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## 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGR) Enzyme of the Sterol Biosynthetic Pathway: A Potential Target against Visceral Leishmaniasis

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### Abstract

Sterol biosynthetic pathway is explored for its therapeutic potential for Visceral Leishmaniasis. In *Leishmania*, this pathway produces ergosterol which is absent in host and therefore is a promising strategy to combat proliferation of both extracellular and intracellular forms of the parasite with minimal host toxicity. The present chapter focuses on 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) enzyme which is the rate-limiting enzyme of the ergosterol biosynthesis. HMGR gene of *L. donovani* was biochemically and biophysically characterized for the first time. HMGR over expressing transgenic parasites were generated to evaluate its role in parasite growth and infection ability. A series of statins like atorvastatin, simvastatin and mevastatin were evaluated for its therapeutic efficacy and mode of action elucidated. Atorvastatin and mevastatin were found to be killing both the promastigote and amastigote forms of the parasite without exhibiting host cytotoxicity. Besides, non-statin class of molecules like resveratrol and glycyrrhizic acid were also analyzed for antileishmanial potential. Two antidepressants, ketanserin and mianserin were found to kill both *L. donovani* promastigotes and intracellular amastigotes with no apparent toxicity to the host cells. Since targeting of the sterol biosynthetic pathway enzymes may be useful therapeutically, the present work may have implications in treatment of Leishmaniasis.

**Keywords:** Visceral Leishmaniasis, HMGR, ergosterol, statins, antidepressants

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### 1. Introduction

A variety of *Leishmania* species are reported to cause disease, which afflicts about 12 million people in 98 countries of which Indian subcontinent, Sudan and Brazil are the major

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regions with higher incidence of Leishmaniasis. The World Health Organization (WHO) has considered Leishmaniasis to be one of the six priority diseases of its special programme for Research and Training in tropical diseases. Visceral Leishmaniasis (VL) being a neglected tropical disease has been of concern for several years. Antimonial compounds remain the first line drug for VL treatment with amphotericin B and pentamidine being the second line drugs. However, both the classes have high toxicity and serious side effects. Drug resistance, toxicity and long-term treatment profile are some of the issues which plague the treatment regimen. In the wake of this problem, there are increasing efforts to identify vaccine candidates and drug target candidates with equal focus on drug repositioning. Till date, several enzymes of various crucial metabolic pathways such as the pentose phosphate pathway, trypanothione biosynthesis pathway and sterol biosynthetic pathway have been explored in parasites [1]. With the whole genome sequence of *Leishmania donovani* now available, it has become feasible to identify new genes and explore its essentiality in parasite survival and host infectivity. Structural analysis of identified enzymes would throw light on potential active site for designing pharmacophore. Based on this, *in silico* ligand screening is performed to identify potential compounds from already existing library. This would further lead to design and synthesis of new chemical entities whose potency can be evaluated in cell-based and target-based screening assays.

Sterol biosynthetic pathway is an important metabolic pathway in fungi and trypanosomatids. In recent years, attention has been focused on the sterol metabolism of *Leishmania* as a potential drug target for therapy. In sterol biosynthetic pathway, condensation of two acetyl-CoA units leads to formation of acetoacetyl-CoA, followed by the addition of a third unit to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is further reduced by NADPH to produce mevalonic acid. The mevalonate pathway comprises of three steps and is catalyzed by acetoacetyl-CoA thiolase, and two mitochondrial enzymes HMG-CoA synthase and HMG-CoA reductase, in yeast [2] and in trypanosomatids [3]. Sterols are important components of the cell membrane that are important for cellular function and maintenance of cell structure. Unlike mammalian cells which have cholesterol as the major membrane sterol, trypanosomatids synthesize ergosterol and other 24-methyl sterols that are required for their growth and viability. *Leishmania* parasite contains predominantly ergostane-based sterols such as ergosterol, which differ from cholesterol by the presence of a 24-methyl group at  $\Delta 7$  and  $\Delta 22$  bonds [4]. Therefore, the sterol biosynthetic pathway from *Leishmania* is considered to be an important drug target. Squalene synthase (SQS) catalyzes the first committed step of sterol synthesis by coupling two farnesyl molecules to form squalene. Two quinuclidine derivatives, ER-119884 and E5700, have been shown to be potent antileishmanial and anti-trypanosomal agents. The inhibition of SQS by these compounds decreased endogenous sterol levels of the parasite and caused an anti-proliferative effect on the parasite [5]. Sterol 24-C-methyltransferase (SMT) is unique to the parasite and validated as a potential drug target against trypanosomatid parasites. Azosterols like ketoconazole are known to inhibit SMT in fungi. They were also found to be anti-proliferative in *Leishmania amazonensis* [6].

