

# Antileishmanial effect of mevastatin is due to interference with sterol metabolism

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**Abstract** Visceral leishmaniasis (VL) is one of the most severe forms of leishmaniasis which is fatal if left untreated. Sterol biosynthetic pathway in *Leishmania* is currently being explored for its therapeutic potential. In the present study, we have evaluated the antileishmanial efficacy of mevastatin, a known inhibitor of 3-hydroxy-3-methyl glutaryl-CoA reductase (HMGR) enzyme. Mevastatin inhibited *Leishmania donovani* promastigotes and intracellular amastigotes with an 50 % inhibitory concentration (IC<sub>50</sub>) value of 23.8±4.2 and 7.5±1.1 μM, respectively, without exhibiting toxicity towards host cell line. Mevastatin also inhibited recombinant *L. donovani* HMGR (*LdHMGR*) enzyme activity with an IC<sub>50</sub> value of 42.2±3.0 μM. Kinetic analysis revealed that the inhibition of recombinant *LdHMGR* activity by mevastatin was competitive with HMG-CoA. Mevastatin-treated parasites exhibited 66 % reduction in ergosterol levels with respect to untreated parasites. Incubation of mevastatin-treated *L. donovani* promastigotes with ergosterol resulted in revival of cell growth, whereas cholesterol supplementation failed to cause reversal in cell death. To further prove the specificity of mevastatin for HMGR enzyme, HMGR-overexpressing parasites were used which showed almost threefold resistance to mevastatin. It also induced morphological changes in the parasite accompanied by lipid body accumulation. Hence, antileishmanial effect of mevastatin was due to the inhibition of HMGR, which eventually leads to reduction in ergosterol levels and hence parasite death. The present

study may have implications in the treatment of visceral form of leishmaniasis.

**Keywords** Antileishmanial effect · Mevastatin · Sterol metabolism

## Introduction

*Leishmania* is an obligate pathogenic parasite which causes worldwide human morbidity and mortality. They are transmitted to humans by the bite of the female sandfly of the genus *Phlebotomus* (Murray et al. 2005). The estimated incidence of leishmaniasis is 0.4 million cases of the visceral leishmaniasis (VL) and 1.2 million cases of the cutaneous form (Alvar et al. 2012). Antimonial compounds remain the drug of choice for the treatment of leishmaniasis despite reports of high toxicity and other severe side effects. Amphotericin B and pentamidine are second-line drugs which are also reported to possess serious side effects (Croft and Olliaro 2011). Miltefosine, an oral drug approved for VL in India, has also shown severe side effects. Till date, there is no vaccine against leishmaniasis, and control of VL is entirely dependent upon chemotherapy which itself is not free from its own drawbacks. Increasing cases of resistance to commonly available drugs has posed a serious problem for the disease treatment. Therefore, identification of novel drug targets and effective drugs with less toxicity and therapeutic switching of available drugs are few strategies being implied to combat leishmaniasis.

Sterol biosynthetic pathway has been extensively explored in trypanosomatids for the development of new drugs (de Souza and Rodrigues 2009; Roberts et al. 2003). Currently, the enzymes of this pathway are also being studied in *Leishmania* because of its unique disparity with its mammalian counterpart (Dinesh et al. 2014b). In fungi and

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*Leishmania*, this pathway produces ergosterol which in host is replaced by cholesterol (Jimenez-Jimenez et al. 2008). In eukaryotic cells, sterols are important components which are responsible for membrane fluidity and permeability. They serve as precursor and regulator of cell cycle and development (Roberts et al. 2003). One of the enzymes which are currently being explored in our laboratory is 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) which has statin as one of the well-known inhibitors. Statins are potent inhibitors of cholesterol biosynthesis which are extensively used in the treatment of hyperlipidemia and also considered useful for the prevention of cardiovascular diseases (Taylor et al. 2013). Statins impede cholesterol synthesis by inhibiting HMGR, which is a rate-limiting enzyme in sterol synthesis. Statins have been found to inhibit the growth of many protozoan parasites like *Plasmodium falciparum* and Trypanosomatidae family such as *Trypanosoma cruzi* and various *Leishmania* species (Montalvetti et al. 2000; Pradines et al. 2007; Urbina et al. 1993; Yokoyama et al. 1998). However, recent studies on statins have been widely investigated for their clinical benefits in various diseases like colon, lung, kidney, bone cancer, and central nervous system, as well as diabetes (Liao 2005) apart from exhibiting immunomodulatory effects (Danesh et al. 2003). We had previously demonstrated the antileishmanial potential of inhibitors belonging to statin class of compounds like atorvastatin, simvastatin, and antidepressants like ketanserin and mianserin via targeting HMGR enzyme (Dinesh et al. 2014a, b; Singh et al. 2014).

Mevastatin also known as compactin belongs to the family of statins which are selective inhibitors of HMGR (Fig. 1a). It is the first member of the statin family which is reported as an inhibitor of cholesterol biosynthesis in *Penicillium citrinum* (Endo et al. 1976). Since the discovery of mevastatin in 1976, various other statins have been propelled by distinct pharmaceutical companies (Korlipara 2002; Teo and Burton 2002). Mevastatin has shown inhibition against *P. falciparum* parasite in a stage-dependent manner by altering the biosynthesis of dolichol, dolichyl-P (dolichyl phosphate), and isoprenyl-PP (isoprenyl pyrophosphate) species (Couto et al. 1999). Inhibition of mevalonate pathway by mevastatin is reported to induce apoptosis in human myeloma cell line (Shipman et al. 1998). In mice, it was reported to upregulate the endothelial nitric oxide synthase mRNA and protein levels by inhibiting HMGR. It also reduces the cerebral injury in mice at 20 mg/kg dosage (Amin-Hanjani et al. 2001). Furthermore, it has been shown to inhibit the growth of theca-interstitial cells by interfering with mevalonic acid production thus leading to reduction of both basal- and insulin-induced activation (Kwintkiewicz et al. 2006). Considering the above-cited emerging therapeutic uses of mevastatin, we have made an attempt to find out the antileishmanial potential of mevastatin for the first time and have elucidated its mode of action.

