

Statins: 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) Reductase Inhibitors Demonstrate Anti-Atherosclerotic Character due to Their Antioxidant Capacity

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Abstract Atherosclerosis is a chronic inflammatory disease of multiple etiologies. It is associated with the accumulation of oxidized lipids in arterial lesions leading to coronary heart disease. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (commonly known as statins) are widely used in cardiovascular disease prevention to lower the cholesterol. The antioxidant activity of HMG-CoA reductase inhibitors was studied by lipid peroxidation inhibition assay, DPPH, and hydroxyl radical scavenging-activity methods. The lovastatin (93%) and simvastatin (96%) showed significant action of lipid peroxidation inhibition compared to other HMG-CoA reductase inhibitors. The DPPH radical and hydroxyl radical scavenging activity of simvastatin was 38% and 33%, respectively. The oxidative modification of serum lipid due to reactive oxygen species causes atherosclerosis. This study revealed the importance of lovastatin and simvastatin to prevent oxidative stress-related cardiovascular diseases.

Keywords HMG-CoA reductase inhibitors · Statins · Antioxidant · Lipid peroxidation · DPPH · Hydroxyl radical

Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase converts HMG-CoA to mevalonate; it is a key catalytic step in the biosynthesis of cholesterol. Inhibition of HMG-CoA reductase results in decreased synthesis of cholesterol and other products

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downstream of mevalonate [1]. HMG-CoA reductase inhibitors (statins) are extensively used as cholesterol-lowering agents because of their ability to block hepatic conversion of HMG-CoA to L-mevalonate and production of isoprenoid geranylgeranylpyrophosphate [2, 3]. For the patients with hyperlipidemia, pravastatin, fluvastatin, and simvastatin are generally used in clinical therapy [4]. Administration of HMG-CoA reductase inhibitors results in decreased risk of coronary and cerebrovascular events and increased survival rates in patients with coronary artery disease [5].

It is proposed that oxidative modification of low-density lipoprotein (LDL) in the arterial wall plays an important role in the development and progression of atherosclerosis. Fluvastatin is known to decrease the rate of oxidation of LDL in the propagation step and scavenges hydroxyl and superoxide radicals [6, 7]. Inhibition of lipid peroxidation was observed by the reduced amount of serum thiobarbituric acid reactive substances in cholesterol-fed rabbits [8]. HMG-CoA reductase inhibitors at a dose comparable to the dose used clinically in humans has protective effects against chemically induced oxidative damage to biological molecules [9].

It is now recognized that free radical species are responsible for numerous types of cell damage, membrane peroxidation, DNA modification, enzyme inactivation, and oxidation of proteins [10]. Free radical-mediated lipid peroxidation, oxidative stress, and antioxidants continue to be widely discussed. An unavoidable consequence of respiration is the formation of free radicals such as superoxide anion (O_2^-) and hydroxyl radical (OH^\cdot). Uncontrolled, generated free radicals are very unstable and react rapidly with other chemical groups or substances in the body, leading to cell or tissue injury. Moreover, uncontrolled lipid peroxidation is involved in the occurrence of numerous chronic diseases [11, 12]. It is known that a chronic imbalance between formation of reactive oxygen species and antioxidant capacity characterizes many disease states such as reperfusion injury, atherosclerosis, carcinogenesis, and premature aging [13]. HMG-CoA reductase inhibitors are the drugs of choice for lowering cholesterol and reducing cardiovascular events to a greater extent than can be explained by their effect on lipids, and are attributed to possessing pleiotropic actions in reducing atherosclerosis [14]. However, the comparative antioxidant effect of different HMG-CoA reductase inhibitors is known moderately. The evaluation of antioxidant properties of therapeutic molecules is of particular importance for their potential application to prevent the damage induced by free radicals. In the present study, we evaluated the antioxidant effects of various HMG-CoA reductase inhibitors on the levels of lipid peroxidation and free radical species.