One of the enzymes of the sterol biosynthetic pathway which is focused in this chapter is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC:1.1.1.34). HMGR catalyzes the NADPH

dependent synthesis of mevalonate from HMG-CoA and is a rate limiting step [7]. There are two classes of HMG-CoA reductase: class I (eukaryotic HMGRs) and class II (prokaryotic HMGRs). The class I HMGR has an N-terminal membrane domain and is present in eukaryotes and several archaea. Class II HMGR lacks this domain and occurs in *Pseudomonas mevalonii*, *Archaeoglobus fulgidus*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Streptomyces* [8–11]. *L. major* HMGR enzyme lacks the N-terminal domain and is the only eukaryote with soluble HMGR protein. Among kinetoplastids, HMGR has been earlier characterized in *L. major* and *Trypanosoma cruzi* [12, 13]. Given that ergosterol is an important component of *Leishmania* membrane, we focused our research on identification and validation of HMGR from *L. donovani* as a potential drug target candidate.

*L. donovani* HMGR gene was identified via a BLAST search of the genome using *L. major* HMGR sequence (www.ebi.ac.uk/parasites/LGN) as the template. *LdHMGR* gene was amplified, cloned in pET30a (+) vector and sequenced (GenBank accession no. JX036280.1). *LdHMGR* exhibited only 25.2% identity (35% similarity) with human HMGR. This signifies that host HMGR is significantly different from parasite HMGR. HMGR enzyme is constitutively expressed in *Leishmania* promastigotes as shown by western blot analysis [14, 27].

### 1.1. Functional analysis of *LdHMGR* overexpressors

Next, we were interested to see whether HMGR has any role in parasite growth and infectivity. For this, HMGR was cloned in a *Leishmania* specific overexpression vector. HMGR overexpression in *L. donovani* promastigotes was confirmed by measuring of HMGR activity, estimation of ergosterol levels and western blot analysis confirmed the overexpression of HMGR gene [15].

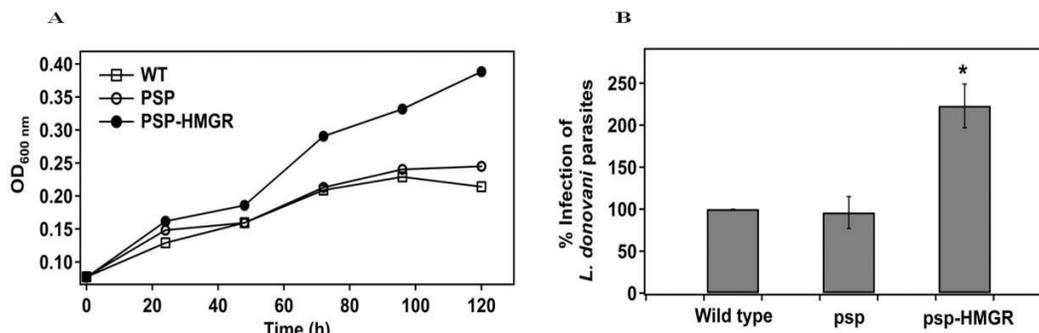
### 1.2. Growth curve analysis of HMGR transfectants

The growth profile of transfected and wild-type parasites *in vitro* was studied by measuring OD at 600 nm of the plated cells for every 24 h. We monitored the growth of parasites and *LdHMGR* transfectants exhibited ~ 1.5 fold increase in growth than compared to wild-type and psp vector transfected parasites (**Figure 1A**).