## Materials and methods

### Materials

Mevastatin (2S)-2-methyl-(1S,7S,8S,8aR)-1,2,3,7,8,8a-hexahydro-7-methyl-8-[2-[(2R,4R)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthalenyl butanoate), protease inhibitor cocktail, and (R,S)-3-hydroxy-3-methylglutaryl coenzyme A sodium salt hydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mevastatin was dissolved in 20 % ethanol, and stock solution of this drug was stored at  $-20^{\circ}\text{C}$ . Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco/BRL, Life Technologies Scotland, UK.

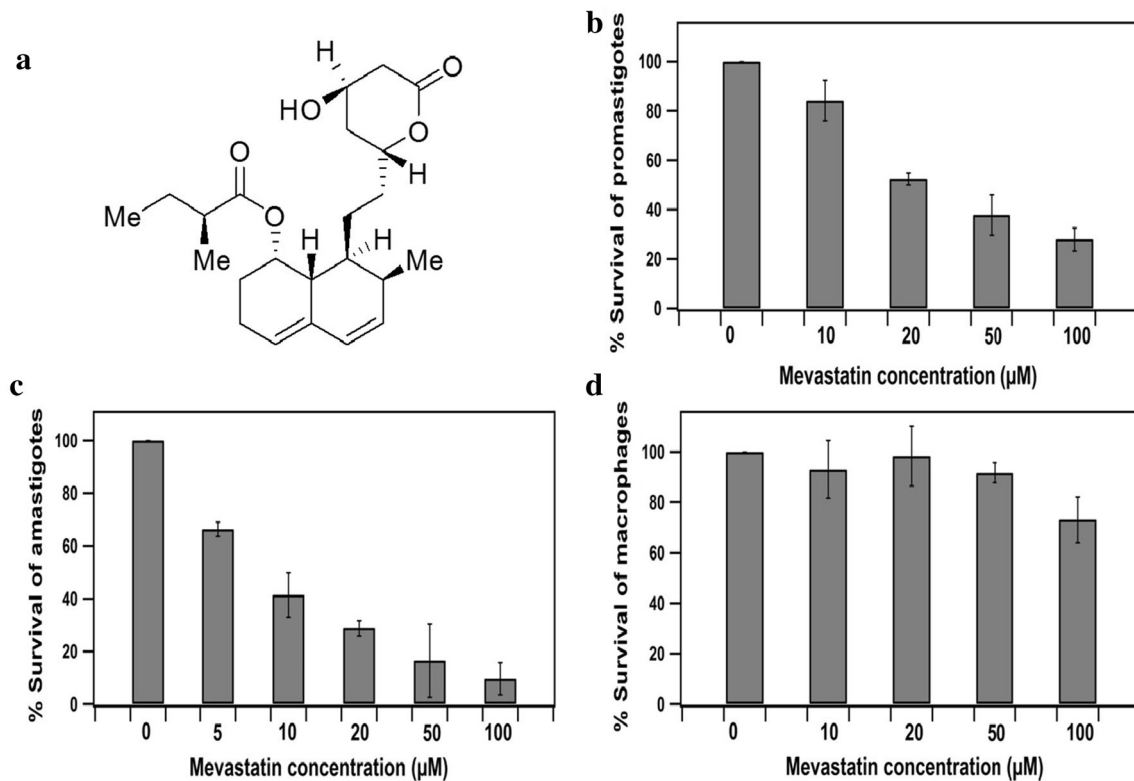
### Parasite and macrophages

Promastigote form of *L. donovani* wild-type (WT, MHOM/80/IN/Dd8) were cultured at  $24^{\circ}\text{C}$  in RPMI-1640 HEPES-modified medium supplemented with 0.2 % sodium bicarbonate, 100  $\mu\text{g}/\text{mL}$  penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 100  $\mu\text{g}/\text{mL}$  gentamycin, and 10 % heat-inactivated FBS. The medium was maintained at pH 7.2. Promastigotes, transfected with psp $\alpha$  hygro $\alpha$  shuttle vector and psp $\alpha$  hygro $\alpha$ -*LdHMGR* episomal overexpression construct, were routinely maintained in 60  $\mu\text{g}/\text{mL}$  hygromycin antibiotic as reported earlier (Singh et al. 2014). These mutant parasites were used in all subsequent experiments. THP-1 cells were routinely cultured in RPMI-1640 medium supplemented with 10 % FBS and maintained at  $37^{\circ}\text{C}$  in a humidified atmosphere with 5 %  $\text{CO}_2$ .

### Evaluation of in vitro antileishmanial activity

Drug sensitivity of *L. donovani* promastigotes was assayed colorimetrically by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described earlier (Mosmann 1983). Briefly,  $2 \times 10^5$  log phase promastigotes of *L. donovani* were incubated for 48 h and treated with increasing concentrations of mevastatin (10–100  $\mu\text{M}$ ) at  $24^{\circ}\text{C}$  for 48 h. The percentage viability of promastigotes was calculated relatively by considering 100 % viability in untreated promastigotes. The results were expressed as mean  $\pm$  SD of at least three independent experiments. Miltefosine was used as the control drug.

Effect of mevastatin on intracellular *L. donovani* amastigotes was assessed via parasite rescue and transformation assay (Jain et al. 2012). Approximately  $2 \times 10^5$  THP-1 monocytes were differentiated into macrophages by treating with 20 ng/mL of phorbol myristate acetate (PMA). Macrophages were allowed to adhere for 48 h and infected with  $2 \times 10^6$  *L. donovani* promastigotes at  $37^{\circ}\text{C}$  in 5 %  $\text{CO}_2$  for 24 h. Unadhered parasites were removed by washing with RPMI medium and the internalized parasite form, i.e., amastigotes



**Fig. 1** (a) Molecular structure of mevastatin (Compactin). Antileishmanial efficacy of mevastatin. Effect of mevastatin on in vitro growth of leishmania (b) promastigotes, (c) amastigotes of *L. donovani*

wild-type (WT), and (d) THP-1 macrophage cell line. The results were represented as mean±standard deviation of three independent experiments

were treated with mevastatin at different concentrations. Parasite-infected macrophages were lysed and amastigotes were reverted back to the promastigotes by growing under suitable conditions. Finally, MTT assay was performed as described earlier. Miltefosine was used as the standard drug. Assay was performed in triplicates, and results were representative of three independent experiments.

