Mechanism of Antioxidative Activity of Fluvastatin–Determination of the Active Position

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In order to clarify the mechanism of action for the antioxidative activity of fluvastatin sodium (FLV, (±)-sodium (3*RS*, 5*RS*, 6*E*)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxy-6-heptanoate) and its derivatives, reaction of the corresponding methyl ester of FLV with di-*tert*-butyl diperoxyoxalate was examined, and the corresponding keto derivative was isolated from the reaction mixture. On the basis of this result, it was concluded that the active site is the allylic carbon conjugated with the indole ring.

Key words fluvastatin; 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor; antioxidative activity; spin trapping; hydrogen-atom abstraction

Hypercholesterolemia drugs such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors lower the level of plasma cholesterol by inhibiting the key enzyme in the biosynthetic pathway from HGM-CoA to mevalonic acid, a precursor of cholesterol.[1,2] Recently, it was reported that this type of medicine is effective for prevention of arteriosclerosis and myocardial infraction.[3,4] The medical effectiveness of medicines for these diseases is considered to be due to their antioxidative activity. Accordingly, other antioxidative compounds such as probucol have also received much attention.[5,6]

Recently, it was shown that fluvastatin sodium (FLV, (±)-sodium (3*RS*, 5*RS*, 6*E*)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxy-6-heptanoate), one of the HMGCoA reductase inhibitors, has *in vivo* and *in vitro* antioxidative activity.[7—13] For example, FLV scavenged hydroxyl radical and superoxide anion,[11—13] inhibited microsomal lipid peroxidation, [11,12] reduced the susceptibility of low density lipoprotein (LDL) to lipid peroxidation,[7—10] and surpressed progression of arteriosclerosis.[9] However, in spite of many studies on the antioxidative activity of FLV, the mechanism for the antioxidative behavior of FLV remains unresolved, and we considered that it is very important to determine the active position. In the present study, we performed hydrogen-atom abstraction from the corresponding methyl ester of FLV (FLV-Me) using *tert*-butoxyl radicals generated by thermolysis of di-*tert*-butyl diperoxyoxalate (DBDP) and isolated a keto form of FLV-Me (FLV-K-Me) from the reaction mixture. On the basis of this result, we could unambiguously determine that the active site for the antioxidative ability of FLV is the allylic carbon conjugated with the indole ring.

Experimental

Materials and Reagents FLV-Me was prepared by Tanabe R and D Service Co., Ltd (Tokyo, Japan). FLV-R-Me was prepared by Tanabe Seiyaku Co., Ltd. (Osaka, Japan). DBDP was prepared by the reported method.[14] Phenyl-*N*-*tert*-butyl nitrone (PBN) and 2,2,6,6-tetramethylpiperidinyl-*N*-oxyl (TEMPO) were purchased from Sigma Aldrich. Column chromatography was carried out on silica gel (BW127ZH, Fuji Silysia Chemical Co., Ltd., Aichi, Japan), and TLC plates (Kieselgel 60F_{254}) were purchased from Merck.

Measurements ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker DRX-500 spectrometer with tetramethylsilane as an internal standard; chemical shifts are given in ppm (δ). Liquid chromatography (LC)-MS spectra were taken on a Hitachi M-1000 LC-API mass spectrometer. ESR spectra were measured with a Bruker ESP300 spectrometer equipped with 100 kHz field modulation. HPLC was carried out on a Shimadzu LC-6A equipped with a SPD-6A UV detector, a CTO-6A column oven, and a C-R5A computer system. The column was a Shiseido Capcellpak C_{18}, and the mobile phase was phosphate buffer saline (50 mmol/l, pH 6.5) : CH_3CN (11 : 9).

