Free Radical Research, Vol. 35, pp. 789–801
Reprints available directly from the publisher
Photocopying permitted by license only

Antioxidative Effects of Fluvastatin and its Metabolites Against Oxidative DNA Damage in Mammalian Cultured Cells

ATSUMUNE IMAEDA[a,*], TORU TANIGAWA[b], TOMONORI AOKI[a], YASUSHI KONDO[c], NAOTO NAKAMURA[b] and TOSHIKAZU YOSHIKAWA[b]

[a]*Safety Research Laboratory, Tanabe Seiyaku Co., Ltd., 3-16-89, Kashima, Yodogawa-ku, Osaka 532-8505, Japan;* [b]*1st Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamikyo-ku, Kyoto 602-0841, Japan;* [c]*Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 3-16-89 Kashima, Yodogawa-ku, Osaka 532-8505, Japan*

Accepted by Professor E. Niki

(Received 7 February 2001; In revised form 12 May 2001)

We investigated the effects of fluvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, on reactive oxygen species (ROS) and on oxidative DNA damage *in vitro*, as well as the effects of the main fluvastatin metabolites (M2, M3, and M4) and other inhibitors of the same enzyme, pravastatin and simvastatin. The hydroxyl radical and the superoxide anion scavenging activities of fluvastatin and its metabolites were evaluated using an electron spin resonance spectrometer. Fluvastatin and its metabolites showed superoxide anion scavenging activity in the hypoxanthine–xanthine oxidase system and a strong scavenging effect on the hydroxyl radical produced from Fenton's reaction. Protective effects of fluvastatin on ROS-induced DNA damage of CHL/IU cells were assessed using the single-cell gel electrophoresis assay. CHL/IU cells were exposed to either hydrogen peroxide or *t*-butylhydroperoxide. Fluvastatin and its metabolites showed protective effects on DNA damage as potent as the reference antioxidants, ascorbic acid, trolox, and probucol, though pravastatin and simvastatin did not exert clear protective effects. These observations suggest that fluvastatin and its metabolites may have radical scavenging activity and the potential to protect cells against oxidative DNA damage. Furthermore, ROS are thought to play a major role in the etiology of a wide variety of diseases such as cellular aging, inflammation, diabetes, and cancer development, so fluvastatin might reduce these risks.

Keywords: Fluvastatin; Reactive oxygen species; Oxidative DNA damage; Antioxidant; Electron spin resonance; Single-cell gel electrophoresis assay

INTRODUCTION

An increased plasma concentration of low-density lipoprotein (LDL) has been considered to be a major risk factor for atherosclerosis. The impaired removal of plasma LDL in these patients results in

*Corresponding author. Tel.: +81-6-6300-2748. Fax: +81-6-6300-2696. E-mail: a-ima@tanabe.co.jp

abnormal persistence of LDL in the plasma, allowing more time for oxidative modification to occur.[1] Oxidative modification of LDL has been reported to be one of the important steps in the progression of atherogenesis.[2,3] Fluvastatin (FV, Fig. 1) is a highly potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase used as a hypolipidemic drug.[4] FV showed protective effects against the oxidation of LDL *in vitro*,[1] and a reduction in the level of serum thiobarbituric acid-reactive substances (TBARS) in cholesterol-fed rabbits.[1,6] Thus FV may help to reduce the risk of atherosclerosis by protecting LDL from oxidative modification as well as reducing plasma LDL levels.[7]

Recently, we reported on the protective effects of FV and its metabolites (M2, M3, and M4, Fig. 1)[4,5] on oxidative DNA cleavage from reactive oxygen species (ROS) and advanced glycation end-products (AGE) *in vitro*. Protective effects were assessed in terms of the DNA breakage rate in a single-stranded phage DNA system, and in the Ames test with *Salmonella typhimurium* TA102 and TA104 test strains. FV and its metabolites showed strong protective effects on ROS-induced DNA strand breakage and FV reduced the mutagenesis by ROS and AGE in *S. typhimurium* TA102 and TA104.[8,9] Thus the results of the previous study indicated that FV and its metabolites have the ability to scavenge ROS[10,11] and protect against oxidative DNA damage,[8] which is an etiological factor underlying a wide variety of diseases.

In the present study, therefore, we investigated the hydroxyl radical ($HO^{\cdot}$) and superoxide anion ($O_2^{\cdot-}$) scavenging activities of FV and its metabolites (M2, M3, and M4) using an electron spin resonance (ESR) spectrometer. Moreover, we confirmed the protective effects of FV on ROS-induced DNA damage of CHL/IU cells using the single cell gel electrophoresis assay (SCG assay), which is a sensitive method of measuring the extent of DNA damage in individual cells *in vivo* and *in vitro*.[12–16] We used CHL/IU cells to assess the validity of the protective effects of FV on ROS-induced phage DNA damage and mutagenesis in *S. typhimurium*. Protective effects of the major metabolites (M2, M3, and M4) of FV in humans also were investigated. In addition, we compared the protective effects of FV on oxidative DNA damage with those of two other HMG-CoA reductase inhibitors, pravastatin (PV) and simvastatin (SV) (Fig. 1).

MATERIALS AND METHODS

Reagents

FV was donated from the Department of Pharmacology, Novartis Pharma (Ibaraki, Japan). FV metabolites (M2, M3, and M4) and Probucol (PB) were synthesized by the Discovery Research Laboratory, Tanabe Seiyaku (Saitama, Japan) (Fig. 1). Hypoxanthine (HX), *t*-butylhydroperoxide (*t*-BuOOH), dimethyl sulfoxide (DMSO), and hydrogen peroxide (H_2O_2) were purchased from Katayama Chemical Industries (Osaka, Japan). Fe^{2+}, Trolox (TX), ascorbic acid (AsA), diethylenetriaminepentaacetic acid (DTPA), and xanthine oxidase (XO) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Daiichi Chemical (Tokyo, Japan) (Table I).

Superoxide (DMPO-OOH Signal) Scavenging Activity

Superoxide anion ($O_2^{\cdot-}$) scavenging activities were measured according to the methods described in a previous article.[17,18] The $O_2^{\cdot-}$ was produced from the HX and XO system and trapped by the spin-trapping agent DMPO. FV and its metabolites (M2, M3, and M4) were dissolved in distilled water. Measurements of levels of $O_2^{\cdot-}$ were made with a JEOL-JES-FR 80 ESR spectrometer (JEOL, Tokyo) under the following conditions: magnetic field, 335.8 mT; microwave power, 8.0 mW; modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; sweep