### Mammalian cytotoxicity studies

THP-1 monocytes were cultured in RPMI-1640 medium with 10 % FBS at 37 °C in 5 % CO<sub>2</sub>-humidified incubator. Cells were seeded at  $2 \times 10^5$  cells in 96-well microplate, differentiated by adding PMA (20 ng/mL), and incubated at 37 °C with 5 % CO<sub>2</sub> for 48 h. After incubation, unadhered cells were removed by washing with serum-free medium. The cells were incubated at 37 °C for 48 h in a humidified atmosphere of 5 % CO<sub>2</sub> after addition of different concentrations of mevastatin and miltefosine ranging from 10 to 100 μM (10, 20, 50, and 100 μM). The cells were subsequently treated with MTT dye and incubated at 37 °C for 4 h. The cells were then centrifuged at 3,000g, and the pellets were dissolved in DMSO. Viability of the cells was analyzed with a plate reader at a wavelength of 540 nm.

### Evaluation of the effect of mevastatin on HMGR activity in total cell lysates of *L. donovani* promastigotes

The total cell lysate was isolated from *L. donovani* promastigotes by using freeze–thaw lysis method. Briefly,  $1 \times 10^8$  cells were seeded and incubated for 48 h. The 50 % inhibitory concentration (IC<sub>50</sub>) of mevastatin (23 μM) was added, and the cells were further incubated at 24 °C for 48 h. The cells were harvested at 6,000g for 10 min and washed with phosphate buffer pH 7.4. The cell pellet was resuspended in lysis buffer [50 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.2), 1 mM DTT, 2 mM PMSF, and 0.5 mg/mL leupeptin] and incubated on ice for 10 min. The cells were lysed by repeated freeze–thaw cycles in liquid nitrogen. The supernatant was collected after centrifugation at 12,000g for 30 min at 4 °C from untreated and mevastatin-treated samples to analyze HMGR activity by a method described earlier (Hurtado-Guerrero et al. 2002). Protein amount was determined by bicinchoninic acid method using bovine serum albumin as the protein standard (Smith et al. 1985).

### Evaluation of the effect of mevastatin on recombinant HMGR activity

Recombinant *L. donovani* HMGR was overexpressed in *Escherichia coli* and purified using Nickel affinity

chromatography (Dinesh et al. 2014b). In order to evaluate the effect of mevastatin on recombinant *LdHMGR* enzyme, activity of purified enzyme was assayed in the presence of increasing concentrations of mevastatin. Briefly, the reaction mixture contained 50 mM  $\text{KH}_2\text{PO}_4$ , 50 mM KCl, 5 mM DTT, 1 mM EDTA, 0.27 mM NADPH, 0.27 mM HMG-CoA, and recombinant enzyme in a final volume of 200  $\mu\text{L}$  at pH 7.2. The HMG-CoA-dependent oxidation of NADPH was monitored at 340 nm (Singh et al. 2014).

For inhibition kinetics, HMGR enzyme was preincubated with inhibitor for 5 min. Reaction was monitored at 340 nm at regular time intervals until the rate of reaction became constant to detect the disappearance of NADPH. In order to determine the rate of reaction, different concentrations of the substrate HMG-CoA (6.25 to 1,200  $\mu\text{M}$ ) were added to the enzyme in the presence of 42 and 100  $\mu\text{M}$  of mevastatin. Double reciprocal plots were generated for the calculation of various kinetic parameters. The parameters were evaluated by nonlinear regression using Michaelis-Menten equation. One unit (1 U) of HMGR is defined as the amount of enzyme which catalyzes the oxidation of 1  $\mu\text{mol}$  of NADPH per minute.

#### Effect of mevastatin on ergosterol levels of *L. donovani* promastigotes

Neutral lipids were isolated and separated by high-pressure liquid chromatography (HPLC) as previously described (Singh et al. 2014). Approximately  $1 \times 10^6$  *L. donovani* promastigotes were incubated at 24 °C for 48 h and treated with mevastatin for 48 h. Promastigotes were harvested, washed with phosphate-buffered saline (PBS), and resuspended in a mixture of methanol/chloroform/water and incubated on ice for 1 h. After centrifugation, the supernatant was collected and a mixture of water/chloroform (1:1 v/v) was added. The mixture was vortexed for 45 s and centrifuged at 3,000g. The organic layer containing sterols was collected. Samples were prepared and subjected to HPLC method with a  $\text{C}_{18}$  column (Phenomenex) equipped with UV detector wavelength of 282 nm. Estimation of ergosterol was carried out by HPLC as described previously (Ng et al. 2008). Ergosterol (Sigma) was used as a standard.

#### Exogenous supplementation of sterols for reversal of mevastatin-mediated growth inhibition

Reversal of mevastatin-mediated growth inhibition of *L. donovani* promastigotes was studied as described previously (Dinesh et al. 2014b). The drug-treated cells were exogenously supplemented with different concentrations of cholesterol (50, 100, and 200 mmol/L) and ergosterol (50, 100, and 200 mmol/L). MTT assay was performed to estimate the viability of *L. donovani* promastigotes upon rescue by cholesterol and ergosterol. All the assays were performed in triplicates,

and solvent alone and solvent plus ergosterol were used as controls.

#### Effect of mevastatin on *LdHMGR*-overexpressing promastigotes

To determine the specificity of *LdHMGR* towards mevastatin, we had evaluated its effect on HMGR-overexpressing transgenic parasites which were generated by transfecting the psp $\alpha$  hygro $\alpha$ -*LdHMGR* construct into promastigotes as reported previously (Singh et al. 2014). Wild-type promastigotes were taken as control. Effect of mevastatin on the growth of wild-type and *LdHMGR* overexpressors was evaluated by MTT assay.

#### Alterations in parasite morphology by scanning electron microscopy

*L. donovani* untreated and mevastatin-treated promastigotes ( $\text{IC}_{50}$  and  $\text{IC}_{90}$ ) were harvested and washed twice in ice-cold PBS followed by 4 % (w/v) paraformaldehyde in PBS. The cells were fixed in 4 % (w/v) paraformaldehyde containing 2.5 % glutaraldehyde (Sigma) in PBS and allowed to adhere to poly-L-lysine-coated coverslips at room temperature for 12 h. Samples were dehydrated for 10 min at each step of an ascending ethanol series, mounted on metallic stub by a bioadhesive carbon tape, and sputtered with a thin gold layer. Finally, morphological changes in the parasites were visualized by scanning electron microscope (S-3400N, Hitachi, Japan).