Reaction of FLV-Me with DBDP A solution of FLV-Me (50 mg, 0.117 mmol) and DBDP (117 mg, 0.50 mmol) in benzene (10 ml) was stirred at 40 °C for 1 h under a nitrogen stream. The reaction mixture was evaporated under reduced pressure, and the residue was chromatographed on silica gel with benzene : ethyl acetate (3 : 1). The light yellow zone was collected and concentrated to give FLV-K-Me in 23% yield (11.4 mg, 0.0269 mmol). Recrystallization from hexane gave pale yellow prisms with mp 99—100 °C. IR (KBr): 1744 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$): 7.75 (1H, d, J=16 Hz, –CO–CH=C<u>H</u>–), 7.57 (1H, d, J=8 Hz, aromatic), 7.50 (1H, d, J=8 Hz, aromatic), 7.38 (2H, m, aromatic), 7.28 (1H, t, J=8 Hz, aromatic), 7.17 (2H, m, aromatic), 7.10 (1H, t, J=8 Hz, aromatic), 6.29 (1H, d, J=16 Hz, –CO–C<u>H</u>=), 4.95 (1H, septet, J=7 Hz, –C<u>H</u>Me$_2$), 4.50 (1H, m, –CH$_2$–C<u>H</u>(OH)–CH$_2$–), 3.71 (3H, s, –COOC<u>H</u>$_3$), 3.54 (1H, d, J=4 Hz, –CH(O<u>H</u>)–), 2.72 (2H, d, J=6 Hz, –CH(OH)–C<u>H</u>$_2$–CO–), 2.55 (2H, d, J=6 Hz, –OCOC<u>H</u>$_2$–), 1.70 (6H, d, J=7 Hz, –CH(C<u>H</u>$_3$)$_2$). ^{13}C-NMR ($CDCl_3$): 198.8 (s), 172.7 (s), 162.5 ($J_{C\text{-}F}$=238 Hz), 137.5 (s), 132.4 (d), 132.4 (overlapped), 132.3 (*d*, $J_{C\text{-}F}$=8 Hz), 131.0 (s), 128.8 (s), 128.0 (d), 124.5 (d), 121.7 (s), 121.1 (d), 120.9 (d), 116.3 (*d*, $J_{C\text{-}F}$=21 Hz), 112.7 (d), 65.1 (d), 52.2 (q), 48.5 (d), 47.4 (t), 41.0 (t), 22.3 (q). LC-MS (APCI) (rel. int. %) *m/z*: 424 (M+H)$^+$(100), 322 (32). *Anal*. Calcd for $C_{25}H_{26}FNO_4$: C, 70.91; H, 6.19; N, 3.31. Found: C, 70.67; H, 6.22; N, 3.25.

Reaction of FLV-Me with DBDP in the Presence of TEMPO The reaction of FLV-Me with DBDP in benzene was carried out in the presence of TEMPO in the same manner as above. Thus, a solution of FLV-Me (50 mg, 0.117 mmol), DBDP (117 mg, 0.50 mmol), and TEMPO (39 mg, 0.25 mmol)

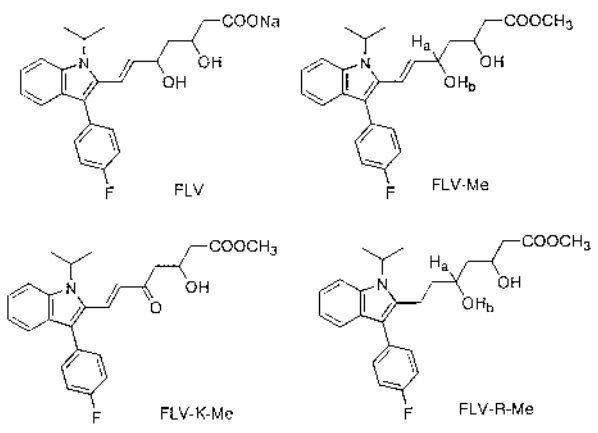

Chart 1

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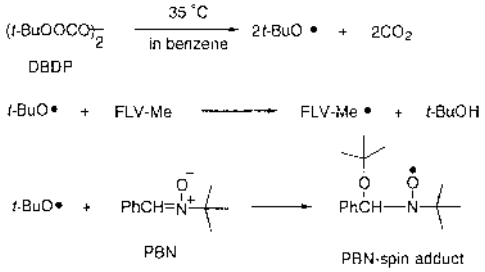