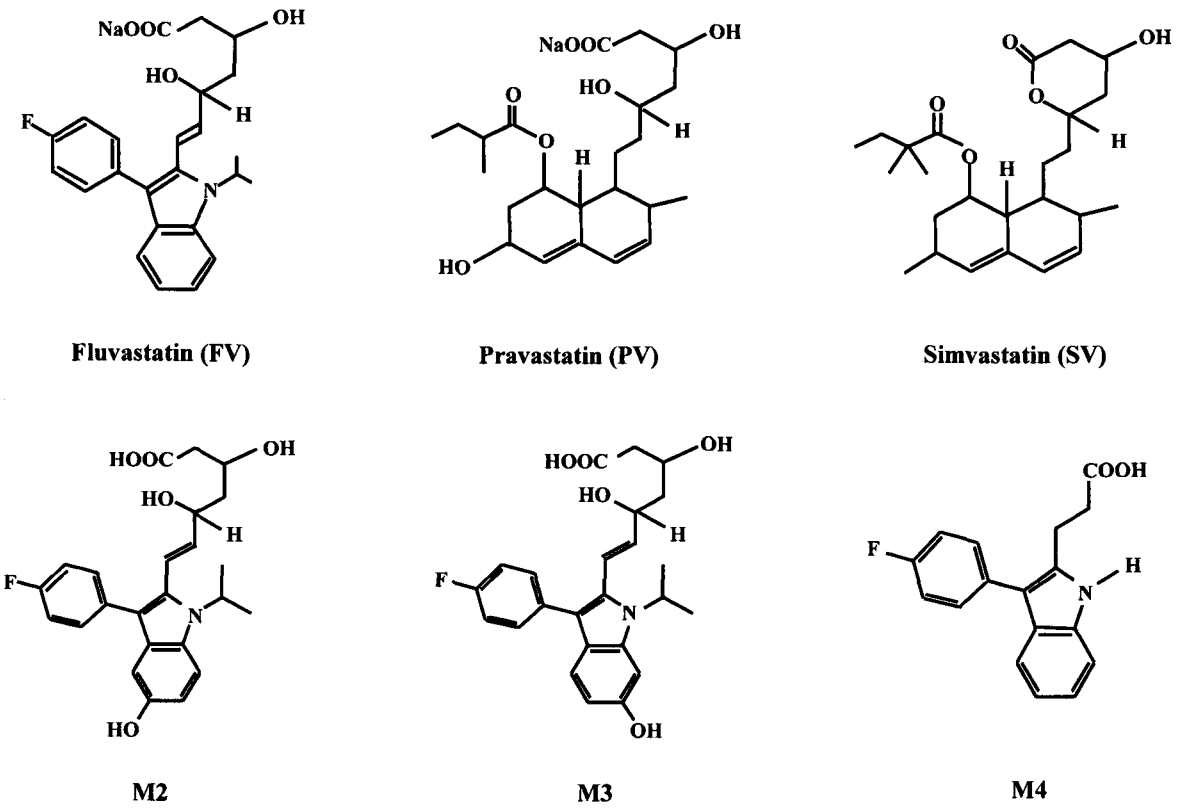

FIGURE 1 Structures of fluvastatin and its major metabolites (M2, M3, and M4), pravastatin, and simvastatin.

width, 5.0 mT; sweep time, 10 mT/min; response time, 0.03 s; and receiver gain, ×400.

Under the conditions described, 20 μl each of 1 mM HX, 1 mM DTPA, 1 M DMPO, and sample were added to 100 μl of 100 mM phosphate-buffered saline (PBS; pH 7.4). Next, 20 μl of XO (final concentration, 0.08 U/ml) were added, the cell was inserted into the ESR instrument, and measurement was started after exactly 1 min. The first DMPO-OOH signal intensities were measured relative to the intensity of the MnO signal, the internal standard to correct the measurement error.

TABLE I Test materials used in this study

Test material	Abbreviation or formula
Fluvastatin	FV
Fluvastatin metabolites	M2, M3, M4
Pravastatin	PV
Simvastatin	SV
Ascorbic acid	AsA
Trolox	TX
Probucol	PB
Hydroxyl radical	$HO^{\cdot}$
Superoxide radical	$O_2^{\cdot-}$
Hydrogen peroxide	H_2O_2
tert-butylhydroperoxide	*t*-BuOOH
Hypoxanthine	HX
Xanthine oxidase	XO
Diethylenetriaminepentaacetic acid	DTPA
5,5-dimethyl-1-pyrroline-*N*-oxide	DMPO

Hydroxyl Radical (DMPO-OH Signal) Scavenging Activity

For detection of hydroxyl radical ($HO^{\cdot}$)-scavenging activity, we used a previously reported ESR spin-trapping method.[17,19] $HO^{\cdot}$ was produced from the Fenton's reaction involving Fe^{2+} and H_2O_2. The resulting $HO^{\cdot}$ were trapped with DMPO. FV and its metabolites (M2, M3, and M4)

were dissolved in distilled water. The reaction mixture consisting of 100 μl of 100 mM PBS (pH 7.4) and 100 μl of 0.2 mM DTPA, 20 μl each of 0.5 mM Fe^{2+}, 100 mM DMPO, and the sample was prepared. Next, 20 μl of H_2O_2. (final concentration, 1 mM) were added to the reaction mixture, and the level was measured after 1 min using an ESR spectrometer. ESR conditions were the same as described above, except for the microwave power of 6.0 mW. The second DMPO-OH signal intensities were measured as the intensity relative to the MnO signal.

Cell Culture and Treatments

CHL/IU cells derived from a neonatal Chinese hamster were used as the indicator cell line. Cells, purchased from Dainippon Pharmaceutical, were suspended in culture medium containing 10% DMSO and stored in −196°C liquid nitrogen. Prior to treatment, the frozen cells were returned to culture and maintained by subculture for 3–5 days, in a CO_2 incubator at 37°C in a 5% CO_2 atmosphere. Eagle's MEM supplemented with 10% inactivated fetal calf serum was used as the culture medium.

Cells (2.7×10^5) were seeded on a tissue culture dish (60 mm in diameter containing 5 ml of medium) and incubated for about 24 h. After replacement with 2.7 ml of fresh medium, 0.15 ml of test solution and 0.15 ml of ROS generation solution were added to the culture for 1 h. The 0.15 ml aliquot of culture medium and vehicle were added to the untreated and solvent control cultures instead of the test solution, respectively. After exposure, the cells were washed with saline and analyzed immediately for DNA damage.

Single-cell Gel Electrophoresis Assay

DNA single strand breakage in individual cells was detected using the single cell gel electrophoresis assay (SCG assay).[12–16] After treatment, CHL/IU cells were resuspended at the concentration of 2.0×10^4 cells/100 μl in 1.0% low-melting agarose in saline and immediately pipetted onto agarose-coated slides. The slides were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sarkosyl, 5% dimethyl sulfoxide, and 1% Triton X100 [pH 10.0]) for 60 min. The slides were then placed on an electrophoretic tray with an alkaline buffer (300 mM NaOH and 1 mM EDTA; pH > 13) for 10 min to allow the DNA to unwind; electrophoresis was then performed at 300 mA and 1.0 V/cm for 15 min in the same alkaline buffer maintained at 4°C. Following electrophoresis, the slides were neutralized with Tris-HCl buffer (pH 7.4) for 10 min and rinsed with distilled water prior to staining with ethidium bromide. The DNA was visualized using a Nikon Labophot fluorescence microscope and the data analyzed using a specialized SCG analysis system (Komet 3.0, Kinetic Imaging, Liverpool, UK). Tail length, measured from the trailing edge of the cell as defined in Ashby *et al.*,[20] percentage of DNA in the tail (tail DNA), and tail moment (the product of tail length and percentage of tail DNA) were

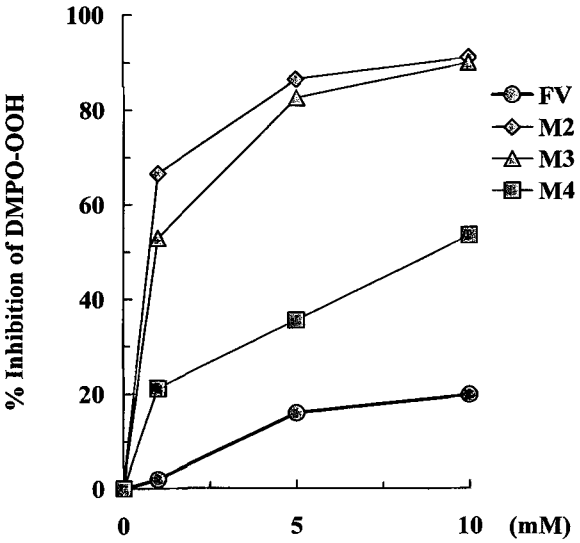

FIGURE 2 Effect of FV and its metabolites (M2, M3, and M4) on O_2^- generation produced by the HX–XO system. The reaction mixture contained 0.1 mM HX, 0.1 M DMPO, 0.08 U/ml XO, and 1–10 mM FV or its metabolite in 50 mM PBS (pH 7.4, with 0.1 mM DTPA). M2, M3, and M4 designate principal FV metabolites. FV, fluvastatin; HX, hypoxanthine; XO, xanthine oxidase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid.

recorded for 50 cells per slide with two slides per variable investigated.

