An *in Vitro* Study of the Hydroxyl Radical Scavenging Property of Fluvastatin, an HMG-CoA Reductase Inhibitor

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We investigated the *in vitro* hydroxyl radical scavenging activity of fluvastatin, a 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor. Fluvastatin showed hydroxyl radical scavenging activity as potent as that of dimethylthiourea and α -tocopherol, which are well-known respectively, as a hydroxyl radical scavenger and a natural antioxidant. Since this effect was not observed in other HMG-CoA reductase inhibitors, such as pravastatin and simvastatin, the scavenging effect of fluvastatin on hydroxyl radicals would not be a common property of HMG-CoA reductase inhibitors, but is derived from the unique chemical structure of fluvastatin. The hydroxyl radical scavenging activities of human metabolites of fluvastatin were also determined. All the tested metabolites possessing the fluorophenyl indole moiety showed activity. Among them, the metabolites which possess a phenolic hydroxyl group on the indole moiety showed stronger effects than that of fluvastatin. We suggest that the fluorophenyl indole moiety of fluvastatin is important for manifestation of the activity and that the phenolic hydroxyl group enhances the potency.

Key words hypolipidemic drug; antioxidant; radical scavenger; fluvastatin; hydroxyl radical; HMG-CoA reductase inhibitor

Accumulated evidence supports the notion that the elevated plasma level of low-density lipoprotein (LDL) is a major risk factor of atherosclerotic diseases.¹⁾ HMG-CoA reductase inhibitors such as fluvastatin, pravastatin and simvastatin are clinically used as hypolipidemic drugs.²⁾ These drugs effectively lower the serum LDL-cholesterol level and reduce the risk for the development of atherosclerosis. The hypothesis that oxidative modification of LDL plays an important role in atherogenesis was presented.³⁾ In this hypothesis, oxidized LDL is recognized and taken up by macrophages *via* the scavenger receptor pathway. Unlike the LDL receptor, the scavenger receptor is not regulated by intracellular cholesterol concentration, and the unregulated oxidized LDL uptake leads to foam cell formation. A number of studies have indicated that synthetic or natural antioxidants protect LDL from oxidative modification *in vitro* and reduce the development of atherosclerosis.^{4–6)} In addition, recent studies have revealed that the administration of fluvastatin delays the oxidation of plasma lipids in patients with hyperlipidaemia *in vivo*.^{7,8)} Furthermore, cholesterol-fed rabbits which were administered fluvastatin showed a reduction in the level of serum thiobarbituric acid-reactive substances (TBARS).⁹⁾ In spite of the above *in vivo* evidence, no direct demonstration of the antioxidative property of fluvastatin has so far been reported. We suppose that the antioxidative effects of fluvastatin *in vivo* are mainly derived from the free radical scavenging activity of fluvastatin itself and its metabolites.

Among the reactive oxygen species, hydroxyl radicals are thought to be the most hazardous and closely related to the oxidation of LDL in patients with hyperlipidaemia.¹⁰⁾ In the present study, we investigated the *in vitro* hydroxyl radical scavenging activity of fluvastatin and its major human metabolites, M-2, M-3, M-4 and M-7.^{2,11)} (Fig. 1).

Materials and Methods

Chemicals Fluvastatin sodium, pravastatin sodium and simvastatin (open acid form) were donated by Tsukuba Research Institute, Novartis Pharma Co., Ltd. (Ibaraki, Japan). The metabolites of fluvastatin (M-2, M-3,

M-4 and M-7) were synthesized at the Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd. (Saitama, Japan). All other reagents used were of the highest grade commercially available.

Hydroxyl Radical Scavenging Activity Hydroxyl radical scavenging activity of test compounds was determined by the method reported by Russell *et al.*¹²⁾

Briefly, a solution of methyl orange and a suspension of zinc oxide were prepared in 5 mM sodium borate buffer (pH 9.2) and mixed to give a final concentration of 40 μ M and the equivalent of 6 mM, respectively. Each test compound was prepared in a volume of dimethylformamide (DMF) and added to the above solution to give a final concentration of 0.1% DMF. The mixed solutions were placed 20 cm away from a 100-watt light source for 2 h. Blank control solutions were also prepared by the same procedure and stored in the dark for 2 h. All the aliquots were centrifuged at 1500 $\times g$ for 5 min to remove suspended zinc oxide. The decrease in absorbance of each sample at the wavelength of 465 nm was recorded in comparison with the blank, which was stored in the dark. The total amounts of hydroxyl radicals generated in this system were determined by the decrease in absorbance of the sample which contains only 0.1% DMF for the control sample. As the decrease in absorbance of methyl orange reflects the amount of hydroxyl radicals which were not scavenged by the test compounds, the hydroxyl radical scavenging activity of test compounds was determined in comparison with the control sample.

Statistics All data were expressed as the means $\pm$ S.E. of 4 experiments. Statistical comparison among the groups was made by analysis of variance (ANOVA) followed by Fisher's PLSD test. Probability below 5% was considered statistically significant.

Fig. 1. Chemical Structures of Fluvastatin and Its Major Metabolites in Human

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Fig. 2. Effects of HMG-CoA Reductase Inhibitors on Hydroxyl Radicals

The activities of fluvastatin (FLV), pravastatin (PRV) and simvastatin (SIV) in scavenging hydroxyl radicals were determined, as described in the text.

Data are expressed as the means $\pm$ S.E. ($n=4$). *: significantly different from control. $p < 0.05$.

Fig. 3. Comparison of Fluvastatin with α -Tocopherol and DMTU for Hydroxyl Radical Scavenging Activity

The activity of fluvastatin ($\circ$), α -tocopherol ($\square$) and DMTU ($\blacksquare$) to scavenge hydroxyl radicals was determined as described in the text. Data are expressed as the means $\pm$ S.E. ($n=4$).