Materials and Method

Preparation of Sample

The HMG-CoA reductase inhibitors: rosuvastatin/crestor, simvastatin/zocor/lipex, pravastatin/pravachol/selektine/lipostat and lovastatin/mevacor/altocor (Ranbaxy, New Delhi and Goa, India), and atorvastatin/lipitor/torvast (Cadila Health Care Limited, Baddi, Himachal Pradesh, India) were purchased from local pharmacy. 2,2,-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Chemicals, USA. Trichloroacetic acid (TCA) and dimethyl sulfoxide (DMSO) was purchased from Merck Specialties Private Limited, Mumbai, India. Thiobarbituric acid (TBA) L-ascorbic acid and Tris were obtained from Hi-Media Laboratories, Mumbai, India. The powdered HMG-CoA reductase inhibitor samples were prepared at 1-mg/ml concentration in distilled water. The prepared sample was used in different concentration for determination of antioxidant activity assay purpose.

Inhibition of Lipid Peroxidation

Lipid peroxidation inhibitory activity of HMG-CoA reductase inhibitors was measured according to the method of Dhale et al. [15]. Egg lecithin (3 mg/ml in phosphate buffer, pH 7.4) was sonicated (Microsonix Microson Ultrasonic Cell Disruptor) for 30 min to obtain small membrane liposome vesicles. Different concentrations of HMG-CoA reductase inhibitors (33–132 µg/ml) were added to 0.5 ml of liposome mixture, and the lipid peroxidation was induced by adding 10 µl of FeCl₃ (400 mM) and 10 µl L-ascorbic acid (200 mM). After 60 min of incubation at 37 °C, the reaction was inhibited by adding 1 ml of HCl (0.25 N) containing 15% of TCA and 0.375% of TBA. This reaction mixture was kept in a boiling water bath for 15 min, then cooled and centrifuged at 5000 rpm for 5 min. The absorbance of supernatant was measured at 532 nm. Blank and control were maintained without liposome and sample, respectively. All the tests were carried out by maintaining appropriate blanks and controls. Inhibition of lipid peroxidation activity was calculated as follows:

$$\text{inhibition (\%)} = \left(\frac{\text{control} - \text{sample}}{\text{control}} \right) \times 100.$$

DPPH Radical Scavenging Activity

Radical scavenging activity of HMG-CoA reductase inhibitors was estimated using DPPH radicals [16]. In a total of 2 ml of reaction mixture, different concentrations (25–100 µg/ml) of HMG-CoA reductase inhibitors were mixed with Tris HCl buffer (100 mM, pH 7.4) and 1 ml DPPH (500 µM in ethanol). The mixture was shaken vigorously and incubated at room temperature for 30 min. The absorbance of the resulting solution was measured by UV-Visible Spectrophotometer (UV-Visible 2450, Shimadzu, Japan) at 517 nm. Blank and control without DPPH and sample were maintained, respectively. All the experiments were carried out by maintaining appropriate blanks and controls. The radical scavenging activity was measured using the following formula:

$$\text{radical scavenging activity (\%)} = \left(1 - \frac{A_{\text{sample (517 nm)}}}{A_{\text{control (517 nm)}}} \right) \times 100.$$

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity was determined according to the method of Singh et al. [17]. Various concentrations of HMG-CoA reductase inhibitors (28–114 µg/ml) were taken in different test tubes. To these, 250 µl of iron EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 125 µl of EDTA (0.018%), and 250 µl of DMSO (0.85% v/v in 0.1 M of phosphate buffer, pH 7.4) were added. The reaction was initiated by the addition of 125 µl of ascorbic acid (0.22%). After 15 min of incubation at 85 °C, the reaction was inhibited by adding a 250 µl of ice-cold TCA (17.5%) and 750 µl of Nash reagent. The absorbance was measured at 412 nm after 30 min of incubation at room temperature. The reaction mixture without ascorbic acid and sample were served as blank and control, respectively. Hydroxyl radical scavenging activity (%) was measured using following formula:

$$\text{inhibition (\%)} = \left(\frac{\text{control} - \text{sample}}{\text{control}} \right) \times 100.$$

Statistical Analysis

All determinations were carried out in triplicate. Data was analyzed using Microsoft Excel Windows Vista (Microsoft Co., Redmond, WA, USA), and statistical analysis was performed with statistical analysis software SPSS-10 (SPSS Inc., Chicago, IL, USA). Statistical differences between means were determined by one-way ANOVA, and post-hoc analysis was performed by Duncan's test for multiple comparisons. *P* values of <0.05 was considered significant.