#### Accumulation of lipid bodies by fluorescence microscopy

Approximately  $2 \times 10^6$  *L. donovani* promastigotes were treated with an  $\text{IC}_{50}$  (23.3  $\mu\text{M}$ ) and  $\text{IC}_{90}$  (100  $\mu\text{M}$ ) concentration of mevastatin for 48 h. Control and treated parasites were harvested and washed twice in ice-cold PBS. Lipid bodies were labeled with 10  $\mu\text{g}/\text{ml}$  of Nile Red (Sigma) and incubated for 30 min at room temperature. Cells were fixed with 4 % paraformaldehyde in PBS and allowed to adhere to poly-L-lysine-coated coverslips. Formation of lipid bodies in the parasites was observed under fluorescent microscope (Leica, Germany) at a magnification of  $\times 100$ . The images were recorded with a DC300F Leica camera.

#### Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism software version 5.0. To compare two different groups, one-way analysis of variance (ANOVA) was used. Only values of  $p \leq 0.05$  were considered as significant.



## Results

### In vitro activity of mevastatin against *L. donovani* promastigotes, intracellular amastigotes, and evaluation of mammalian cytotoxicity

Effect of mevastatin on the survival of *L. donovani* promastigotes was assayed by incubating the parasites with different concentrations of mevastatin (0–100  $\mu\text{M}$ ), and percentage viability was calculated by performing MTT assay. Solvent-treated samples were taken as controls in the present study. It was observed that mevastatin induced dose-dependent inhibition of promastigote growth. The  $\text{IC}_{50}$  in promastigotes was found to be  $23.8 \pm 4.2$   $\mu\text{M}$  (Fig. 1b). Miltefosine was taken as reference drug. Mevastatin inhibitory effect on intracellular amastigotes was evaluated by transformation rescue assay. The inhibition of amastigotes was also concentration dependent, and the calculated  $\text{IC}_{50}$  value was  $7.5 \pm 1.1$   $\mu\text{M}$  (Fig. 1c). The  $\text{IC}_{50}$  of miltefosine, standard drug used, was 14  $\mu\text{M}$  against promastigotes and 4  $\mu\text{M}$  against amastigotes, respectively, which correlated well with previously published results (Corral et al. 2014). Cytotoxicity of mevastatin against host cells in vitro was determined using THP-1-differentiated macrophages and showed no inhibitory effect till 100  $\mu\text{M}$  (Fig. 1d). Therefore, the selectivity index for mevastatin was calculated to be  $>13.3$   $\mu\text{M}$ .

### Mevastatin inhibits *Ld*HMGR in a competitive manner

To evaluate specific inhibition of mevastatin towards recombinant *Ld*HMGR, the enzyme was purified from *E. coli* using conditions as mentioned earlier (Dinesh et al. 2014b). Mevastatin caused dose-dependent reduction in enzyme activity with the  $\text{IC}_{50}$  value of  $42.2 \pm 3.0$   $\mu\text{M}$  upon treatment with different concentration of mevastatin ranging from 10 to 100  $\mu\text{M}$  (Fig. 2a). To know the inhibitory mechanism of mevastatin, enzyme inhibition studies were performed at different time intervals. The results indicated that the enzyme follows classical Michaelis-Menten kinetics with increasing concentration of HMG-CoA as the substrate (Fig. 2b). The calculated  $K_m$  and  $V_{\text{max}}$  were found to be  $38.4 \pm 6.5$   $\mu\text{M}$  and  $1.1 \pm 0.1$  units/mg, respectively, and were similar to the results reported earlier (Dinesh et al. 2014b). At 42  $\mu\text{M}$  mevastatin concentration,  $K_m$  increased to  $81.26 \pm 1.7$   $\mu\text{M}$ , and it increased further to a  $K_m$  value of  $122.6 \pm 9.8$   $\mu\text{M}$  at 100  $\mu\text{M}$  of mevastatin. Since the  $V_{\text{max}}$  of the enzyme remained constant with an increase in apparent  $K_m$ ; it suggested that mevastatin could be acting as a competitive inhibitor of HMGR. The effect of mevastatin ( $\text{IC}_{50}$  concentration) on the total cell lysate of promastigotes was also evaluated, and  $\sim 40$  % reduction in the HMGR activity in mevastatin-treated promastigote cell lysate compared to untreated cell lysate was observed (Fig. 2c).