The parameter used to describe the extent of DNA damage was the tail moment, which is the product of the length of the tail and intensity of its fluorescence. The measurements from 100 randomly selected cells per treatment condition were averaged.

Effects on DNA Damage Induced by ROS In Vitro

Protective effects against oxidative DNA damage induced by ROS were measured by the SCG assay.[12–16] Each reagent was dissolved in distilled water, except for TX and PB. TX was dissolved in 1 M $NaHCO_3$ and PB in DMSO. FV and its metabolites (M2, M3, and M4) were diluted to adjust the final concentrations to 0.4, 0.8, 4, and 8 μM in the reaction mixture. AsA and TX were used as well-known antioxidants. H_2O_2 *t*-BuOOH were used as the ROS generating system. Each reagent was dissolved in distilled water. H_2O_2 was diluted to adjust the final concentrations to 0.5, 1, 2, 4, and 8 μM and *t*-BuOOH was diluted to adjust the final concentrations to 0.125, 0.25, 0.5, 1, and 2 mM in the reaction mixture.

Duplicate plates were prepared and observed for each dose. The protocol was performed at least twice for reliability, and satisfactory results were obtained between repeated tests *in vitro*.

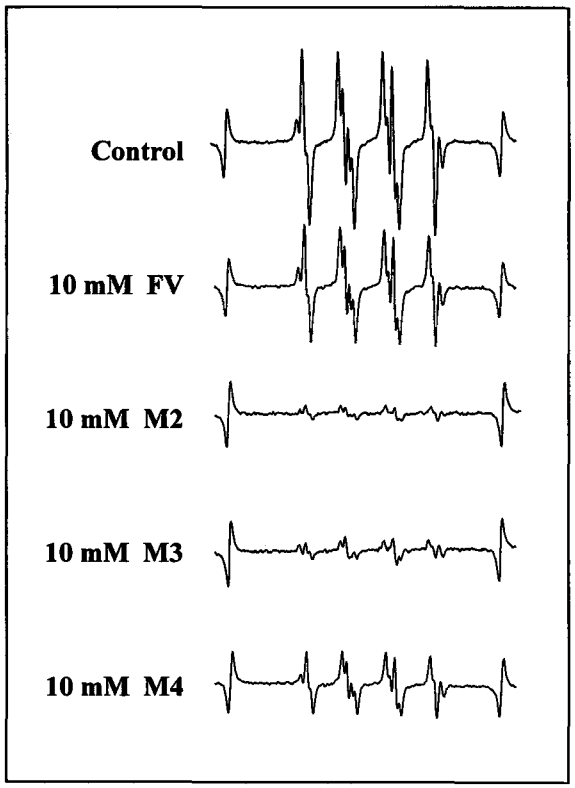

FIGURE 3 Typical ESR signal for the O_2^- scavenging effect of FV and its metabolites (M2, M3, and M4). The reaction mixture is described in the legend of Fig. 2. M2, M3, and M4 designate principal FV metabolites. FV, fluvastatin.

STATISTICAL ANALYSIS

Statistical significance among the groups was analyzed by Dunnett's multiple comparison test. The statistical significance of the differences was evaluated by Dunnett's parametric test when variances were equal ($p \geqq 0.05$), by a Bartlett's

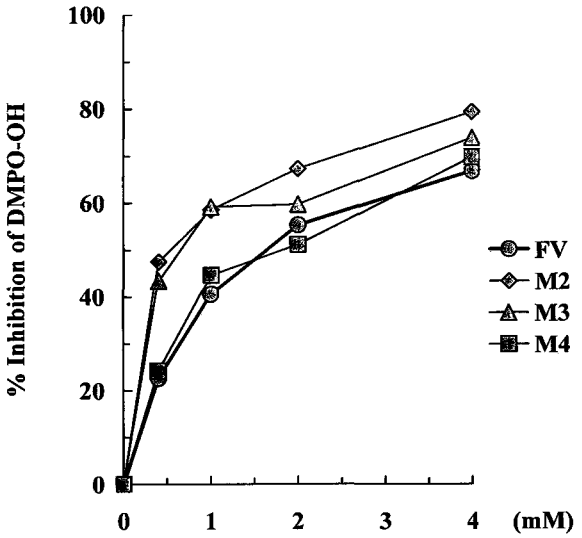

FIGURE 4 Effect of FV and its metabolites (M2, M3, and M4) on the generation of $HO^{\cdot}$ produced by Fenton's reagent. The reaction mixture contained 0.05 mM Fe^{2+}, 1 mM H_2O_2, 10 mM DMPO, and 0.4–4 mM FV or its metabolite in 50 mM PBS (pH 7.4, with 0.1 mM DTPA). M2, M3, and M4 designate principal FV metabolites FV, fluvastatin; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid.

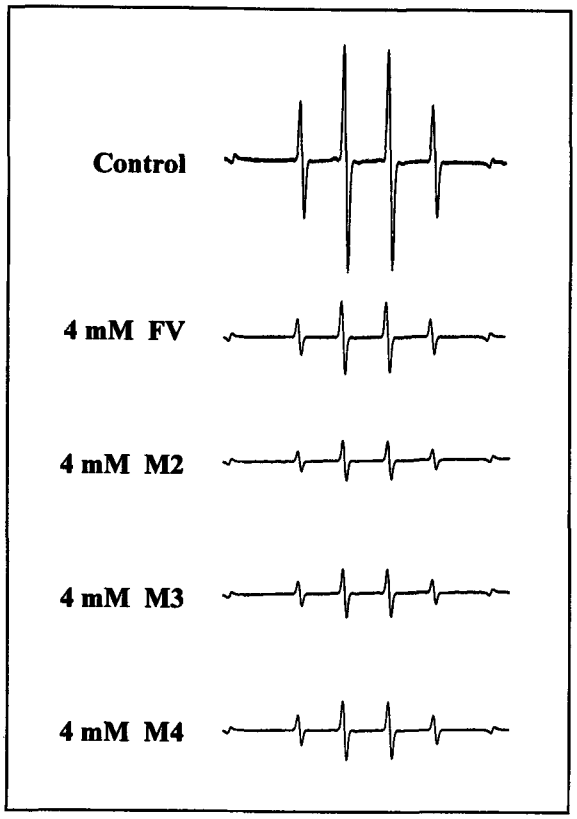

FIGURE 5 Typical ESR signal for the HO· scavenging effect of FV and its metabolites (M2, M3, and M4). The reaction mixture is given in the legend of Fig. 4. M2, M3, and M4 designate principal FV metabolites. FV, fluvastatin.

test for homogeneity of variance, or Dunnett's non-parametric test when variances were unequal ($p < 0.05$). Results were considered to be statistically significant at a probability level of $p < 0.05$ or 0.01.