Results and Discussion

Peroxidation of lipids is a complex process that involves formation and propagation of lipid radicals, and in presence of oxygen, the lipid hydroperoxides are formed as primary products of lipid oxidation. The oxidation hypothesis of atherosclerosis suggests that the initiating event in the development of atherosclerosis is an oxidative modification of LDL that significantly increases its uptake into the arterial intima [18]. The antioxidant activity of different HMG-CoA reductase inhibitors (Fig. 1) to effectively prevent atherosclerosis was investigated and compared in this study using different in vitro model systems.

Figure 2 shows the comparative inhibition of lipid peroxidation of five HMG-CoA reductase inhibitors. Henriksen et al. [19] proposed that modified LDL could account for the accumulation of lipid within macrophages, a critical early step in the formation of the atherosclerotic plaque. Oxidation of LDL is a free radical-driven chain reaction where polyunsaturated fatty acids are converted to lipid peroxides and easily decompose to biologically active aldehydes. Macrophages avidly accumulate LDL particles modified by oxidation or acetylation through a number of scavenger receptors. This leads to the

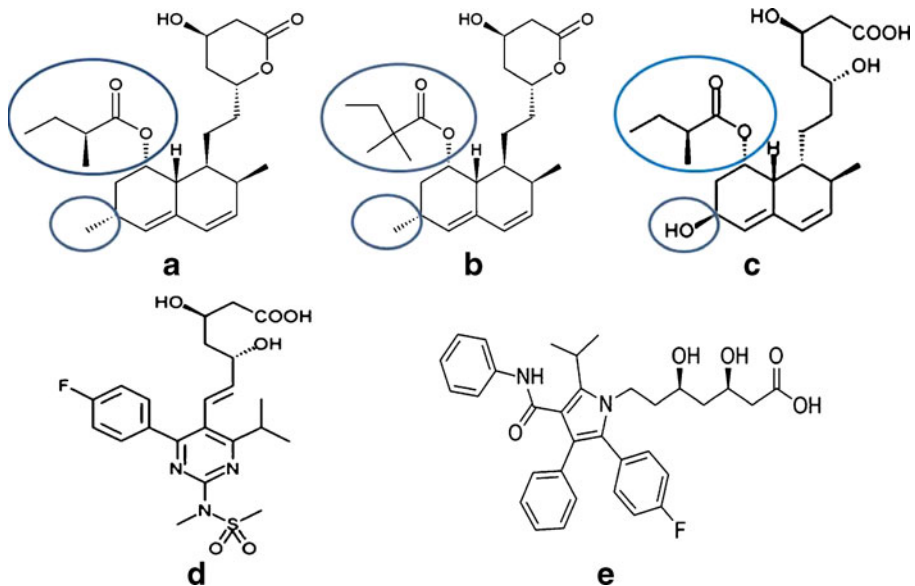


Fig. 1 HMG-CoA reductase inhibitors used for antioxidant assay: **a** lovastatin, **b** simvastatin, **c** pravastatin, **d** rosuvastatin, and **e** atorvastatin