### 1.3. Role of HMGR in parasite infection ability

The transfectants were tested for their ability to infect THP-1 differentiated macrophages. The stationary phase of wild-type and HMGR overexpressing promastigotes were used to infect THP-1 differentiated macrophages. The percentage of infection with wild type was considered as 100% and relatively percentage of infection was calculated for psp and HMGR overexpressors. The HMGR transfectants exhibited ~2 fold change in the infectivity compared to wild-type parasites (**Figure 1B**).

In other organisms like yeast, it was reported that combined overexpression of genes (*ERG1* and *ERG11*) leads to significant increase in the amount of total sterols by threefold in comparison with a wild-type strain in yeast. The HMG-CoA reductase controls the entering



**Figure 1.** Functional analysis of *LdHMGR* overexpressors. (A) Growth analysis of wild-type (WT), psp and *LdHMGR* overexpressing parasites; (B) evaluation of infection efficiency of wild-type (WT), psp and *LdHMGR* overexpressors. Data were expressed as mean  $\pm$  standard deviations from three independent experiments. \* $p \leq 0.05$ .

of intermediates on the pre-squalene part of the pathway and Erg1p and Erg11p seem to control the transfer of intermediates into the post-squalene part of sterol biosynthetic pathway [16]. Other studies reported that overexpression of HMGR in yeast leads to increased linalool production which is a plant monoterpene which display antiparasitic, antimicrobial and antiviral properties as well as a plethora of promising health benefits [17].

## 2. HMGR inhibition profiles

### 2.1. Evaluation of antileishmanial effect of class I statin (simvastatin), class II statin (atorvastatin) and mevastatin

The inhibitors used in the present study were atorvastatin, simvastatin and mevastatin. The concentrations of atorvastatin and mevastatin at which 50% growth of *L. donovani* promastigotes was inhibited ( $IC_{50}$ ) were  $19.4 \pm 3.07 \mu\text{M}$  and  $23.8 \pm 4.2 \mu\text{M}$  respectively [14, 18, 25]. The  $IC_{50}$  value of simvastatin was  $73.2 \pm 3.7 \mu\text{M}$  and at  $100 \mu\text{M}$  it caused only 63% inhibition. The cytotoxicity of atorvastatin, simvastatin and mevastatin against THP-1 differentiated macrophages was determined by using MTT assay. The  $IC_{50}$  value of three drugs were found to be above  $100 \mu\text{M}$ , that is, noncytotoxic to host macrophage cell line. Miltefosine inhibited promastigote growth with an  $IC_{50}$  value of  $14.6 \mu\text{M}$ . Atorvastatin was found to inhibit *L. donovani* promastigotes at low micromolar concentrations compared to mevastatin and simvastatin. The concentrations of atorvastatin, simvastatin and mevastatin at which 50% growth of *L. donovani* amastigotes was inhibited ( $IC_{50}$ ) were  $6.75 \pm 0.353 \mu\text{M}$ ,  $21.5 \pm 4.94 \mu\text{M}$  and  $7.5 \pm 1.1 \mu\text{M}$  respectively. The amastigotes were approximately threefold more sensitive to atorvastatin and resveratrol than promastigotes. Miltefosine was taken as the reference drug, and its  $IC_{50}$  value for amastigotes was  $3.9 \pm 1.27$  [14, 25]. The  $IC_{50}$  values are depicted in **Table 1**.

The inhibitors were screened for their ability to inhibit the catalytic efficiency of recombinant *LdHMGR*. The  $IC_{50}$  value of atorvastatin, simvastatin and mevastatin was found to be half maximal at around  $315 \pm 2.12 \text{ nM}$ ,  $43.66 \pm 31.5 \mu\text{M}$  and  $42.2 \pm 3.0 \mu\text{M}$  respectively. Atorvastatin ( $1 \mu\text{M}$ ) resulted in  $93.5 \pm 7.2\%$  inhibition of the recombinant HMGR. **Table 1** shows the  $IC_{50}$  values of the statins on recombinant HMGR.

