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Discovery of novel anti-leishmanial agents targeting LdLip3 lipase

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ABSTRACT

Leishmaniasis is a neglected tropical disease, caused by several species of *Leishmania*. Being an opportunistic lipid-scavenging pathogen, *Leishmania* relies extensively on lipid metabolism especially for host–pathogen interaction, utilizing host lipids for energy and virulence. The rational approach is to target lipid metabolism of the pathogen focusing lipid-catabolizing lipases. The LdLip3 lipase is considered as drug target as it is constitutively expressed in both promastigote and amastigote forms. Since the LdLip3 structure is not known, we modeled its three-dimensional structure to implement structure-based drug discovery approach. Similarity-based virtual screening was carried out to identify potential inhibitors utilizing NCI diversity set on ZINC database including natural products. Implementing computational and experimental approaches, four anti-leishmanial agents were discovered. The screened molecules ZINC01821375, ZINC04008765, ZINC06117316 and ZINC12653571 had anti-leishmanial activity with IC_{50} (% viable promastigotes *vs.* concentration) of 5.2 ± 1.8 μM, 13.1 ± 2.6 μM, 9.4 ± 2.6 μM and 17.3 ± 3.1 μM, respectively. The molecules showed negligible toxicity toward mouse macrophages. Based on the contact footprinting analysis, new molecules were designed with better predicted free energy of binding than discovered anti-leishmanial agents. Further validation for the therapeutic utility of discovered molecules can be carried out by the research community to combat leishmaniasis.

1. Introduction

Leishmaniasis is considered as an emerging disease with about 12 million infected people worldwide [1]. Several species and sub-species of *Leishmania*, an intra-macrophage obligate parasite causes clinically heterogeneous leishmaniasis. The fatal visceral leishmaniasis is linked with *Leishmania donovani*, *L. infantum* and *L. chagasi*. *L. donovani* is predominant in India [2,3]. Leishmaniasis is widespread in 98 countries with 350 million people at risk. Unfortunately, it has been underreported for several decades and still has a neglected status [4,5]. The available therapeutics is associated with high toxicity, high cost and low efficacy (Supplementary Table 1) [6,7]. Emergence of HIV coinfection, drug-resistance and disease spreading worldwide worsens the current scenario that urges the need for new/novel drugs to combat leishmaniasis. Few research organizations stress on the control measures along with the identification of new drug targets and drugs [8,9].

Leishmania is a lipid-scavenging pathogen with dimorphic life cycle. The parasite is transmitted into mammalian host by female phlebotomine sand flies and it relies highly on its lipid metabolism to survive in different milieu of the hosts [10]. The lipid metabolism of pathogen plays a vital role in energy storage and pathogenesis [11]. Additional roles of the lipid metabolism have been implicated in cellular signaling as well as organization, dynamics and trafficking of membrane [12]. The systemic metabolic analysis in *Leishmania major* revealed that (i) most of the intracellular reactions participate in lipid metabolism and (ii) 38% of the essential genes are associated with lipid metabolism [13]. Several studies have also highlighted that lipid metabolism was elevated in amastigotes when compared with promastigotes [14–16]. Miltefosine, a FDA-approved anti-leishmanial drug is believed to target lipid metabolism of *Leishmania* [17]. Lipid metabolism of *Leishmania* gained lot of attention in the last decade and is of our interest for the discovery of new drug targets [18].

Lipases are one of the key enzymes in lipid metabolism which act on triglycerides and phospholipids of the host. These macromolecules are abundant in mammalian phagolysosomes where lipid-scavenging pathogens reside [19]. Lipases of pathogens have multitude of functions in many pathophysiological processes including virulence, transmission, life cycle development, modulation of host lipids and host immune responses [20–23]. Lipases from human pathogens notably *Mycobacterium tuberculosis*, *Candida albicans*, *Cryptococcus neoformans* and *Malassezia* species are proven for their role in virulence and survival [24,25].

LdLip3, a lipase from *L. donovani*, is believed to participate in key biological processes that include host lipid degradation, alteration

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Table 1
LdLip3 and its homologs used in the multiple sequence alignment study.

UniProtKB accession	Name	Organism	Identity (%)[a]	Similarity (%)[a]
D7P7V3	LdLip3	*L. donovoni*		
A4I6H7	Li-Lip	*L. infantum*	99	99
Q4Q6I0	Lm-Lip	*L. major*	89	91
P19515	Rm-TGL	*R. miehei*	35	45

[a] Identity and similarity of the corresponding protein against LdLip3.

of phagolysosome membrane and structural remodeling of membrane lipids for the survival and infection of pathogen. LdLip3 might favor the parasite growth and development in the host [26]. Hence, LdLip3 could be an attractive drug target to combat leishmaniasis. Targeting LdLip3 of the pathogen is considered as therapeutic strategy in the present study and attempts were made to identify novel potential inhibitors that might preferentially kill *Leishmania*. The three dimensional (3D) model of LdLip3 was predicted which was further utilized for the structure-based drug discovery (SBDD). The similarity-based virtual screening of representative chemical scaffolds was opted for the identification of potential inhibitors [27–29]. Furthermore, the anti-leishmanial activity of the screened hits was validated with experimental assay, resulting in the identification of four novel lead-like molecules. The identified novel anti-leishmanial agents could serve as potential candidates for drug development against leishmaniasis that might address infectious diseases caused by Trypanosomatida protozoans.