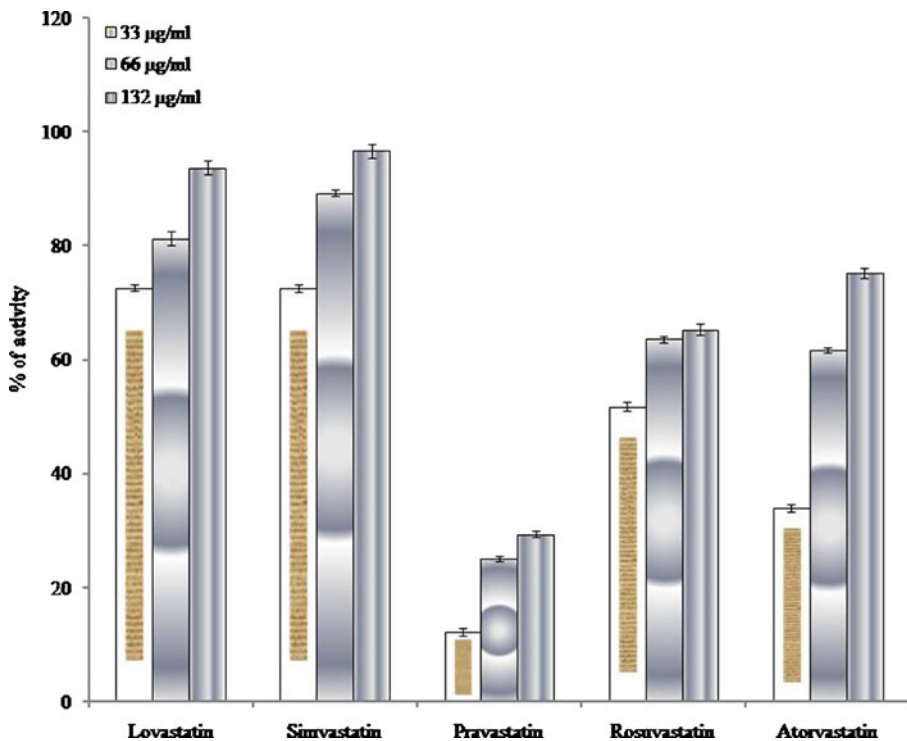


Fig. 2 Lipid peroxidation inhibition of HMG-CoA reductase inhibitors was measured at different concentration. Values are mean \pm SEM of triplicates shown by vertical bars with different concentration. Mean values with different vertical bars were significantly different by Duncan's test at $p < 0.05$

accumulation of foam cells and development of the atherosclerotic plaque [18, 20]. During lipid peroxidation process, at 132 $\mu\text{g/ml}$, simvastatin and lovastatin inhibited 96% and 93% peroxidation, respectively, while rosuvastatin inhibited 65% lipid peroxidation at the same concentration. Higher inhibitory activity of lipid peroxidation was observed with increased concentration of HMG-CoA reductase inhibitors. The lipid peroxidation inhibition experiment was measured by estimating the malnaldehyde formed during the reaction, production of low concentration of malnaldehyde suggested the importance of HMG-CoA reductase inhibitors. The lipid peroxidation was induced by the Fe^{3+} and ascorbic acid. The transition metal ions initiate the chain reaction of hydroperoxide and alkoxy radical formation [21].

The protective effect of HMG-CoA reductase inhibitors on the formation of thiobarbituric acid reactive substances may be due to its ability to scavenge free radicals, but other mechanisms such as chelation of transient metal ions cannot be eliminated. Free iron or copper ions, in fact, are essential for the formation of reactive oxygen species such as hydroxyl radicals generated by the Fenton reaction [9, 22]. In the above experiments, the lipid peroxidation and hydroxyl radicals were induced by metal ion such as Fe^{2+} ; this indicate that the inhibition of lipid peroxidation and hydroxyl radical scavenging activity apparently are due to metal chelation or they could be due to the chain termination of peroxy and hydroxyl radical formation reaction. The unique feature of lovastatin and simvastatin as an antioxidant to inhibit the lipid peroxidation and scavenging hydroxyl radicals, induced by Fe^{2+} , was confirmed by antioxidative tests.

