International Journal of Biological Macromolecules 83 (2016) 78–96

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Establishment of correlation between *in-silico* and *in-vitro* test analysis against *Leishmania* HGPRT to inhibitors

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ARTICLE INFO

Article history:
Received 18 September 2015
Received in revised form
13 November 2015
Accepted 18 November 2015
Available online 23 November 2015

Keywords:
rLdHGPRT
FlexX
Docking
Leishmania
QSAR

ABSTRACT

Hypoxanthine Phosphoribosyltransferase (HGPRT; EC 2.4.2.8) is a central enzyme in the purine recycling pathway of all protozoan parasites. Protozoan parasites cannot synthesize purine bases (DNA/RNA) which is essential for survival as lack of de-novo pathway. Thus its good target for drug design and discovery as inhibition leads to cessation of replication. PRTase (transferase enzyme) has common PRTase type I folding pattern domain for its activities. Genomic studies revealed the sequence pattern and identified highly conserved residues catalyzed the reaction in protozoan parasites. A recombinant protein has 24 kDa molecular mass (rLdHGPRT) was cloned, expressed and purified for testing of guanosine monophosphate (GMP) analogous compounds *in-vitro* by spectroscopically to the rLdHGPRT, lysates protein and MTT assay on *Leishmania donovani*. The predicted inhibitors of different libraries were screen into FlexX. The reported inhibitors were tested *in-vitro*. The 2′-deoxyguanosine 5′-diphosphate (DGD) (IC_{50} value 12.5 μM) is two times more effective when compared to guanosine-5′-diphosphate sodium (GD). Interestingly, LdHGPRT complex has shown stable after 24 ns in molecular dynamics simulation with interacting amino acids are Glu125, Ile127, Lys87 and Val186. QSAR studies revealed the correlation between predicted and experimental values has shown R^2 0.998. Concludes that inversely proportional to their docked score with activities.

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1. Introduction

The genus *Leishmania* is the causative agent of leishmaniasis, a severe parasitic disease of considerable importance in terms of both diversity and complexity. It has been reported that the number of persons at risk of contracting leishmaniasis is approximately 350 million and that there are 2.3 million new cases every year [1]. All *Leishmania* species having digenetic life cycle and alternates between the flagellated mobile promastigotes to non-flagellated and non-motile amastigotes [2]. Visceral leishmaniasis (VL), also known as Kala-azar, black fever, and Dum-Dum fever, is the most severe form of leishmaniasis [3]. Currently, the treatment of choice for VL in India is amphotericin B [4] in its various liposomal preparations (AmBisome [5], Abelcet [6], Amphocil [7]), and miltefosine is the first oral drug for the treatment of this disease [8]. Most of the compounds used to treat visceral leishmaniasis are highly toxic in nature and potentially mutagenic or carcinogenic [9]. These undesirable effects have led to the desire to develop new antileishmanial drugs that are selective for the metabolic machinery of the parasites. One of the most prominent metabolic discrepancies between *Leishmania* and their hosts is the pathway by which *Leishmania* synthesize purine nucleotides [10]. The mammalian cells synthesize purine nucleotides from amino acids and one-carbon moieties, whereas protozoan parasites are incapable of de novo purine synthesis [10–12].

Abbreviations: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; HPRT, hypoxanthine phosphoribosyltransferase; APRT, adenosine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; CL, cutaneous leishmaniasis; MCL, muco-cutaneous leishmaniasis; VL, visceral leishmaniasis; PKDL, post-Kala-azar dermal leishmaniasis; BLAST, basic local alignment search tool; PDB, protein data bank; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RMSD, root mean square deviation; GMP, guanosine monophosphate; GD, guanosine-5′-diphosphate sodium; AG, acycloguanosine; DGD, 2′-deoxyguanosine 5′-diphosphate; GCM, guanosine 3′,5′-cyclic monophosphate.

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http://dx.doi.org/10.1016/j.ijbiomac.2015.11.051
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Purine salvage pathway is an important for survival of parasitic organism which is fully or partial depend on host organism. They have variety of functions include vital cellular and metabolic processes including energy production, cell signaling, synthesis of vitamin-derived cofactors and nucleic acids, and as determinants of cell fate. As unlike in the host cells (mammals and insect host), *Leishmania* have lacking *de-novo* pathway for synthesis of purine [13]. The obligate nature of parasite offers an attractive target for drug discovery. Metabolic, biochemical and genetic studies have revealed that *Leishmania donovani* promastigotes funnel a variety of exogenous purine into hypoxanthine indicating that the enzyme hypoxanthine-guanine Phosphoribosyltransferase (HGPRT) plays a central role in this purine acquisition process [14]. One of the most important biocatalytic activities of HGPRT is to recycle purine inside parasitic cells [15]. The salvage pathway recovers purines (adenine and guanine) from the degradation products of nucleotide metabolism and from hypoxanthine and xanthine [16]. In *Leishmania*, three PRTase (phosphoribosyltransferases) are involved in the recycling of purine bases, hypoxanthine-guanine PRTase (EC 2.4.2.8) [17], adenine PRTase (EC 2.4.2.7) and xanthine PRTase (EC 2.4.2.22), all of which are potential targets for drugs. Of these three PRTases, one enzyme (EC 2.4.2.8) exhibits activity with hypoxanthine and guanine (Hyp-Gua Phosphoribosyltransferase) [18]. It has been established by co focal and immunoelectron microscopy that the HGPRT protein of *L. donovani* is localized solely to the glycosome [16].