RESULTS

$O_2^{\cdot-}$ (DMPO-OOH) Scavenging Activity of FV

The ESR signal was detected by an ESR spectrometer at room temperature. The 12 characteristic lines of the signal were observed 1 min after the addition of XO. The g value and hyperfine coupling constant were $g = 2.018$, $a_N = 1.43$, $a_H = 1.17$, and 0.13 mT, which could be assigned to a DMPO-OOH adduct. Figure 2

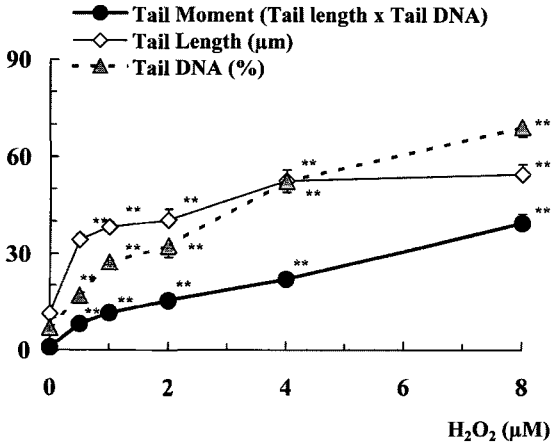

FIGURE 6 Tail moment, tail length, and tail DNA of H_2O_2-treated CHL/IU cells using SCG assay. The cells were treated for 1 h with 0.5–8 μM H_2O_2 and immediately assayed for DNA damage with SCG assay. The tail moment is the product of the length of the tail and the amount of DNA present within the tail. The data represent mean±S.E. $^{**}p < 0.01$ as compared with control (0 μM H_2O_2).

indicates the $O_2^{\cdot-}$ scavenging effects of FV and its metabolites (1, 5, and 10 mM) using the method of ESR spin-trapping. The inhibition rate of DMPO-OOH was 1.9 and 19.8%, respectively, by adding to 1.0 or 10 mM of FV. At the doses of 1, 5, and 10 mM M2, the percent inhibition of DMPO-OOH was 66.5, 86.4, and 91.1, respectively. M3 reduced $O_2^{\cdot-}$-induced DMPO-OOH adduct to a similar extent. The inhibition rate of DMPO-OOH by M4 (1.0 and 10 mM) was 21.2 and 53.5%, respectively. FV, M2, M3, and M4 attenuated the DMPO-OOH signal in a dose-dependent manner. Slight inhibition was observed in the DMPO-OOH signal with FV, and M2 and M3 significantly scavenged $O_2^{\cdot-}$ radicals compared with the control. A typical ESR signal is shown in Fig. 3.

HO·(DMPO-OH) Scavenging Activity of FV

The ESR signals composed of representative four line signals with a relative intensity of 1:2:2:1 and a hyperfine splitting constant of $a_N = a_H = 1.50$ mT, which were characteristic of those of the DMPO-OH spin adduct, appeared. Figure 4 shows the DMPO-OH signal scavenging effect of

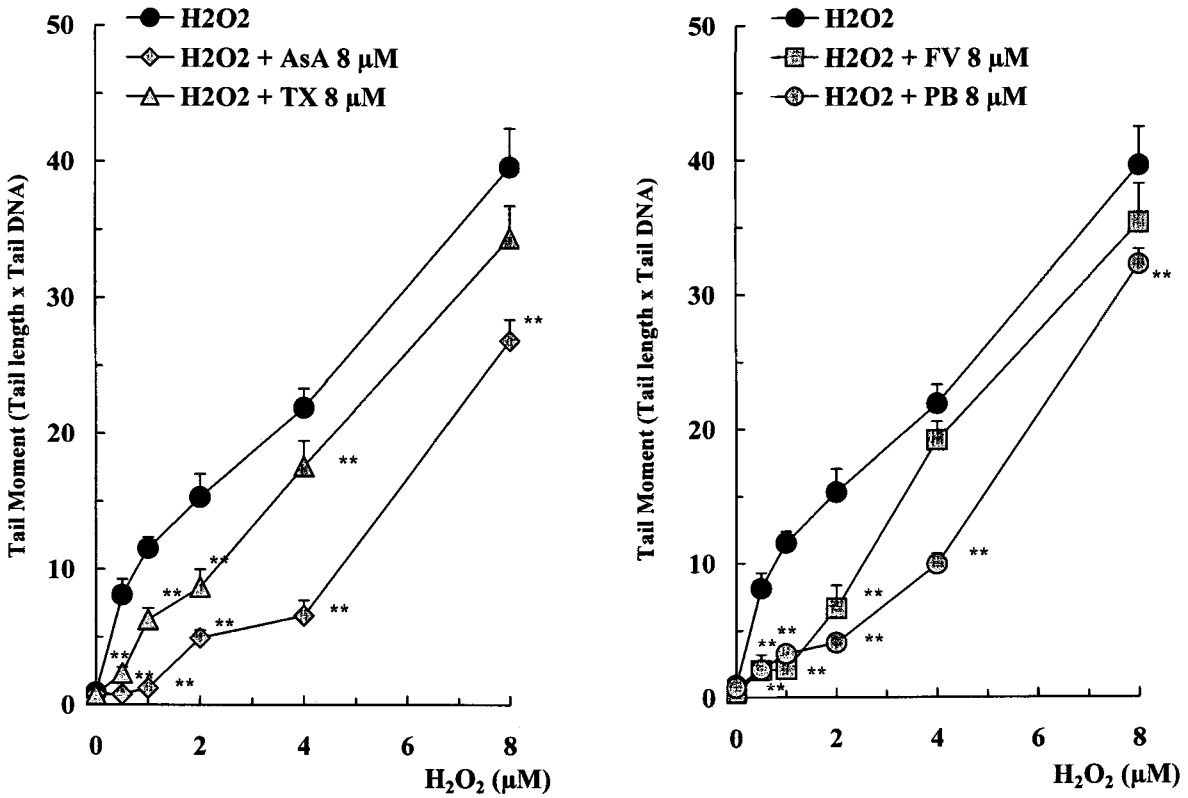

FIGURE 7 The effect of FV and antioxidants of H_2O_2-induced DNA damage. The cells were treated for 1 h with 0.5–8 μM H_2O_2 in the absence or presence of 8 μM FV and various antioxidants, and immediately assayed for DNA damage with SCG assay. Results are expressed as the tail moment, which is the product of the length of the tail and the amount of the tail DNA, and represents the mean of at least two separate experiments, each performed in duplicate. FV, fluvastatin; AsA, ascorbic acid; TX, trolox; PB, probucol. The data represent mean ± S.E. $^{**}p < 0.01$ as compared with control (H_2O_2 only).

FV and its metabolites (0.4, 1, 2, and 4 mM) using the method of ESR spin-trapping. At the doses of 0.4, 1, 2, and 4 mM FV, the percent inhibition of DMPO-OH was 22.7, 40.6, 55.4, and 66.8, respectively. M4 reduced HO·-induced DMPO-OH adduct to a similar extent. M2 and M3 significantly scavenged HO· compared with the control. These results indicated that the HO· scavenging activity of FV, M2, M3, and M4 was dose dependent. A typical ESR signal is shown in Fig. 5.