Results

The hydroxyl radical scavenging activity of the compounds was determined by the methyl orange–zinc oxide system.¹²⁾ The data shown in Figs. 2 to 4 are the amounts of hydroxyl radicals which were not scavenged by the test compounds. Figure 2 shows the hydroxyl radical scavenging activities of fluvastatin, pravastatin and simvastatin. Fluvastatin (3–30 μ M) showed potent hydroxyl radical scavenging activity in a dose-dependent manner. In contrast, pravastatin and simvastatin, the other HMG-CoA reductase inhibitors, did not show any effect on the amount of hydroxyl radicals at the highest concentration of 30 μ M.

In order to evaluate the potency of the scavenging effect of fluvastatin on hydroxyl radicals, we compared its effect with those of dimethylthiourea (DMTU) and α -tocopherol, a well-known hydroxyl radical scavenger and a natural antioxidant, respectively. As shown in Fig. 3, the potency of fluvastatin in scavenging hydroxyl radicals was comparable to these reference compounds.

The hydroxyl radical scavenging activities of major human metabolites of fluvastatin were also determined. Figure 4 shows the hydroxyl radical scavenging activities of fluvastatin and its metabolites. All the metabolites tested showed strong activity, except for M-7. Among them, metabolites M-2 and M-3, which possess a phenolic hydroxyl group on the indole moiety, showed about 3 times more potent effects than

Fig. 4. Comparison of Fluvastatin with Its Human Metabolites for Hydroxyl Radical Scavenging Activity

The ability of fluvastatin ($\circ$), M-2 ($\triangle$), M-3 ($\blacktriangle$), M-4 ($\square$) and M-7 ($\blacksquare$) to scavenge hydroxyl radicals was determined as described in the text. Data are expressed as the means $\pm$ S. E. ($n=4$).

fluvastatin itself.

Discussion

We investigated the hydroxyl radical scavenging activity of the HMG-CoA reductase inhibitor fluvastatin. Our results clearly indicate that fluvastatin and its metabolites have potent scavenging activity against hydroxyl radicals *in vitro*.

Fluvastatin is the first totally synthesized HMG-CoA reductase inhibitor. Its chemical structure, including the fluorophenyl indole moiety (Fig. 1), differs from that of pravastatin and simvastatin, which were obtained by chemical modification of fungal metabolites.²⁾ Although fluvastatin showed potent hydroxyl radical scavenging activity, the other HMG-CoA reductase inhibitors did not show any effect. Therefore, the hydroxyl radical scavenging activity of fluvastatin was not considered to be a common property of HMG-CoA reductase inhibitors, but was likely to be derived from fluvastatin's unique chemical structure.

In view of the findings that all the tested metabolites with the fluorophenyl indole moiety showed scavenging activity against the hydroxyl radicals, and that the metabolites M-2 and M-3 having a phenolic hydroxyl group on the indole moiety showed more potent effects than fluvastatin itself, the fluorophenyl indole moiety was suggested to be important for the manifestation of hydroxyl radical scavenging activity, and the phenolic hydroxyl group was considered to enhance this activity. It has been well documented that indole derivatives show some antioxidative properties,^{13,14)} and that phenolic hydroxyl groups enhance the antioxidative property of the aromatic compounds.¹⁵⁾ Therefore, these observations are consistent with previous reports.

We determined the hydroxyl radical scavenging activity of our compounds using the methyl orange–zinc oxide system.¹²⁾ The quantity of hydroxyl radicals generated in this system changed slightly depending on the chemical conditions used. The optimal concentration of the compounds scavenging hydroxyl radicals may also change in response to the quantity of hydroxyl radicals generated in the system. For these reasons, we compared the potency of fluvastatin with that of two well-known standard compounds, DMTU and α -tocopherol. As a result, the ability of fluvastatin to scavenge hydroxyl radicals was as potent as these reference compounds. It is well known that free radical scavengers and/or

antioxidants such as DMTU and α -tocopherol protect cells and tissues from oxidative injury.^{6,16} Therefore, fluvastatin and its metabolites may also show protective effects on oxidative tissue injury, similarly to DMTU and α -tocopherol.

In this experiment, the assay was performed under an alkaline condition (pH 9.2). Therefore, there is a possibility that the antioxidative profiles of these compounds under physiological pH are somewhat different from this condition. However, in our separate experiments, fluvastatin and its metabolites effectively inhibited hydrogen peroxide (H_2O_2)-hemin-mediated LDL oxidation, which was first reported by Balla *et al.*,¹⁷ under the physiological pH *in vitro*. It is considered that hydroxyl radicals and other radicals participate in this oxidation. Therefore, we consider that these compounds also show radical scavenging and antioxidative effects, even at physiological pH *in vivo*.

Our results present one explanation why the administration of fluvastatin delayed the oxidation of plasma lipids of patients with hyperlipidaemia,^{7,8} and why it reduced the level of plasma TBARS in cholesterol-fed rabbits.⁹

Although the exact mechanism of the oxidative modification of LDL *in vivo* remains unclear, the oxidation of LDL in the arterial wall *in vivo* is thought to be initiated by metal ions,¹⁰ lipoxygenase,¹⁸ myeloperoxidase,¹⁹ peroxynitrite²⁰ and other endogenous substances. Reactive oxygen species such as hydroxyl radicals may also be closely related to the oxidation of LDL.¹⁰ Therefore, combination of the hydroxyl radical scavenging activity with the hypolipidemic property of fluvastatin may augment its therapeutic effect on patients with complications of hyperlipidaemia with increased risk factors for oxidative stress.

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