Free radicals are highly reactive, short-lived, and toxic molecules that have one or more unpaired electrons. Over production of free radicals within tissue can damage DNA, proteins, lipids, and carbohydrates [23]. The significant difference in the DPPH radical scavenging activity of HMG-CoA reductase inhibitors was not observed (Fig. 3). Highest DPPH radical scavenging activity (38%) was observed by simvastatin and lowest by rosuvastatin (25%). The mechanism of antioxidant activity of HMG-CoA reductase inhibitors is by donating the electrons to the free radicals. Even the antioxidant mechanism of HMG-CoA reductase inhibitors was evidenced by reduced hypertrophy in the neonatal rat cardiac myocytes treated with angiotensin II [24].

The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells. In the presence of transition metal ion like Fe^{2+} or Cu^{2+} , H_2O_2 can break down and release more highly reactive hydroxyl radicals and participate in self-propagating chain reaction with membrane lipids [22]. Hydroxyl radical scavenging activity is estimated by generating the hydroxyl radical using ascorbic acid–iron EDTA. The hydroxyl radical scavenging activity of HMG-CoA reductase inhibitors observed was dose dependent (Fig. 4). Simvastatin and rosuvastatin scavenged 33% and 23% hydroxyl radicals at 114 $\mu\text{g}/\text{ml}$ of concentration, respectively. Lovastatin and atorvastatin scavenged 11% hydroxyl radicals. Although all oxyradicals are potentially toxic, their chemical reactivity towards biological targets is different; accordingly, the relative efficiency of an antioxidant can vary depending on the oxidant [25].

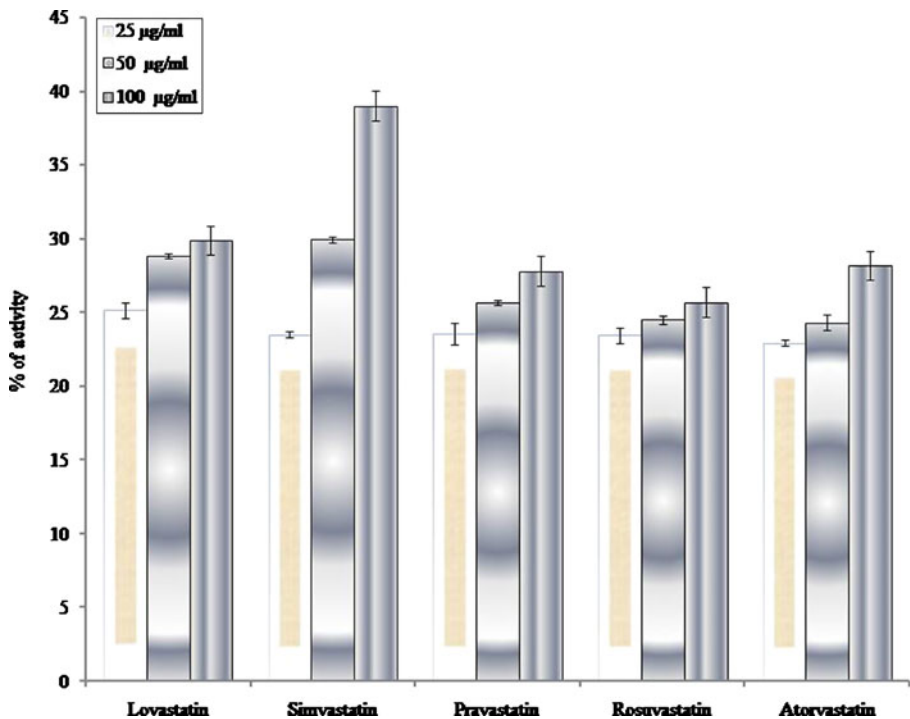


Fig. 3 DPPH radical scavenging activity of HMG-CoA reductase inhibitors was measured at different concentration. Values are mean \pm SEM of triplicates shown by vertical bars with different concentration. Mean values with different vertical bars were significantly different by Duncan's test at $p < 0.05$