Inhibitors	IC <sub>50</sub> values (μM)				
	<i>L. donovani</i> promastigotes	<i>L. donovani</i> amastigotes	THP-1 differentiated macrophages	SI values	rHMGR
Atorvastatin <sup>a</sup>	19.4 ± 3.07	6.75 ± 0.353	>100	>14.8	0.315 ± 2.12
Simvastatin <sup>a</sup>	73.2 ± 3.7	21.5 ± 4.94	>100	>4.65	43.66 ± 31.5
Mevastatin <sup>a</sup>	23.8 ± 4.2	7.5 ± 1.1	>100	>13.3	42.2 ± 3.0
Miltefosine <sup>b</sup>	14.6 ± 1.7	3.9 ± 1.27	43.6 ± 5.5	11.17	—

<sup>a</sup>Dinesh et al. [14, 25].  
<sup>b</sup>Dinesh et al. [18].

**Table 1.** Antileishmanial effect of statins.

## 2.2. Evaluation of antidepressants as HMGR inhibitors

Tricyclic drugs, antidepressants and antipsychotics are reported to be toxic to both the promastigote and amastigote forms of *Leishmania* [19]. Imipramine, a tricyclic antidepressant belonging to the same class of cationic amphiphilic drugs, when administered orally was found to be active against both antimony-sensitive and antimony resistant clinical isolates of *L. donovani* [20].

Ketanserin is a serotonin 5<sub>2</sub>-receptor antagonist which is used as an antihypertensive agent. The IC<sub>50</sub> value of ketanserin for *L. donovani* promastigotes was 37.8 μM and intracellular amastigotes was 28.5 μM. It was however found to be noncytotoxic up to a concentration of 100 μM, when tested on differentiated THP-1 cells. Miltefosine inhibited amastigote growth with an IC<sub>50</sub> value of 3.4 μM which correlated with the previously reported data [21]. However, the standard drug killed the macrophage cells at an IC<sub>50</sub> value of 43.6 μM. This was well correlated with the already published results on the effect of miltefosine on THP-1 and J774A.1 cell line [22, 23]. These results showed that ketanserin displayed antileishmanial activity at non-cytotoxic concentrations. We evaluated the effect of ketanserin on recombinant *Ld*HMGR and found its IC<sub>50</sub> value to be 43 ± 2.5 μM. This data showed that ketanserin binds to the *Ld*HMGR enzyme active site and inhibits its activity (**Table 2**) [15].

Mianserin hydrochloride is a noradrenergic and specific serotonergic antidepressant (NaSSA) with a tetracyclic structure and is used for the treatment of depressive illness and depression associated with anxiety [24]. Mianserin strongly blocks postsynaptic 5-HT<sub>2</sub> receptors and only weakly blocks post synaptic 5-HT<sub>1</sub> and 5-HT<sub>3</sub> receptors and blocks moderately presynaptic α<sub>2</sub> receptors [24]. The effect of mianserin was investigated on the proliferation rate of *L. donovani* promastigotes and amastigotes. The dose-dependent antileishmanial effect of mianserin against *L. donovani* promastigotes resulted in significant reduction in viable parasites compared to the untreated parasites. The concentration of mianserin at which 50% of the promastigote and amastigote growth was inhibited was 21 ± 3.7 μM and 46.4 ± 5.2 μM respectively. Mianserin up to 100 μM failed to cause any toxic effect on viability of THP-1 differentiated macrophages indicating that mianserin selectively inhibits *Leishmania* promastigotes. Mianserin inhibited recombinant *L. donovani* HMGR enzyme with an IC<sub>50</sub> value of 19.8 ± 3.1 μM (**Table 2**) [14, 25].