In drug discovery, it is common to have activity data for group of compounds acting upon a particular protein but knowledge of the 3D structure of the active site was remains unknown. In the absence of such 3D information, one may attempt to build a hypothetical model of the active site that can provide insight into the nature of the active site. Knowledge of the 3D structure of a protein is essential to understand how a protein performs its function. The protein structure can be determined at a high resolution by either experimental methods such as X-ray crystallography and NMR or computational analysis [19]. In the absence of crystallographic structures, the structure of protein can be closely matched using a variety of advanced homology modeling methods those have been developed [20]. These methods can provide reliable models of proteins that share 30% or more sequence identity with a known structure [21–24]. Thus, computational analysis and test compounds was screened against HGPRT by virtual screening and enzymatic assay will be helpful for the development of promising compounds that may inhibit the survival of the *Leishmania* parasite in the host.

In the present study, we have designed and developed some compounds against the *Leishmania* parasite; these compounds have shown inhibition against LdHGPRT. A homology model of the LdHGPRT with 221 amino acids was constructed to obtain an in-depth idea of this protein's structural and functional characteristics. The construction of 3D model of the *L. donovani* HGPRT based on available 3D structure of the HGPRT from *L. tarentolae* (PDB ID: 1PZM_A) by homology modeling. The predicted structures were refined by taking advantages of the CHARMM parameters and energy minimization studies and were evaluated using the DOPE (Discrete Optimized Protein Energy) score, PROCHECK and Verify3D to analyze the structural integrity (data not shown) [25]. GMP (guanosine monophosphate), analogues and currently prescribed antileishmanial compounds were docked into the active site of 3D model protein. Now, we have cloned, expressed and purified of HGPRT protein of *L. donovani*. Finally, the *in-vitro* test analysis was performed of these compounds based on spectroscopically (enzymatic assay) and MTT assay on *Leishmania* culture. The predicted compounds need to validates *in-vivo* activities for further studies.

2. Material and methods

2.1. Genomic analysis of HGPRT of L. donovani

2.1.1. Parasite culture, RNA extraction and cDNA preparation

Promastigotes of Indian *L. donovani* strain MHOM/IN/83/AG83 was obtained from culture bank of Rajendra Memorial Research Institute of Medical Sciences (ICMR), Patna, India. The cryo-cells were revived and grown in RPMI1640 medium (Sigma–Aldrich) supplemented with 10% Fetal Calf Serum (FCS: Sigma–Aldrich) in BOD incubator at 24 ± 1 °C. Promastigotes at stationary phase were collected by centrifugation at 8000 rpm for 5 min. Total RNA was isolated by using an RNA isolation kit (Qiagen), according to the manufacturer's instructions and further purified with RNeasy columns (Qiagen, Inc., Valencia, CA). RNA was used to synthesize cDNA with the help of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Foster City, CA). A pair of primer was designed based on *Leishmania major* hypoxanthine-guanine phosphoribosyltransferase (HGPRT, accession no-XM_001682973.1) sequence: Forward primer-5′-ATCCGTGGCAACACCGCTGAGGCCACGA-3′ and Reverse primer 5′-ATGGGCAAGGATAAGGTGCACATGAA-3′. The reaction was performed in 25 μl of the solution 1 μg cDNA, 20 pmol each of forward and reverse primers, 4 mM $MgCl_2$, 0.4 mM dNTPs, 1× PCR buffer, 1.25 unit of Taq DNA polymerase (Roche) and H_2O up to 25 μl. PCR amplification was carried out within 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 60 s and extension at 72 °C for 60 s and after final extension at 72 °C for 5 min in a thermal cycler (ABI). PCR product was submitted to electrophoresis using 1.2% agarose gel (Agarose: Molecular grade, TAE 1X buffer) (TAE: Tris-base, Acetic acid, EDTA) with EtBr. The DNA band was visualized under an ultraviolet light (UV Transilluminator) and photographed using Chem doc (Biorad).

2.1.2. Purification and sequencing of PCR product

The PCR product was purified using PCR product purification kit (Qiagen) to remove unused dNTPs, enzyme and salt. The ABI Prism BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) was used for the sequencing of the PCR product. The sequencing reaction mixture contained 4 μl of Big Dye premixture, 0.5× buffers, 3.2 pmol of sequencing primer, and approximately 150 ng of PCR product template in a total volume of 20 μl. Sequencing PCR was carried out with the same forward primer. PCR amplification was carried out at conditions 96 °C for 60 s, followed by 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 05 s and extension at 60 °C for 4 min in a thermal cycler (ABI). The product was processed, dried and resuspended in 19 μl formamide and then loaded in ABI 3130xL genetic analyzer for sequencing following the manufacturer's recommendations. The sequencing results were analyzed with Sequencer software under the condition of signal/noise > 98%. The translated sequence was analyzed based on its closed sequences of other species by multiple sequence alignment and phylogenetic analysis.

2.2. Cloning, expression and purification of HGPRT of L. donovani

2.2.1. Chemical requirements

All the chemicals and solvents used were of AR-grade and LR-grade and obtained from Sigma–Aldrich, Qualigens, Rankem, S D Fine-Chem, HiMedia and Merck were used without further purification.

2.2.2. Cloning, expression and purification of rLdHGPRT

L. donovani strains Ag83 (MHOM/IN/83/Ag) were cultured in the medium M199 supplemented with 10% heat

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