### Mevastatin alters ergosterol levels in *L. donovani* promastigotes

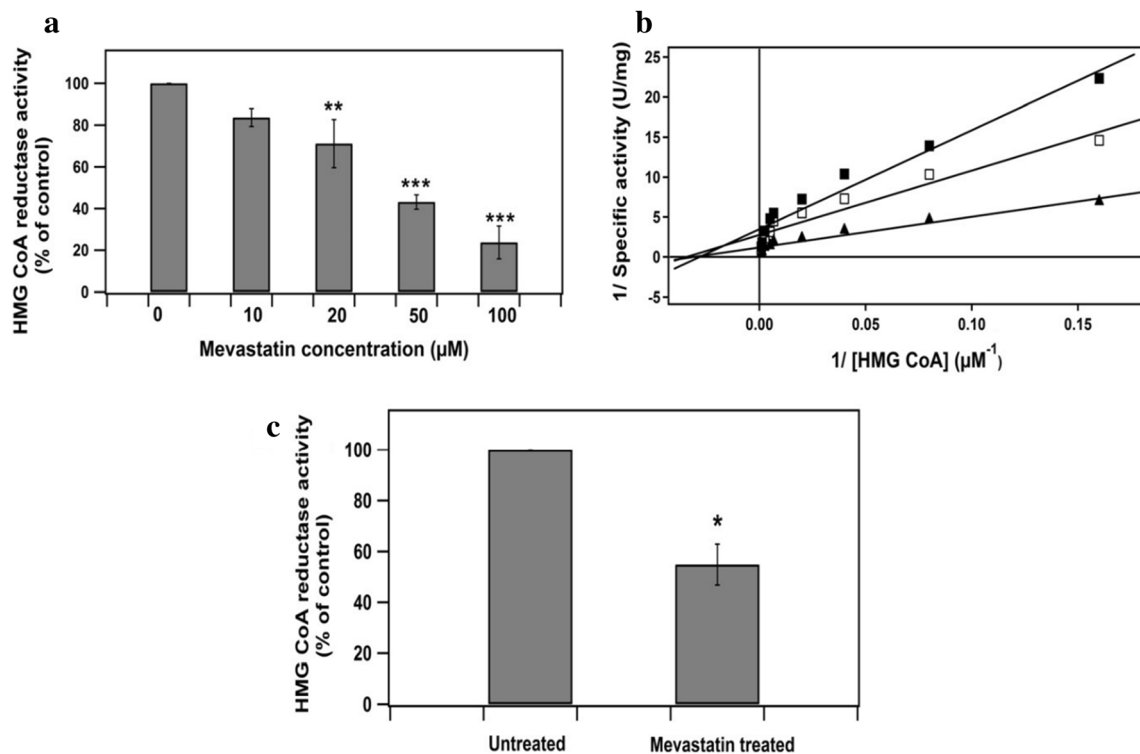
To evaluate the effect of mevastatin on ergosterol levels, neutral lipids were isolated from drug-treated and untreated promastigotes and subjected to HPLC. Figure 3a represents standard ergosterol peak obtained at a retention time of  $\sim 11.8$  min which correlated with earlier reported data (Dinesh et al. 2014a). Subpanels b and c of Fig. 3, respectively, showed the chromatogram separation of ergosterol isolated from control and mevastatin-treated samples with a peak at a retention time of 11.8 min. Results demonstrated  $\sim 66$  % inhibition of ergosterol levels in mevastatin-treated cells compared to the untreated cells (Fig. 3d), thereby demonstrating that mevastatin interferes with the ergosterol biosynthetic pathway.

### Ergosterol but not cholesterol reverses mevastatin-mediated growth inhibition of *L. donovani* promastigotes

To further confirm whether the antiproliferative effect of mevastatin was due to the depletion of ergosterol levels, the promastigotes were treated with mevastatin at its  $\text{IC}_{50}$  value for 48 h. Subsequently, the cells were supplemented with ergosterol and cholesterol exogenously to see the restoration of inhibition upon supplementation of sterols by MTT assay. Reversal data revealed that 200 mmol/L of ergosterol causes significant revival of the growth (80 %), whereas the same concentration of cholesterol supplementation had no effect on the revival of the growth (Fig. 3e). Thus, it is clearly evident that mevastatin-mediated *Leishmania* cell death was only due to the depletion of ergosterol levels. Ergosterol and cholesterol alone did not cause any cytotoxicity to the cells.

### HMGR overexpression renders *L. donovani* promastigotes resistant to mevastatin inhibition

To further prove the specificity of mevastatin towards the HMGR enzyme, effect of mevastatin on the viability of HMGR-overexpressing *Leishmania* promastigotes was carried out by using HMGR-overexpressing transgenic parasites as reported earlier. The wild-type and HMGR-overexpressing parasites were treated with a range of mevastatin concentration (10–100  $\mu\text{M}$ ) and incubated for 48 h. HMGR overexpressors exhibited an  $\text{IC}_{50}$  value of  $68 \pm 4.1$   $\mu\text{M}$ , i.e.,  $\sim 3$ -fold higher than the  $\text{IC}_{50}$  value of wild-type promastigotes (Fig. 4). Our results confirm that the overexpression of HMGR leads to decreased sensitivity towards mevastatin, and hence HMGR is a specific target for mevastatin.



**Fig. 2** Inhibitory effect of mevastatin on HMGR activity. **(a)** Effect of mevastatin on recombinant *Ld*HMGR enzyme. **(b)** Inhibition kinetics of HMGR in presence of mevastatin. Double reciprocal plot showing competitive mode of inhibition of recombinant *Ld*HMGR by mevastatin. The *bottom line* is a control curve without inhibitor. The

remaining *curves*, *middle line*, and *top line* are for 42 and 100 µM concentration of mevastatin, respectively. **(c)** Effect of mevastatin on HMGR activity in total cell lysate of promastigotes. Data was expressed as mean±standard deviations from three independent experiments. \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , and \*\*\* $p \leq 0.001$

### Mevastatin leads to alteration in parasite morphology

Changes in the morphology of the promastigotes upon mevastatin treatment were investigated using SEM. Figure 5a–c shows the elongated and flagellated shape of the untreated parasites. Subpanels d–f and g–i of Fig. 5 show parasite morphology at an  $IC_{50}$  value of 23.8 µM of mevastatin and  $IC_{90}$  value of 100 µM of mevastatin, respectively. At the  $IC_{50}$  value, the parasite starts losing its elongated shape, and at  $IC_{90}$ , it assumes a rounded morphology. This revealed that mevastatin treatment results in alteration in parasite morphology.