2. Materials and methods

2.1. Homology modeling

The protein sequence of LdLip3 (UniProt ID: D7P7V3) and its representative homologs from leishmanial species were collected from UniProtKB (www.uniprot.org). The retrieved sequences were subjected to multiple sequence alignment with ClustalW and subsequently analyzed with ESPript to identify functionally important residues, motifs and protein domains [30,31] (Table 1).

The appropriate template for homology modeling was identified by searching the structural homologs using 'BLASTP' program against Protein Data Bank (PDB). Lipase from *Rhizomucor miehei* (Rm-TGL) in its inhibitor bound conformation (PDB id: 4TGL) was used as template for structure modeling based on its sequence and functional homology [32]. The first 20 residues of LdLip3 were not modeled due to lack of structural information of Rm-TGL. Initially hundred models were generated with MODELLER9v7 which were then ranked on the basis of their discrete optimized protein energy (DOPE) and restraint violation (molpdf) scores [33,34]. The best model having reasonable DOPE and molpdf scores with acceptable statistics with PROCHECK [35] and Errat plot [36] was validated using NIH SAVES server. Protein fold of LdLip3 model was evaluated using knowledge-based energy profile of ProSA program [37,38]. The best model of LdLip3 from structure validation studies was analyzed for stability using GROMOS96 43a1 force field under periodic boundary conditions in GROMACS4.5.5 package [39–41]. The system was solvated with simple point charge (SPC) water model. The net charge of system was neutralized by replacing water molecules that are at least 3.50 angstroms (Å) from the protein surface with four chlorine ions. The system was energy minimized to remove bad contacts using steepest descent algorithm. The solvent was equilibrated for 500 ps in NVT ensemble by restraining the solute atoms through a harmonic force constant of 1000 kJ nm^{-2}. Then the system was equilibrated by restraining all the bonds for 500 ps in NPT ensemble which was followed by 500 ps of equilibration without any restraints. Production run was carried out for 20 ns with NPT ensemble using 2 fs of integration time. Simulations were carried out with velocity rescaling thermostat at 300 K in which protein and non-protein atoms were coupled to separate temperature coupling baths and pressure was controlled at 1 atm using Parrinello-Rahman barostat. The linear constraint solver (LINCS) algorithm was used to constrain the bonds involving hydrogen atoms [42,43]. Particle Mesh Ewald (PME) summation method was used for calculating the long-range electrostatic interactions with 12 Å cut-off. Conformations of LdLip3 model from the production run were analyzed as time-dependent function to check the conformational stability of energy minimized LdLip3 model in the solvent system.

2.2. Similarity-based virtual screening

2.2.1. Virtual screening

The success of virtual screening in the identification and enrichment of lead and lead-like molecules are well reported [44–48]. Virtual screening of small molecules was performed using the energy minimized model of LdLip3 with AutoDock4.2 using lamarckian genetic algorithm (LGA) [49,50]. NCI diversity set II was used to identify structurally diverse molecular scaffolds that contain 1880 chemical molecules (2654 molecular structures). The choice of NCI diversity set explores the vast chemical space and meanwhile greatly reduces the computational time as it contains only the representative scaffolds and the task can be carried out with limited computational resources. LdLip3 model was preprocessed by adding hydrogen atoms to all the residues followed by addition of Gasteiger-Marsili charges. Then non-polar hydrogen atoms were merged into their respective heavy atoms and atom types were fixed using AutoDockTools [51]. All the small molecules were similarly processed using 'prepare_ligand4' python script available with AutoDockTools distribution. For docking simulations, a grid box of $90 \times 90 \times 90$ points was constructed with grid spacing of 0.375 Å keeping catalytic serine (Ser168) at the center of grid box that covers the entire active site of LdLip3 model. Grid maps were generated for all the atom types present in the grid box along with electrostatic and desolvation maps using the AutoGrid utility. The whole docking procedure was then repeated with the human structural ortholog – monoglyceride lipase (MGL), to find the potential inhibitors that could be effective toward LdLip3 than human MGL (PDB id: 3HJU) [52]. Molecular docking of NCI diversity set was performed with an initial population size of 300 for 20 independent LGA runs. In each run, the best individual from each generation was propagated to the next generation and remaining docking parameters were set to default. Each molecule was clustered on the basis of positional root-mean-square deviation (RMSD) between the docked positions to predict the optimal binding of the molecules with LdLip3.

The ten top hits were chosen as initial data set based upon their difference in binding free energy better than −2 kcal/mol and interactions of the top hits with leishmanial-specific residues of LdLip3 to carry out similarity screening. Similar molecules (tanimoto coefficient > 0.6) of the ten top hits from ZINC and its natural product database resulted in 22,919 molecules including isomers which were collected and preprocessed [53,54]. Similarity-based virtual screening was intended to understand the structure–activity relationships and to identify molecules with better free energy of binding than hits from initial screening. Virtual screening process was repeated with these similar molecules on both LdLip3 model and human MGL with an initial population size of 300 and 20 independent LGA runs with stringent evaluations of 30,000,000. Docking results were visually analyzed with AutoDockTools and PyMOL to understand the docking poses and interactions between the potential inhibitors and LdLip3 [55]. The top twenty hits were re-docked with 100 independent LGA runs to increase the reliability of the docking predictions (reliability test). The best conformation