Chart 2

in benzene (10 ml) was stirred at 40 °C for 1 h under a nitrogen stream, and the mixture was evaporated under reduced pressure. The residue was then chromatographed on silica gel with benzene : ethyl acetate (3 : 1), and the light yellow zone was collected and concentrated to give FLV-K-Me in 52% yield (25.8 mg, 0.061 mmol). Melting point, IR and ^{1}H-and ^{13}C-NMR spectra agreed completely with those of the above sample.

Spin Trapping of *tert*-Butoxyl Radical by PBN in the Presence of FLV-Me Benzene solutions (1.0 ml) containing PBN (10 mmol/l), DBDP (5.0 mmol/l), FLV-Me (0—10.0 mmol/l) in ESR tubes were degassed with three freeze-pump-thaw cycles, and the tubes were sealed. After standing at 35 °C for 30 min, ESR measurements were carried out. The results are shown in Fig. 1.

Competitive Reaction of FLV-Me and FLV-R-Me with *tert*-Butoxyl Radical A solution of FLV-Me (50.0 mg, 0.118 mmol), FLV-R-Me (50.4 mg, 0.118 mmol), DBDP (55 mg, 0.236 mmol), and TEMPO (36.9 g, 0.236 mmol) in benzene (10 ml) was stirred at 35 °C under a nitrogen stream. Every 30 min, 0.1 ml of the reaction mixture was taken and diluted to 10 ml with the mobile phase. Twenty μl of the sample solution was used for HPLC measurements. The results are shown in Fig. 2.

Results and Discussion

Activity of FLV-Me as a Scavenger for *tert*-Butoxyl Radicals Since oxygen-centered free radicals such as ·OH or ·OOH play an important role for oxidation *in vivo*, it is desirable that ·OH or ·OOH radicals be used for hydrogen-atom abstraction from FLV. However, it is very difficult to generate these radicals effectively. We therefore chose *tert*-butoxyl radical as an oxygen-centered radical instead of ·OH or ·OOH radical. Since *tert*-butoxyl radical can be effectively generated by thermolysis of DBDP in hydrocarbons such as benzene and hexane.[14] However, FLV is insoluble in benzene and hexane. We therefore used FLV-Me instead of FLV because of its good solubility in benzene.[15] At first, the antioxidative activity of FLV-Me was evaluated using the spin trapping method.[16] A solution of FLV-Me, PBN and DBDP in benzene was placed in an ESR tube and, after the solution was degassed and allowed to stand at 35 °C for 30 min, ESR spectra were measured. DBDP is decomposed in a homolytic mechanism in hydrocarbon solvents at room temperature to give two *tert*-butoxyl radicals and two CO_2 molecules in quantative yields.[17] The generated *tert*-butoxyl radicals react with PBN to give PBN-spin adducts or abstract a hydrogen atom from FLV-Me, as shown in Chart 2. If FLV-Me has no active hydrogen, *tert*-butoxyl radicals are trapped by PBN or decompose to nonradical compounds. In this case, the intensity of the ESR signal due to the PBN-spin adduct would be constant regardless of the concentration of FLV-Me. As found in Fig. 1, the intensity of ESR signal due to the PBN-spin adduct is drastically decreased with an increase in the concentration of FLV-Me, and when the concentrations of FLV-Me are higher than 1.25 mmol/l, the ESR signal intensity is less than 10% of the case of the absence of FLV-Me. This indicates that most *tert*-butoxyl radicals generated are consumed by hydrogen-atom abstraction from FLV-Me, indicating that FLV-Me has high radical scavenging ability.