Inhibitors	IC <sub>50</sub> values (μM)				
	<i>L. donovani</i> promastigotes	<i>L. donovani</i> amastigotes	THP-1 differentiated macrophages	SI values	rHMGR
Mianserin <sup>a</sup>	21.0 ± 3.7	46.4 ± 5.2	>100	>2.15	19.8 ± 3.1
Ketanserin <sup>b</sup>	37.8 ± 3.3	28.5 ± 1.9	>100	>3.5	43.0 ± 2.5
Miltefosine <sup>a</sup>	14.6 ± 1.7	3.4 ± 0.9	43.6 ± 5.5	12.8	—

<sup>a</sup>Dinesh et al. [14, 25].  
<sup>b</sup>Singh et al. [15].

**Table 2.** Antileishmanial effect of antidepressants.

### 3. Natural products as inhibitors of HMGR

The inhibitors used in the present study were resveratrol and glycyrrhizic acid. The concentrations of resveratrol at which 50% growth of *L. donovani* promastigotes was inhibited (IC<sub>50</sub>) was 36.1 ± 3.6 μM. The cytotoxicity of resveratrol against THP-1 differentiated macrophages was determined by using MTT assay. The results showed the IC<sub>50</sub> value of three drugs was found to be above 100 μM, that is, noncytotoxic to host macrophage cell line. The concentrations of resveratrol at which 50% growth of *L. donovani* amastigotes was inhibited IC<sub>50</sub> value was 9.5 ± 2.12 μM [14, 25]. The data are depicted in **Table 3**.

*Glycyrrhiza glabra*, which is popularly known as liquorice is used for the treatment of pulmonary diseases and inflammatory processes [26]. Glycyrrhizic acid (GA), licochalone A and Glycyrrhetic acid which have been reported to exert antileishmanial properties are the major bioactive components in liquorice root [27, 29, 30]. GA exhibits potent antileishmanial and immunomodulatory properties with enhanced parasite clearance [27]. A dose-dependent inhibition of the viability of *L. donovani* promastigotes was observed in the presence of GA. The IC<sub>50</sub> determined from the graph was approximately 34 ± 2.9 μM. GA was found to inhibit intracellular amastigotes with an IC<sub>50</sub> value of 20 ± 4.2 μM. GA did not cause macrophage killing up

Inhibitors	IC <sub>50</sub> values (μM)				
	<i>L. donovani</i> promastigotes	<i>L. donovani</i> amastigotes	THP-1 differentiated macrophages	SI values	rHMGR
Glycyrrhizic acid <sup>a</sup>	34.0 ± 2.9	20.0 ± 4.24	>100	>5.0	24.0 ± 4.3
Resveratrol <sup>b</sup>	36.1 ± 3.6	9.5 ± 2.12	>100	>10.5	46.3 ± 16.4
Miltefosine <sup>a</sup>	15.3 ± 2.1	3.8 ± 1.2	44.2 ± 5.29	11.5	—

<sup>a</sup>Dinesh et al. [28].

<sup>b</sup>Dinesh et al. [14, 25].

**Table 3.** Natural products as inhibitors of HMGR.

to 100  $\mu\text{M}$  concentration. GA was tested against recombinant *LdHMGR* enzyme at the range of 10–100  $\mu\text{M}$  concentration. The  $\text{IC}_{50}$  value was found to be  $24 \pm 4.3 \mu\text{M}$  (Table 3).

In *Leishmania* sterol, biosynthetic pathway produces ergosterol which is absent in host. This makes *LdHMGR* enzyme a potential drug target for designing parasite specific molecules. The present review encompasses functional characterization of *L. donovani* HMGR enzyme and the evaluation of the effect of various HMGR inhibitors as potential candidates for treatment of Leishmaniasis. Inhibitors which showed inhibition of both the extracellular and intracellular forms of the parasites at low micromolar range with no cytotoxicity to host cells are promising antileishmanial candidates. They can be further explored in an experimental animal model of VL to evaluate its anti-VL efficacy.

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