Effects of FV on DNA Damage Induced by H_2O_2 In Vitro

In the first series of experiments, we assessed the sensitivity of the conventional SCG assay to detect the DNA damage of CHL/IU cells. Cells were exposed to H_2O_2 at 37°C at various concentrations. Figure 6 shows a representative SCG assay of DNA damage as a function of H_2O_2 concentration for 1 h. A significant increase in tail length (μm), tail DNA (%), and tail moment (tail length × tail DNA) were observed in CHL/IU cells exposed to H_2O_2 in a dose-dependent manner. On the other hand, the concentration of 8 μM AsA, TX, PB, and FV significantly inhibited H_2O_2-induced tail moment (Fig. 7). Figure 8 shows representative photomicrographs of ethidium bromide-stained nuclei, and indicates that 8 μM FV virtually abolished the extensive DNA cleavage caused by 2 μM H_2O_2.

The effect of FV on DNA damage produced by H_2O_2 was investigated. In these experiments the cells were exposed for 1 h to 2 μM H_2O_2, in the

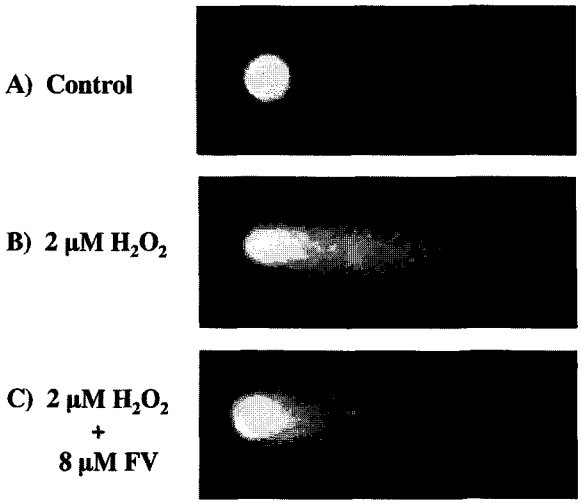

FIGURE 8 Fluorescence photographs of CHL/IU cells after exposure to 2 μM H_2O_2 in the SCG assay. (A) (Control), nuclei of undamaged negative control cells, 200× magnification. (B) (2 μM H_2O_2), severely damaged cell, hence the name "Comet". The fragmented DNA has migrated out of the nucleus (comet head) and streamed out in a long tail towards the anode. Highly fragmented DNA has migrated away from the nuclear cage, leaving a small comet head. (C) (2 μM H_2O_2+8 μM FV), nuclei of moderately damaged cell. FV, fluvastatin.

absence or presence of an increasing concentration of FV, and the level of DNA damage was measured immediately after the treatment using SCG assay. As shown in Fig. 9, the FV effectively reduced DNA damage caused by H_2O_2, and this effect was dose dependent. AsA, TX, and PB reduced H_2O_2-induced DNA damage to a similar extent. M2, M3, and M4, metabolites of FV, inhibited the H_2O_2-induced DNA damage in a dose-dependent manner. In addition, M2 and M3 provided even stronger protection against DNA strand breakage than FV did, and M4 equivalent to the ratio attainable with FV. Protective effects of FV against DNA damage were compared with those of PV or SV. FV exhibited protective effects against H_2O_2-induced breakage, but PV and SV did not.

Effects of FV on DNA Damage Induced By t-BuOOH In Vitro

Cells were exposed to *t*-BuOOH at 37°C at various concentrations. Figure 10 shows a

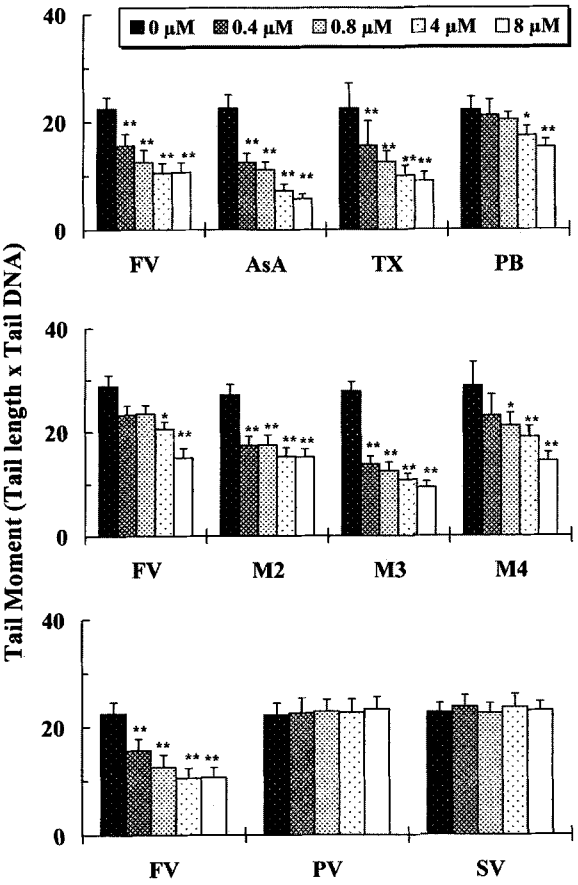

FIGURE 9 The effect of FV, its metabolites (M2, M3, and M4) and antioxidants on 2 μM H_2O_2-induced DNA damage. The cells were treated for 1 h with 2 μM H_2O_2 in the absence or presence of 0.4–8 μM FV, its metabolites (M2, M3, and M4), and various antioxidants, and immediately assayed for DNA damage with SCG assay. Results are expressed as the tail moment and represent the mean of at least two separate experiments. M2, M3, and M4 designate principal FV metabolites in humans. FV, fluvastatin; AsA, ascorbic acid; TX, trolox; PB, probucol; PV, pravastatin; SV, simvastatin. The data represent mean±S.E. $^*p < 0.05$, $^{**}p < 0.01$ as compared with control (H_2O_2 only).

representative SCG assay of DNA damage as a function of *t*-BuOOH concentration for 1 h. A significant increase in tail length, tail DNA, and tail moment were observed in CHL/IU cells exposed to *t*-BuOOH showing a dose-dependent suppressive activity. On the other hand, the concentration of 8 μM AsA, TX, PB, and FV significantly inhibited *t*-BuOOH-induced DNA damage (Fig. 11).

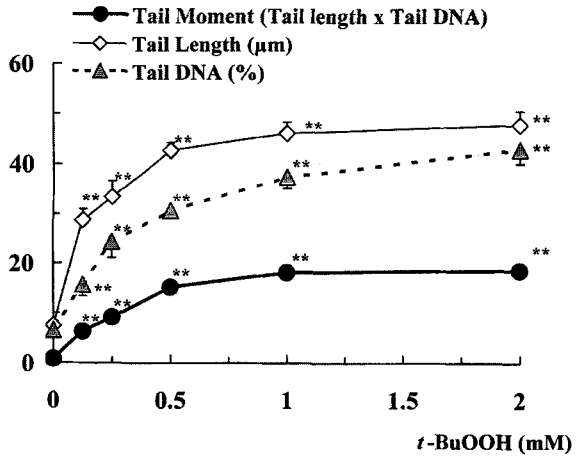

FIGURE 10 Tail moment, tail length, and tail DNA of *t*-BuOOH-treated CHL/IU cells using SCG assay. The cells were treated for 1 h with 0.125–2 mM *t*-BuOOH and immediately assayed for DNA damage with SCG assay. The tail moment is the product of the length of the tail and the amount of DNA present within the tail. *t*-BuOOH, *t*-butylhydroperoxide. The data represent mean±S.E. $^{**}p < 0.01$ as compared with control (0 μM *t*-BuOOH).