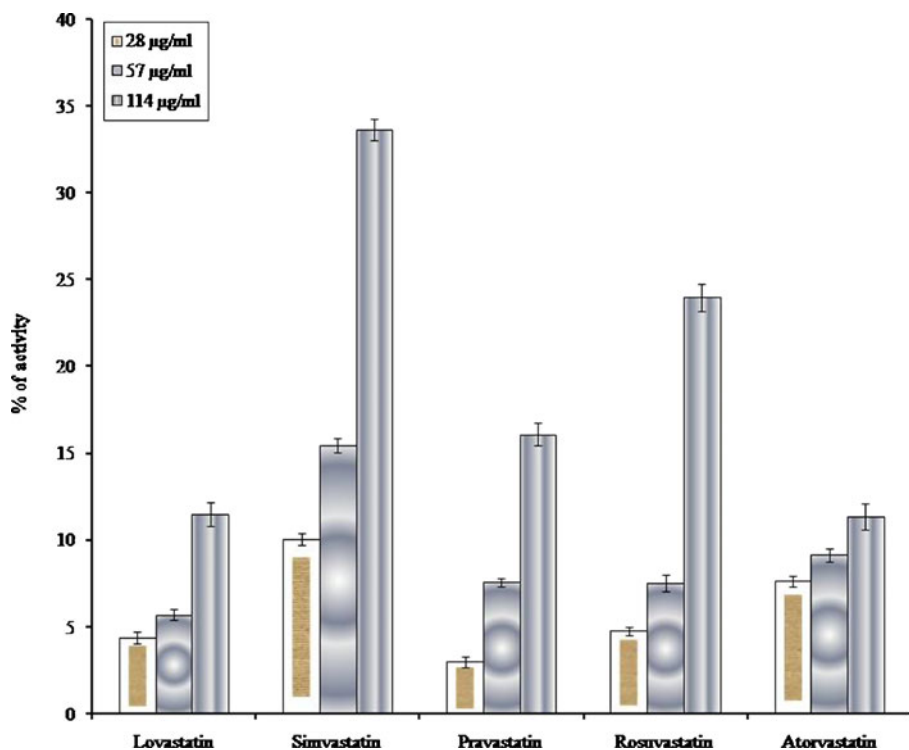


Fig. 4 Hydroxyl radical scavenging activity of HMG-CoA reductase inhibitors was measured at different concentration. Values are mean \pm SEM of triplicates shown by vertical bars with different concentration. Mean values with different vertical bars were significantly different by Duncan's test at $p < 0.05$

Even though there is a structural similarity among these natural HMG-CoA reductase inhibitors (lovastatin, simvastatin, and pravastatin), the difference in antioxidant activity was observed. The structures of the synthetic HMG-CoA reductase inhibitors—atorvastatin and rosuvastatin—are dissimilar and quite different from the natural HMG-CoA reductase inhibitors (Fig. 1). However, all these HMG-CoA reductase inhibitors inhibit the HMG-CoA reductase. It is because HMG-CoA, like moiety, is responsible for HMG-CoA reductase inhibition and is common to both natural and synthetic HMG-CoA reductase inhibitors. Though inhibition of lipid peroxidation and radical scavenging activity of HMG-CoA reductase inhibitors is independent of HMG-CoA, like moiety, it might be dependent on other functional group present. Lovastatin (Fig. 1a) contains a methylbutyric side chain and a 6- α methyl group. Simvastatin (Fig. 1b) contains an additional methyl group in the 2' position of the side chain. Pravastatin (Fig. 1c) has the β -hydroxylactone in the 6-hydroxy sodium salt form, and is the C6-hydroxy analog of mevastatin. [1]. These results suggested that the structural mechanism of antioxidant activity of HMG-CoA reductase inhibitors is yet to be elucidated.

Based on the present findings, HMG-CoA reductase inhibitors may suppress the occurrence atherosclerosis by inhibiting the initiation step in the lipid peroxidation pathway. Our results should assist physicians in finding more suitable drug for their patients suffering from oxidative stress-related atherosclerosis.

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