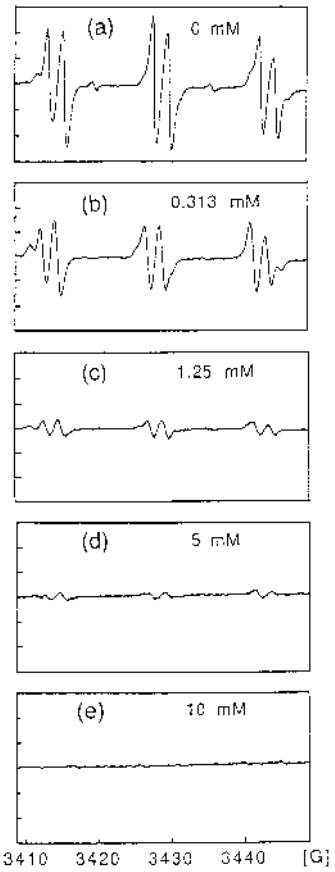

Fig. 1. ESR Spectra from Solutions of FLV-Me (0—10), DBDP (5.0), and PBN (10 mmol/l) in Benzene

FLV-Me: (a) 0, (b) 0.313, (c) 1.25, (d) 5.00, (e) 10.0 mmol/l.

Determination of the Active Position for FLV-Me To determine the active site for the antioxidative activity of FLV-Me the reaction of FLV-Me with DBDP was carried out in benzene in the presence of TEMPO. TEMPO is a very stable free radical which has the ability to trap a wide variety of intermediate radicals. We therefore expected that TEMPO might capture an intermediate radical generated by hydrogen-atom abstraction from FLV-Me by *tert*-butoxyl radicals to give a stable coupled product. The reaction was carried out at 40 °C for 1 h under a nitrogen atmosphere. TLC inspection showed three spots. Two of them were due to FLV-Me and TEMPO, and the third spot (light yellow) was due to the product. ESR measurements of the reaction mixture showed that the concentration of TEMPO was constant within experimental error during the reaction. Isolation of the product was performed by column chromatography with benzene : ethyl acetate (3 : 1), and FLV-K-Me was obtained in 52% yield (see, Chart 3). Crystallization from hexane gave pale yellow prisms with mp 99—100 °C.

The reaction of FLV-Me with DBDP was also carried out in the absence of TEMPO. In this case, TLC analysis showed formation of many minor products other than FLV-K-Me, and FLV-K-Me was obtained in a lower yield (23%).

The structure of FLV-K-Me was determined by flow injection LC-MS and ^{1}H- and ^{13}C-NMR spectroscopy. The flow injection LC-MS gave *m/z* 424 as a $(M+H)^+$ ion. The ^{1}H-

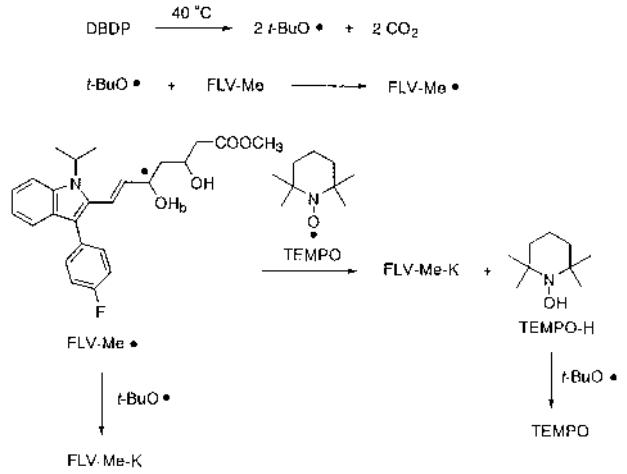

Chart 3

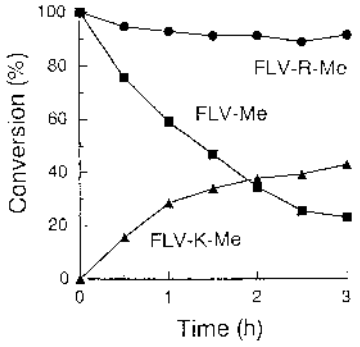

Fig. 2. Plots of the Relative Concentrations of FLV-Me, FLV-R-Me, and FLV-K-Me *vs.* Time

Reaction conditions: [FLV-Me]=11.8 mmol/l; [FLV-R-Me]=11.8 mmol/l; [DBDP]=23.6 mmol/l; [TEMPO]=23.6 mmol/l; benzene 10 ml; temperature, 35 °C.