The effect of FV and DNA damage produced by *t*-BuOOH was investigated. In these experiments, the cells were exposed for 1 h to 0.5 mM *t*-BuOOH, in the absence or presence of an increasing concentration of FV, and the level of DNA damage was measured. The addition of FV markedly reduced the levels of tail moment caused by *t*-BuOOH in a dose-dependent manner (Fig. 12). FV reduced *t*-BuOOH-induced DNA damage; the results were comparable to those with AsA, TX, and PB. M2, M3, and M4 inhibited *t*-BuOOH-induced DNA strand scission similarly to FV and these effects were dose dependent. M2 and M3 were more effective than FV at the same concentration, but no significant difference was evident between M4 and FV. On the other hand, no protective effects of PV and SV were shown in this system.

DISCUSSION

FV, developed as an HMG-CoA reductase inhibitor, and its metabolites have been reported to have antioxidative activities, which are related to their chemical structures.[7,10,21–23] FV showed protective effects against oxidation of LDL.[1,3,6] In the present study, we described the ROS scavenging activities of FV and its human metabolites (M2, M3 and M4), and the protective effects on oxidative DNA damage induced by ROS *in vitro*. In addition, we compared the protective effects of FV against oxidative DNA damage with those of two other HMG-CoA reductase inhibitors, PV and SV.

We reported previously[8,9] on the protective effects of FV on oxidative DNA cleavage from ROS or AGE *in virto*, as well as the effects of major human metabolites of FV. Using a phage DNA single-strand breakage system, we examined the suppressive activity of FV on oxidative DNA damage directly induced by Fenton's reaction, the xantine/XO system, *t*-BuOOH, adriamycin, and AGE. FV and its metabolites showed a strong protective effect comparable to those seen with $HO^{\cdot}$ scavengers, such as thiourea and mannitol. Furthermore, FV reduced the mutagenesis by ROS or AGE in *S. typhimurium* TA102 and TA104 test strains. These results suggested that FV might reduce cytotoxicity caused by ROS-induced DNA damage.

ROS, including free radicals such as $HO^{\cdot}$ and O_2^{-}, are well known to cause DNA damage and induce cytotoxicity.[24] The mechanisms of ROS-induced cellular injury, including membrane lipid peroxidation, oxidation of structural and functional proteins and amino acids, nucleic acid base damage, DNA strand breaks, and DNA strand cross-linkage, may result in reversible or irreversible consequences including cellular repair, proliferation, differentiation, transformation, and apoptosis or cell death.[25] ROS may play a major role as endogenous initiators and promoters of DNA damage and mutation that contribute to cancer, diabetes, heart disease, and other age-related diseases.[25–27] For example, increased oxidative stress in diabetes has been implicated in the pathogenesis of diabetic complications.[28,29] On the other hand, antioxidants may represent an important defense

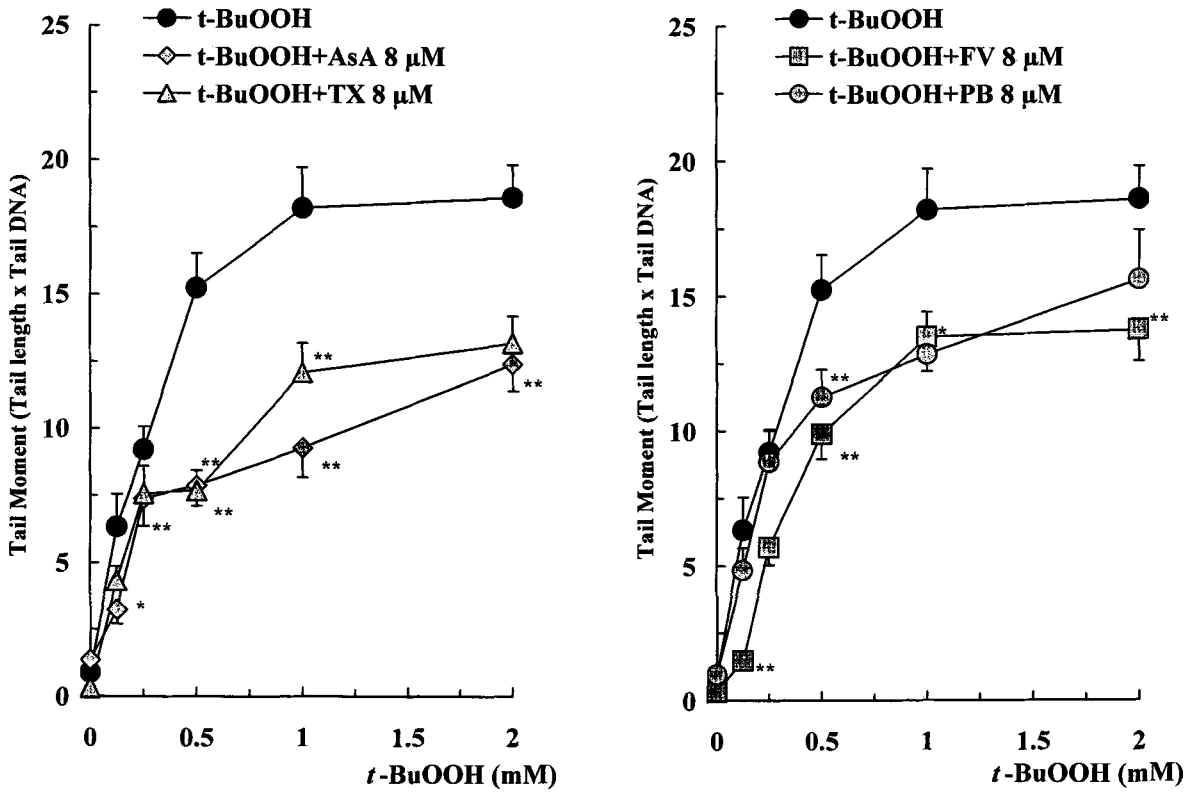

FIGURE 11 The effect of FV and antioxidants on *t*-BuOOH-induced DNA damage. The cells were treated for 1 h with 0.125–2 mM *t*-BuOOH in the absence or presence of 8 μM FV and various antioxidants, and immediately assayed for DNA damage with SCG assay. Results are expressed as the tail moment, which is the product of the length of the tail and the amount of the tail DNA, and represent the mean of at least two separate experiments, each performed in duplicate. *t*-BuOOH, *t*-butylhydroperoxide; FV, fluvastatin; AsA, ascorbic acid; TX, trolox; PB, probucol. The data represent mean±S.E. $^{*}p < 0.05$, $^{**}p < 0.01$ as compared with control (*t*-BuOOH only).

against ROS-induced cellular injury, and many antioxidants, such as cathechins,[30] melatonin,[31] flavonoids,[32] carotenoids,[33] and natural phenolic products,[34] are being studied as anticarcinogens.