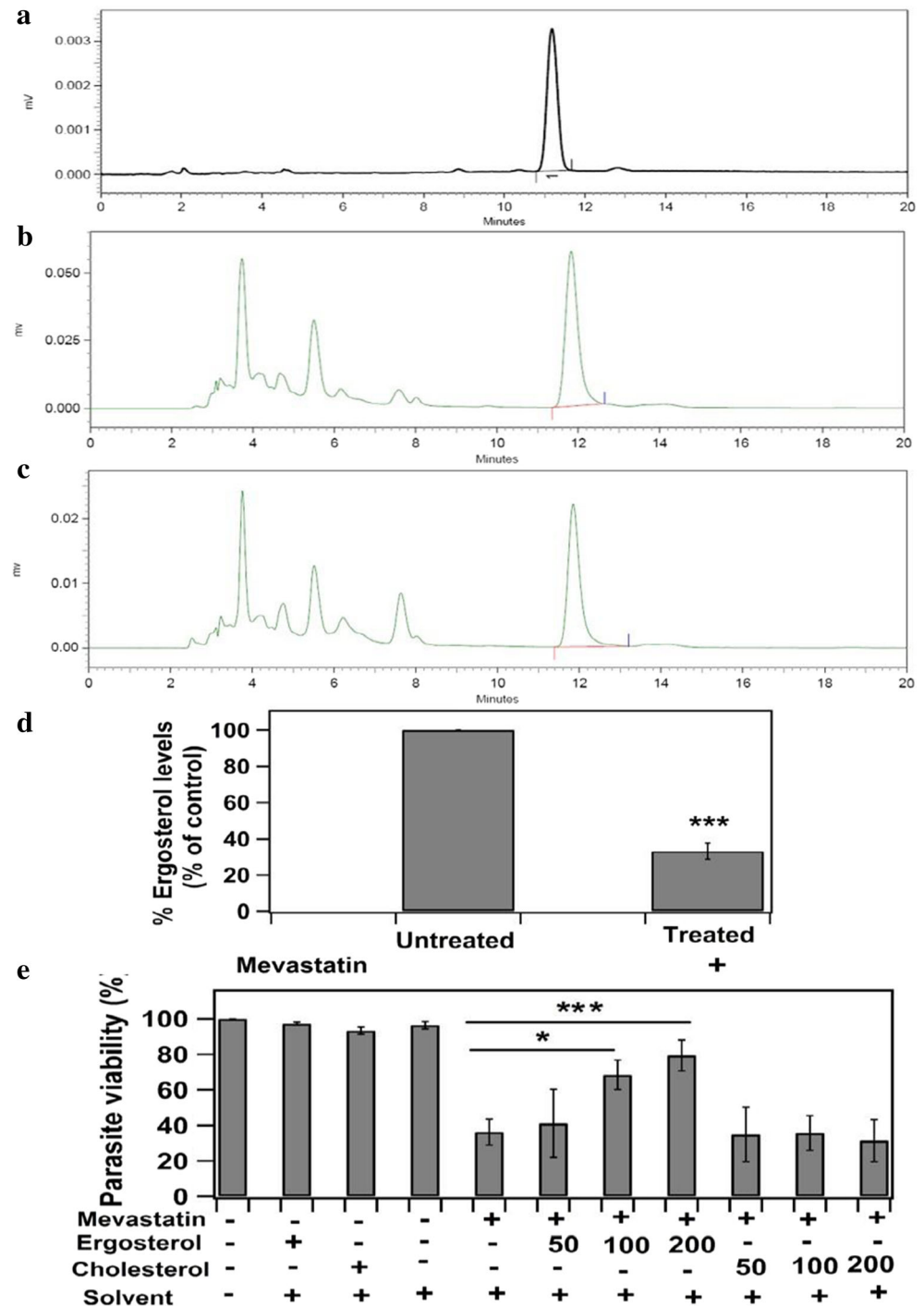
### Mevastatin causes lipid body accumulation

Cytoplasmic lipid body accumulation upon mevastatin treatment was evaluated by Nile Red staining, which binds to neutral lipids. The fluorescence images demonstrated an accumulation of lipid bodies that are randomly distributed throughout the cytoplasm. As shown in Fig. 6c–f, there was a gradual increase in lipid body formation in parasites with the increase in concentration of mevastatin compared to mevastatin-untreated cells (Fig. 6a, b).

### Discussion

In the absence of vaccine for prevention of leishmaniasis, the current treatment regimen is completely based on chemotherapy which is far from satisfactory. Therefore, there is an urgent need to search for new compounds with an antileishmanial potential (Gonzalez-Coloma et al. 2012; Mishra et al. 2013). This is the first report demonstrating the antileishmanial activity of mevastatin. Mevastatin, also known as compactin or ML236B, belongs to polyketide group of drugs which includes anticancer drugs, cholesterol-lowering drugs, and immunosuppressants to name a few (Harsha et al. 2013). Statins are known inhibitors of HMGR which catalyzes the rate-limiting step of the sterol biosynthetic pathway leading to synthesis of cholesterol and ergosterol in humans and in fungi and *Leishmania*, respectively (Stancu and Sima 2001). Besides, statins are also reported to inhibit synthesis of farnesyl pyrophosphate, geranylgeranyl pyrophosphate, guanosine triphosphate-binding protein Ras, and Ras-like proteins (Cordle et al. 2005). Statins have gained lot of attention because of its potential to inhibit the growth of bacteria (Catron et al. 2004), yeast (Song et al. 2003), and protozoa (Wong and Davis 2009). Statins are also reported to exert a broad range of antiviral activity against HIV-1 (Amet et al. 2008), poliovirus

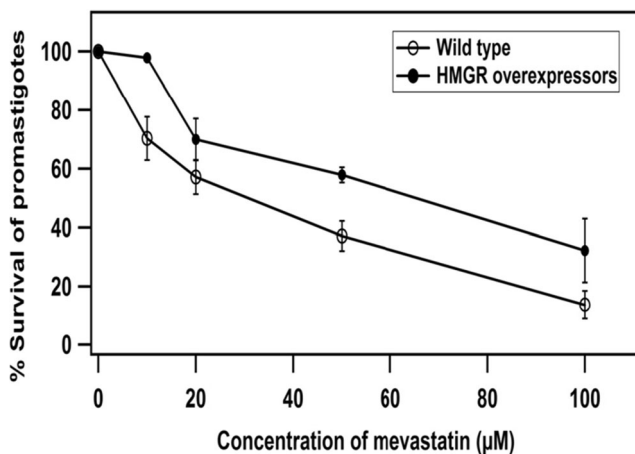
**Fig. 3** Estimation of ergosterol levels in mevastatin-treated *L. donovani* promastigotes. **(a)** Chromatogram profile of ergosterol as internal standard, HPLC profile of *L. donovani* neutral lipid fraction of untreated **(b)** and mevastatin-treated **(c)** promastigotes. A bar graph showing percentage inhibition of ergosterol levels **(d)**. Reversal of mevastatin-mediated inhibition of *L. donovani* promastigotes. **(e)** Reversal was carried out using 50, 100, and 200 mmol/L each of ergosterol and cholesterol. Data was expressed as mean±standard deviations from three independent experiments. \* $p \leq 0.05$ , \*\*\* $p \leq 0.001$



(Liu et al. 2006), and cytomegalovirus (Potena et al. 2004). Simvastatin has been shown to inhibit the growth of both procyclic and epimastigote forms of trypanosome with an  $IC_{50}$  value of 25–50  $\mu$ M (Coppens et al. 1995). Recently, we had reported the antileishmanial efficacy of statins such as atorvastatin and simvastatin (Dinesh et al. 2014b).

