein-ligand docking and *in-vitro* cellular responses, we determined the relationship between mechanism of action and chirality. Based on structure-to-activity relationships, we synthesized the analogues of the potent chiral form. These newly synthesized derivatives showed greater degree of antileishmanial activity in nanomolar range, both in promastigotes as well as intramacrophagic amastigotes. Overall, this study reports the discovery of novel β-carboline-quinazolinone hybrids showing enhanced antileishmanial activity. This study further sheds light on downstream mechanistic effects of selective inhibition of LdTR.

2. Materials and methods

2.1. Parasite culturing

L. donovani Bob strain was a kind gift from Dr. Amitabha Mukhopadhyay (National Institute of Immunology, India). This strain was cultured at 26 °C in M199 media (HIMEDIA, India) supplemented with 40 mM HEPES (Sigma-Aldrich), 2 mM glutamine (Life Technologies, USA), 0.02 mg/mL gentamycin (Life Technologies, USA). Promastigotes in its log phase were used throughout this study, unless specified. Constant parasite count (1×10^6 cells/mL) was maintained for all experiments.

2.2. Synthesis of Compound 1 analogues

2.2.1. General information for 8 compounds

All reagents, starting materials and solvents were obtained from commercially available sources and used without further purification unless otherwise specified. All reactions were performed using oven-dried glassware under nitrogen atmosphere. The progress of the reaction was monitored by thin-layer chromatography (TLC) and the TLC plates were visualized using UV illumination. All proton (^{1}H) and carbon 13 (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded using Bruker AVHDN 400 using $CDCl_3$ (Sigma-Aldrich) and DMSO-D_6 (Sigma-Aldrich) as solvents. The chemical shifts are reported in parts per million (ppm) relative to $CDCl_3$ (δ 0.00 ppm for proton NMR) and DMSO-D_6 (δ 2.50 ppm for proton NMR). Coupling constants are reported in Hz. The splitting patterns are indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet).

Initially, scaffold **1** was synthesized. Tryptophan methyl ester (**2**) was synthesized from tryptophan according to the protocol used in our previous synthesis [14]. This was followed by the Pictet-Spengler lactamization of **2** with levulinic acid and catalytic amount of PTSA (Sigma-Aldrich) in toluene to yield the scaffold **1** in 52% yield. The desired compound was obtained after purification by column chromatography with ethyl acetate-hexane (Sigma-Aldrich) as eluent. Nucleophilic substitution of **1** was done with a variety of alcohols in the presence of diacetoxy-iodobenzene (DIB) (Sigma-Aldrich) in toluene afforded compounds **3**, **4**, **5**, **6**. The average yields ranged from 47 to 65%. When **3** and **4** were subjected to hydrolysis in the presence of NaOH and methanol at RT, they resulted in the formation of **7** and **8** respectively. All the yields for the hydrolysis products were in the range of 76–82%. In addition, **1** was directly hydrolyzed to form the carboxylic acid from the methyl ester (**9**), thus completing the library.

Synthesis of methyl 2-amino-3-(1H-indol-3-yl)propanoate) (**2**): To a stirred solution of tryptophan (24.5 mmol, 1 equiv.) in 75 mL MeOH, thionyl chloride (61.2 mmol, 2.5 equiv.) (Sigma-Aldrich) was added dropwise at 0 °C. The resulting pale yellow solution was refluxed at 65 °C for 2 h. Once TLC confirmed the consumption of starting material, the reaction mixture was evaporated under reduced pressure to yield a white solid. This solid was dissolved in minimum amount of water and it was basified to pH 9 by the addition of aqueous NH_3 at 0 °C. This solution was extracted with ethyl acetate (3 × 100 mL) (Sigma-Aldrich), and then the organic layer was evaporated under reduced pressure to produce an oily yellow liquid in 95% yield.

Synthesis of methyl 11b-methyl-3-oxo-2,3,5,6,11,11b-hexahydro-1H-indolizino[8,7-b]indole-5-carboxylate (**1**): Compound **2** (28.4 mmol, 1 equiv) was taken in toluene (65 mL), levulinic acid (Sigma-Aldrich) (34.09 mmol, 1.2 equiv) and catalytic amount of PTSA (*p*-toluene sulphonic acid) were added, and the mixture was refluxed at 100 °C overnight. A dark brown solution resulted. Thin layer chromatography (TLC) was performed to confirm the consumption of starting material and then the reaction mixture was evaporated under reduced pressure to evaporate the toluene. The resultant brown semi-solid was purified by column chromatography using ethyl acetate-hexane as the mobile phase. The final product was obtained as a brown solid in 52% yield.

2.2.2. General procedure for the synthesis of ***3***, ***4***, ***5***, ***6***

Compound **1** (1.67 mmol, 1 equiv.) was dissolved in the appropriate alcohols (10 mL), and diacetoxy-iodobenzene (Sigma-Aldrich) (DIB) (2.514 mmol, 1.5 equiv.) was added to it. The reaction mixture was stirred at RT overnight. It resulted in the formation of a clear solution. Completion of reaction was checked via TLC and the reaction was quenched with solid $NaHCO_3$. The mixture was decanted and the resulting solution was evaporated under