The ROS scavenging activity of FV and its metabolites (M2, M3, and M4) was examined using an ESR spectrometer. FV and its metabolites chemically scavenged ROS such as $O_2^{\cdot-}$ and $HO^{\cdot}$ generated by the HX–XO system and Fenton's reaction, respectively. FV showed a strong scavenging activity of $HO^{\cdot}$ compared with $O_2^{\cdot-}$. The relative protective effect of M2, M3, and M4 on the $O_2^{\cdot-}$ scavenging activity in the HX–XO system was significantly greater than that of FV, on the order of M2 = M3 > M4 > FV. On the other hand, there was no significant difference in the $HO^{\cdot}$ scavenging activity seen between the groups treated by FV and groups treated by its metabolites at high doses (2, 4 mM). FV has the ability to act not only as an HMG-CoA reductase inhibitor, but also an $O_2^{\cdot-}$ and $HO^{\cdot}$ scavenger. The scavenging activity of FV, then, derives from its unique chemical structure, which includes a double bond conjugated with the fluorophenyl indole moiety.[21] The antioxidative effects of FV are likely to be related to this conjugated double bond.[21] These results suggest that FV might reduce the cytotoxicity caused by ROS-induced DNA damage.

We examined the protective effects of FV and its metabolites on oxidative DNA damage of

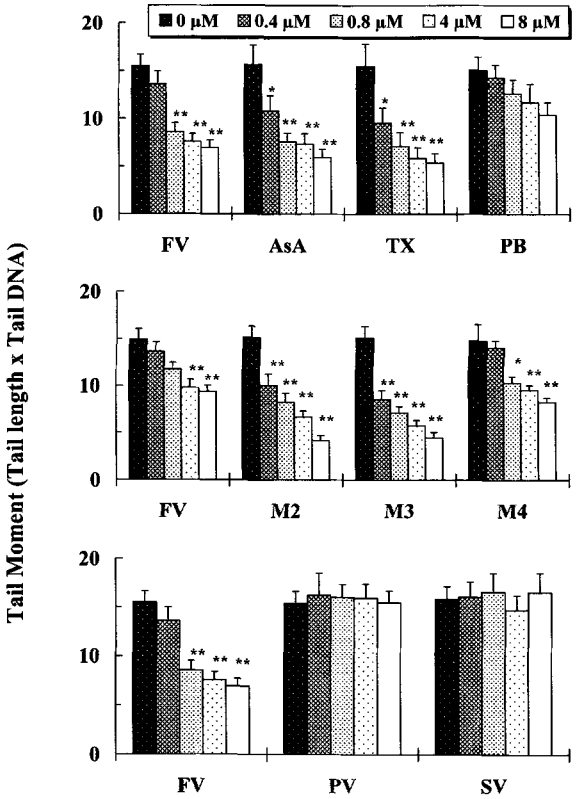

FIGURE 12 The effect of FV, its metabolites (M2, M3, and M4), and antioxidants on 0.5 mM *t*-BuOOH-induced DNA damage. The cells were treated for 1 h with 0.5 mM *t*-BuOOH in the absence or presence of 0.48–8 μM FV, its metabolites (M2, M3, and M4), and various antioxidants, and immediately assayed for DNA damage with SCG assay. Results are expressed as the tail moment and represent the mean of at least two separate experiments. M2, M3, and M4 designate principal FV metabolites in humans. *t*-BuOOH, *t*-butylhydroperoxide; FV, fluvastatin; AsA, ascorbic acid; TX, trolox; PB, probucol; PV, pravastatin; SV, simvastatin. The data represent mean±S.E. $*p < 0.05$, $**p < 0.01$ as compared with control (*t*-BuOOH only).

CHL/IU cells induced by H_2O_2 and *t*-BuOOH using the SCG assay. The alkaline (pH > 13) version of the SCG assay is capable of detecting DNA single-strand breaks, alkali-labile sites, DNA–DNA/DNA–protein cross-linking, and DNA single-strand breaks associated with incomplete excision repair sites.[15] FV exhibited protective effects against oxidative DNA damage of CHL/IU cells induced by both H_2O_2 and *t*-BuOOH. FV reduced H_2O_2-induced DNA damage to the same extent as AsA, TX, and PB, which are well-known antioxidants. PB has a highly lipophilic property and phenolic moieties, which show the antioxidant property partly due to their free radical scavenging effect.[35,36] The relative protective effect of M2 and M3, which are major metabolites of FV, against oxidative DNA damage induced by H_2O_2, was greater than that of FV, in the order of M3 > M2 > M4 = FV. FV caused a reduction in DNA damage induced by *t*-BuOOH, and the protective potency of FV was equal to that of AsA, TX, and PB. The relative protective effect of M2 and M3 against *t*-BuOOH-induced DNA damage was significantly greater than that of FV, in the order of M3 = M2 > M4 = FV. The effect of FV against oxidative DNA damage of CHL/IU cells using the SCG assay supported previous findings that FV has protective effects on ROS-induced phage DNA breakage and mutagenesis in *S. typhimurium*. These results indicated that FV and its metabolites reduced injury to cells caused by ROS-induced DNA damage. This may be due to the scavenging of ROS by FV. In comparison with two other HMG-CoA reductase inhibitors, PV and SV, only FV showed consistently the protective effect on the oxidative DNA damage.

We conclude that FV and its metabolites may act as free radical scavengers and potent antioxidants. FV and, to an even greater effect, its metabolites were shown to be far more effective than two other HMG-CoA reductase inhibitors in protecting cells from the oxidative modification which is mediated by ROS. Therefore, a combination of the ROS scavenging activity with the hypolipidemic property of FV may augment its clinical value in preventing a wide variety of diseases such as cellular aging, inflammation, diabetes, and cancer development resulting from oxidative stress.

References

[1] Hussein, O., Schlezinger, S., Rosenblat, M., Keidar, S. and Aviram, M. (1997) "Reduced susceptibility of low

density lipoprotein (LDL) to lipid peroxidation after fluvastatin therapy is associated with the hypocholesterolemic effect of the drug and its binding to the LDL", *Atherosclerosis* **128**, 11–18.

[2] Leonhardt, W., Kurktschiev, T., Meissner, D., Lattke, P., Abletshauser, C., Weidinger, G., Jaross, W. and Hanefeld, M. (1997) "Effects of fluvastatin therapy on lipids, antioxidants, oxidation of low density lipoproteins and trace metals", *European Journal of Clinical Pharmacology* **53**, 65–69.

[3] Goldstein, J.L., Ho, Y.K. and Brown, M.S. (1979) "Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition", *Proceedings of the National Academy of Sciences USA* **76**, 333–337.

[4] Plosker, G.L. and Wagstaff, A.J. (1996) "Fluvastatin—a review of its pharmacology and use in the management of hypercholesterolaemia", *Drugs* **51**, 433–459.

[5] Dain, J.G., Fu, E., Gorski, J., Nicoletti, J. and Scallen, T.J. (1993) "Biotransformation of fluvastatin sodium in humans", *Drug Metabolism and Disposition* **21**(4), 567–572.

[6] Mitani, H., Bandoh, T., Ishikawa, J., Kimura, M., Totsuka, T. and Hayashi, S. (1996) "Inhibitory effects of fluvastatin, a new HMG-CoA reductase inhibitor, on the increase in vascular ACE activity in cholesterol-fed rabbits", *British Journal of Pharmacology* **119**, 1269–1275.

[7] Suzumura, K., Yasuhara, M., Tanaka, K. and Suzuki, T. (1999) "Protective effect of fluvastatin sodium (XU-62-320), a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, on oxidative modification of human low-density lipoprotein *in vitro*", *Biochemical Pharmacology* **57**, 697–703.