Mevastatin is reported to possess hexahydronaphthalene skeleton substituted with a  $\beta$ -hydroxy- $\delta$ -lactone moiety (Endo 1992). Couto et al. reported that mevastatin alters the

synthesis of N-linked oligosaccharides in *P. falciparum* through the inhibition of dolichol synthesis which is required for isoprenoid pathway. Mevastatin was found to inhibit *P. falciparum* at 120  $\mu$ M (Couto et al. 1999). It was also reported to exert antibacterial activity against *Chlamydia trachomatis* which causes urogenital infections and blindness (Bashmakov et al. 2010). Mevastatin possesses in vitro anti-hepatitis C virus (HCV) activity (Delang et al. 2009). Mevastatin is also reported to abrogate proliferation of ovarian



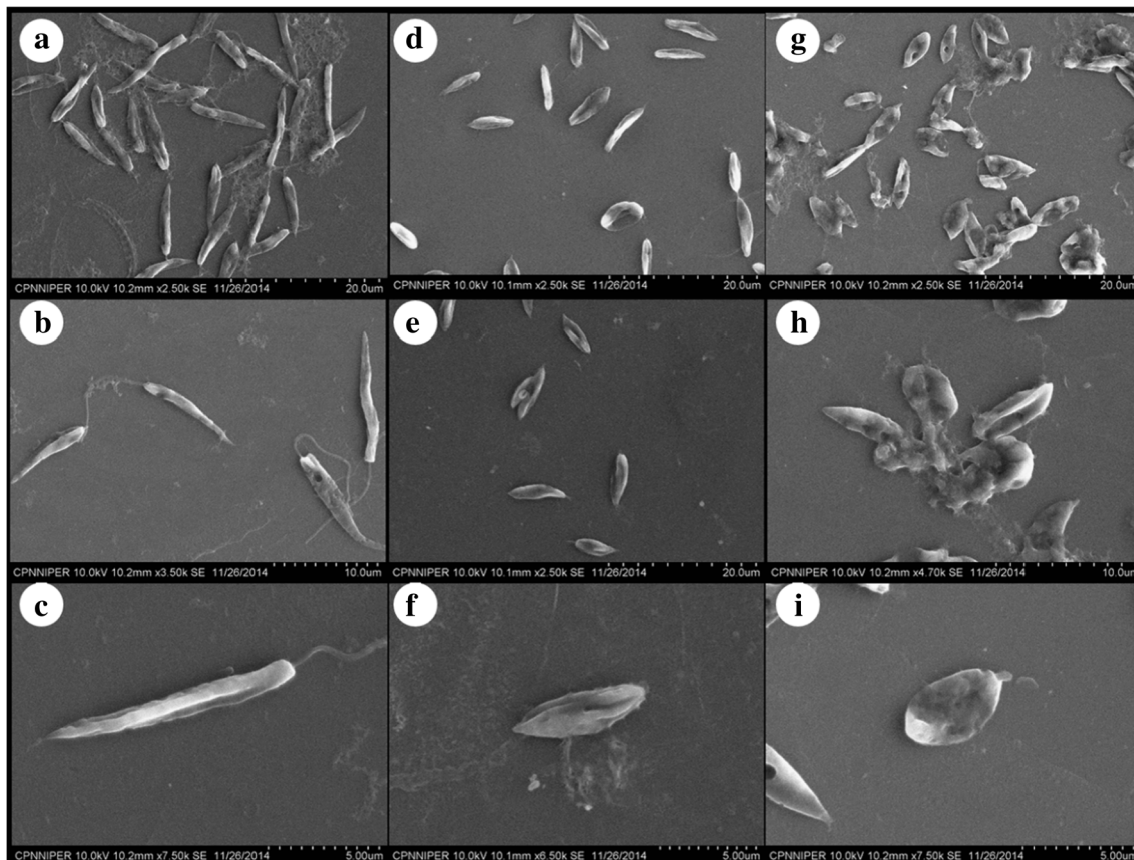
**Fig. 4** Evaluation of the effect of mevastatin on growth of *L. donovani* wild-type and overexpressors of *LdHMGR*. Data was expressed as mean  $\pm$  standard deviations from three independent experiments

theca interstitial cell by inhibiting cholesterol biosynthesis (Izquierdo et al. 2004). It has also exhibited anticancer properties by inhibiting colon cancer cell (Ukomadu and Dutta 2003). In humans, HMGR is specifically inhibited by family of potent competitive and irreversible inhibitors like simvastatin and lovastatin (Goldstein and Brown 1990). An

intrusion with the mevalonate pathway has been already exploited to fight against *Leishmania* parasite.

In the current study, we have demonstrated the effect of mevastatin on mevalonate pathway and its specific inhibition of the parasite HMGR enzyme. We observed that mevastatin inhibits not only the extracellular parasite stage but also the intracellular parasite stage in low micromolar range. The  $IC_{50}$  value of mevastatin was determined as 23.8  $\mu$ M which is very close to the  $IC_{50}$  value of 19.4  $\mu$ M for atorvastatin and is lower when compared to simvastatin (73.2  $\mu$ M) against *Leishmania donovani* promastigotes (Dinesh et al. 2014b). Interestingly, cytotoxicity of mevastatin on macrophage cell line suggested that it has no toxic effect up to 48 h which shows it is parasite selective. The parasite rescue/transformation assay is used to determine the effect of mevastatin on intracellular amastigotes.  $IC_{50}$  value was determined as 7.5  $\mu$ M which suggested that amastigotes, which are important in establishing the infection, exhibited more sensitivity than promastigotes.