NMR spectrum showed that the *N*-isopropyl-3-(4-fluorophenyl)indole group remained unchanged. On the other hand, disappearance of the peak at 4.13 ppm due to the allylic hydrogen (H_a) and that at 3.32 ppm due to the hydroxy proton (H_b) of FLV-Me was observed. The ^{13}C-NMR spectrum showed the appearance of a new peak due to a carbonyl carbon at 198.8 ppm. The formation of the carbonyl group was also confirmed by IR. Elemental analysis was in satisfactory agreement with the calculated values.

A plausible mechanism for formation of FLV-K-Me is shown in Chart 3. *tert*-Butoxyl radical generated by thermolysis of DBDP abstracts the allylic hydrogen-atom (H_a) from FLV-Me to give intermediate FLV-Me· radical. In the presence of TEMPO, the FLV-Me· radical is subject to immediate hydrogen-atom abstraction by TEMPO to give FLV-K-Me and TEMPO-H. Since TEMPO-H is known to be highly reactive for hydrogen-atom abstraction,[18] this compound must be immediately converted to TEMPO by reaction with *tert*-butoxyl radical. The constant concentration of TEMPO can be explained by this mechanism. In the absence of TEMPO, other reactions are anticipated for the FLV-Me·radical. Since the main product is FLV-K-Me, the main reaction is hydrogen-atom abstraction from the FLV-Me·radical by *tert*-butoxyl radical. As other possible reactions, coupling reaction between the FLV-Me· radicals or hydrogen-atom abstraction by FLV-Me· from nonradical compounds or coexisting radicals are expected. However, since the byproducts were not isolated, the detailed mechanism for the decomposition of FLV-Me· radicals is unclear.

Competitive Hydrogen-atom Abstraction from FLV-Me and FLV-R-Me by *tert*-Butoxyl Radical Competitive hydrogen abstraction from FLV-Me and FLV-R-Me by *tert*-butoxyl radical was carried out to clarify whether the double bond conjugated with the indole ring plays an important role or not in the antioxidative activity of FLV-Me. A solution of an equimolar amount of FLV-Me and FLV-R-Me (0.118 mmol), DBDP (0.236 mmol), and TEMPO (0.236 mmol) in benzene (10 ml) was stirred at 35 °C, and the consumption of FLV-Me and FLV-R-Me and formation of FLV-K-Me were followed by HPLC. The relative concentrations of these compounds are plotted as a function of time in Fig. 2.[19] It is obvious that FLV-Me is consumed much more rapidly than FLV-R-Me, indicating that *tert*-butoxyl radical abstracts dominantly a hydrogen-atom from FLV-Me. On the basis of this result, we can conclude that the double bond conjugated with the indole ring plays an important role for the antioxidative activity of FLV-Me. Since the –CH(OH)–CH=CH–indole ring skeleton is common to FLV and FLV-Me, it is most likely that the antioxidative mechanism of FLV is the same as that of FLV-Me.

Conclusions

Hydrogen-atom abstraction from FLV-Me by *tert*-butoxyl radical gave FLV-K-Me. From the formation of FLV-K-Me, it was concluded that the active position in FLV-Me is the allylic carbon having an OH group. Competitive hydrogen-atom abstraction from FLV-Me and FLV-R-Me by *tert*-butoxyl radical showed that the double bond conjugated with the indole ring plays an important role in the high antioxidative activity of FLV-Me.

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