[8] Imaeda, A., Aoki, T., Kondo, Y., Hori, M., Ogata, M., Obayashi, H., Hasegawa, G., Nakamura, N., Tokuda, K., Nishino, H., Yoshikawa, T. and Kondo, M. (2000) "Protective effects of fluvastatin against reactive oxygen species induced DNA damage and mutagenesis", *Free Radical Research* **34**, 33–44.

[9] Imaeda, A., Aoki, T., Kondo, Y. and Hori, M. (2000) "Effects of antioxidants on the mutagenicity of reactive oxygen species-generating systems towards *Salmonella typhimurium* TA102 and TA104 strains", *Environmental Mutagen Research* **22**, 1–7.

[10] Suzumura, K., Yasuhara, M., Tanaka, K., Odawara, A., Narita, H. and Suzuki, T. (1999) "An *in vitro* study of the hydroxyl radical scavenging property of fluvastatin, an HMG-CoA reductase inhibitor", *Chemical and Pharmaceutical Bulletin* **47**, 1010–1012.

[11] Suzumura, K., Yasuhara, M. and Narita, H. (1999) "Superoxide anion scavenging properties of fluvastatin and its metabolites", *Chemical and Pharmaceutical Bulletin* **47**, 1477–1480.

[12] McKelvey-Martin, V.J., Green, M.H.L., Schmezer, P., Pool-Zobel, B.L., DeMeo, M.P. and Collins, A. (1993) "The single cell gel electrophoresis assay (comet assay): a European review", *Mutation Research* **228**, 47–63.

[13] Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995) "The comet assay: a comprehensive review", *Mutation Research* **339**, 37–59.

[14] Hartmann, A. and Spier, G. (1995) "Genotoxic effects of chemicals in the single cell gel (SCG) test with human blood cells in relation to induction of sister chromatid exchanges (SCE)", *Mutation Research* **346**, 49–56.

[15] Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C. and Sasaki, Y.F. (2000) "Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing", *Environmental and Molecular Mutagenesis* **175**, 184–191.

[16] Singh, N.P., McCoy, M.T., Ticc, R.R. and Schneider, E.L. (1988) "A simple technique for quantitation of low levels of DNA damage in individual cells", *Experimental Cell Research* **175**, 184–191.

[17] Yoshikawa, T., Minamiyama, Y., Naito, Y. and Kondo, M. (1994) "Antioxidant properties of bromocriptine, a dopamine agonist", *Journal of Neurochemistry* **62**, 1034–1038.

[18] Miyagawa, H., Yosikawa, T., Tanigawa, T., Yoshida, N., Sugino, S. and Kondo, M. (1998) "Measurement of superoxide dismutase activity by electron spin resonance", *Journal of Clinical Biochemical Nutrition* **5**, 1–7.

[19] Tanigawa, T. (1990) "Determination of hydroxyl radical scavenging activity by electron spin resonance", *Journal of Kyoto Prefectural University of Medicine* **99**, 133–143.

[20] Ashby, J., Hilton, H., Lefevre, P.A. and Browne, M.A. (1995) "The single cell gel electrophoresis assay for induced DNA damage (comet assay): measurement of tail length and moment", *Mutagenesis* **10**, 85–90.

[21] Nakamura, T., Nishi, H., Kokusenya, Y., Hirota, K. and Miura, Y. (2000) "Mechanism of antioxidative activity of fluvastatin-determination of the active position", *Chemical and Pharmaceutical Bulletin* **48**, 235–237.

[22] Nakashima, A., Ohtawa, M., Masuda, N., Morikawa, H. and Bando, T. (1999) "Antioxidative effects of fluvastatin, and its major metabolites", *Yakugaku Zasshi* **119**, 93–99.

[23] Yamamoto, A., Hoshi, K. and Ichihara, K. (1998) "Fluvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, scavenges free radicals and inhibits lipid peroxidation in rat liver microsomes", *European Journal of Pharmacology* **361**, 143–149.

[24] Jiann, G.L., Michael, C.M., William, R., Kenneth, A. and Robert, D. (1996) "The use of single cell electrophoresis assay in detecting DNA single strand breaks in lung cells *in vitro*", *Toxicology and Applied Pharmacology* **141**, 195–204.

[25] Ames, B.A. (1983) "Dietary carcinogens and anticarcinogens", *Science* **221**, 1256–1264.

[26] Anderson, D., Phillips, B.J., Yu, T.W., Edwards, A.J., Ayesh, R. and Butterworth, K.R. (1997) "The effect of vitamin C supplementation on biomarkers of oxygen radical generated damage in human volunteers with low or high cholesterol levels", *Environmental Molecular Mutagenesis* **30**, 161–174.

[27] Wiseman, H. and Halliwell, B. (1996) "Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer", *Biochemical Journal* **313**, 17–29.

[28] Araki, A. (1997) "Oxidative stress and diabetes mellitus: a possible role of alpha-dicarbonyl compounds in free radical formation", *Nippon Ronen Igakkai Zasshi* **34**, 716–720.

[29] Leinonen, J., Rantalaiho, V., Lehtimaki, T., Koivula, T., Wirta, O., Pasternack, A. and Alho, H. (1998) "The association between the total antioxidant potential of plasma and the presence of coronary heart disease and

renal dysfunction in patients with NIDDM", *Free Radical Research* **29**, 273–281.

[30] Ishino, A., Mita, S., Watanabe, S. and Sakagami, H. (1999) "Effect of anticancer drugs, metals and antioxidants on cytotoxic activity of epigallocatechin gallate", *Anticancer Research* **19**, 4343–4348.

[31] Blask, D.E., Sauer, L.A., Dauchy, R.T., Holowachuk, E.W., Ruhoff, M.S. and Kopff, H.S. (1999) "Melatonin inhibition of cancer growth *in vivo* involves suppression of tumor fatty acid metabolism *via* melatonin receptor-mediated signal transduction events", *Cancer Research* **59**, 4693–4701.

[32] Zhao, J., Sharma, Y. and Agarwal, R. (1999) "Significant inhibition by the flavonoid antioxidant silymarin against 12-*O*-tetradecanoylphorbol 13-acetate-caused modulation of antioxidant and inflammatory enzymes, and cyclooxygenase 2 and interleukin-1alpha expression in SENCAR mouse epidermis: implications in the prevention of stage I tumor promotion", *Molecular and Carcinogenesis* **26**, 321–333.

[33] Smith, T.A. (1998) "Carotenoids and cancer: prevention and potential therapy", *British Journal of Biomedical Science* **55**, 268–275.

[34] Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Spiegelhalder, B. and Bartsch, H. (2000) "The antioxidant/anticancer potential of phenolic compounds isolated from olive oil", *European Journal of Cancer* **36**, 1235–1247.

[35] Hiramatsu, M., Liu, J., Edamatsu, R., Ohba, S., Kadowaki, D. and Mori, A. (1994) "Probucol scavenged 1,1-diphenyl-2-picrylhydrazyl radicals and inhibited formation of tiobarbituric acid reactive substances", *Free Radical Biology and Medicine* **16**, 201–206.

[36] Valoti, M., Sipe, H.J., Sgaragli, G. and Mason, R.P. (1989) "Free radical intermediates during peroxidation of 2-*t*-butyl-4-methoxyphenol, 2,6-di-*t*-butyl-4-methylphenol, and related phenol compounds", *Archives of Biochemistry and Biophysics* **269**, 423–432.