Since mevastatin is a known inhibitor of HMGR enzyme, we evaluated its effect on recombinant *LdHMGR* enzyme. Mevastatin inhibits the activity of HMGR in a dose-dependent manner. The  $IC_{50}$  value was determined as

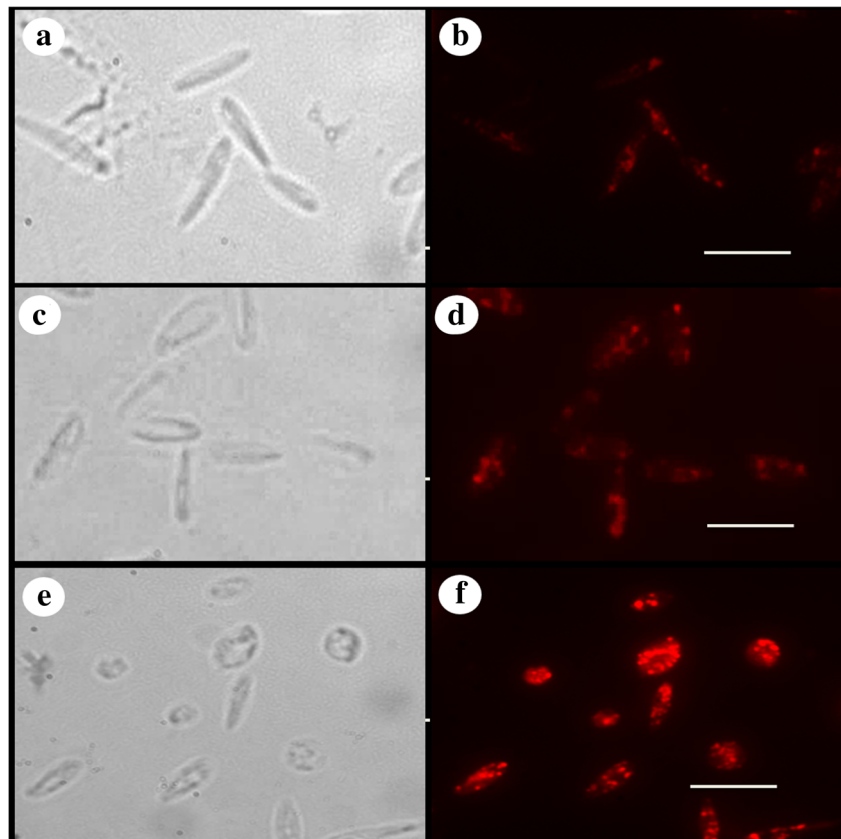


**Fig. 5** Scanning electron microscopy (SEM) of *L. donovani* promastigotes. Control parasites (a–c) and promastigotes that were treated with  $IC_{50}$  (d–f) and  $IC_{90}$  (g–i) concentration of mevastatin for

48 h were visualized by SEM. Untreated parasites show elongated shape with flagella (a–c), whereas mevastatin-treated promastigotes show decrease in size and rounded up morphology (d–i)



**Fig. 6** Analysis of lipid body formation in promastigotes upon mevastatin treatment. Differential interference contrast (DIC) microscopy (**a, c, e**) and fluorescence microscopy using Nile Red (**b, d, f**) of untreated (**a, b**) and treated *L. donovani* promastigotes with  $IC_{50}$  (**c, d**) and  $IC_{90}$  (**e, f**) concentration of mevastatin for 48 h. Image shows the presence of lipid droplets in the cytoplasm of the treated parasites. Scale bar indicates a distance of 10  $\mu$ m



42.2  $\mu$ M which was surprisingly higher than the  $IC_{50}$  concentration determined from the cell-based assay. Kinetic analysis revealed that the inhibition of the *L. donovani* HMGR activity by mevastatin was competitive. The structural similarity between mevastatin and HMG-CoA and the observed competition by these two molecules explained the structure–activity relationship in the inhibition of HMGR (Endo 1992). It was earlier reported that the inhibition of HMGR by mevastatin is competitive with respect to HMG-CoA in case of rat liver enzyme (Endo et al. 1976). Mevastatin-mediated inhibition of yeast HMGR is reported to be competitive with respect to HMG-CoA (Nakamura and Abeles 1985). However, when the effect of mevastatin was tested on the native parasite HMGR enzyme, it caused 70 % inhibition at 20  $\mu$ M of mevastatin concentration. Ergosterol is reported to be essential for survival of *Leishmania* and fungi (Kulkarni et al. 2013). To prove that mevastatin interferes with the sterol biosynthetic pathway, we looked at the alteration in the end product of sterol metabolism, i.e., ergosterol levels. We observed that due to mevastatin treatment, the ergosterol levels drastically reduced (66 %) confirming the specificity of mevastatin to sterol biosynthetic pathway of the parasite. To validate further, various concentrations of ergosterol and cholesterol were added exogenously to the mevastatin-treated cells for the revival of growth. Interestingly, ergosterol caused 80 % reversal of mevastatin-mediated growth inhibition, but cholesterol

supplementation could not overcome the growth inhibition. Previous reports indicated that mevastatin-induced block in cell cycle progression in fibroblast cells was overcome by supplementation of mevalonate not cholesterol (Endo 1992). Apart from causing inhibition of HMGR, mevastatin also caused change in parasite morphology from elongated to rounded shape. Mevastatin-induced morphological changes were also reported in microglial cells and fibroblast cells (Schmidt et al. 1982). Effect of mevastatin on accumulation of lipid storage bodies was confirmed through Nile Red staining by fluorescence microscopy. The increased deposition of lipid bodies may suggest the interference of drug with the phospholipids and sterol content. Similar effect on lipid deposition in parasites of *Leishmania* and *Trypanosome* was earlier reported with various drugs like 22,26-azasterol, S-limonene, and amiodarone (Britta et al. 2014; de Macedo-Silva et al. 2011; Rodrigues et al. 2002).

Our findings suggested that mevastatin specifically inhibits the sterol metabolism of *L. donovani* by targeting HMGR. Based on the above results, the following sequence of events are unfolded upon mevastatin treatment (i) inhibition of HMGR enzyme, (ii) alteration in ergosterol levels, (iii) lipid body accumulation, and (iv) parasite death. In conclusion, the efficacy of mevastatin as a potential candidate for treatment of leishmaniasis has been explored, and its mode of action has

been elucidated. The effect of mevastatin in an experimental animal model of VL will provide significant insights into its anti-VL efficacy.

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