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Effect of FR194738, a potent inhibitor of squalene epoxidase, on cholesterol metabolism in HepG2 cells

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Abstract

(*E*)-*N*-ethyl-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-3-[2-methyl-2-(3-thienylmethoxy)propyloxy]benzylamine hydrochloride (FR194738) inhibited squalene epoxidase activity in HepG2 cell homogenates with an IC_{50} value of 9.8 nM. In the study using intact HepG2 cells, FR194738 inhibited cholesterol synthesis from [^{14}C]acetate with an IC_{50} value of 4.9 nM, and induced intracellular [^{14}C]squalene accumulation. On the other hand, the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor simvastatin reduced both cholesterol and squalene synthesis from [^{14}C]acetate. Incubation with simvastatin for 18 h produced increases in HMG-CoA reductase activity in HepG2 cells, which was related to the degree of reduction in cholesterol synthesis. The HMG-CoA reductase activity increased by 13- and 19-fold at the concentrations of simvastatin that inhibited cholesterol synthesis by 65% and 82%, respectively. In contrast, FR194738 did not increase HMG-CoA reductase activity at the concentrations that inhibited cholesterol synthesis by 24% and 69%, and moderate increase (4.6-fold) was observed at the concentration that inhibited cholesterol synthesis by 90%. These results suggest that non-sterol metabolite(s) derived from mevalonate prior to the squalene epoxidation step in the cholesterol synthetic cascade have a regulatory role in the suppression of HMG-CoA reductase activity. We speculate that FR194738 inhibits cholesterol synthesis with a minimal change of the regulator(s) and would be highly effective in the treatment of hypercholesterolemia.

Keywords: FR194738; Squalene epoxidase; Simvastatin; HMG-CoA reductase; HepG2 cell

1. Introduction

3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase (EC 1.1.1.34) is the major rate-limiting enzyme of the cholesterol biosynthesis pathway and catalyzes the reduction of HMG-CoA to mevalonic acid (Rodwell et al., 1976). Cholesterol and several biologically important non-sterol products, such as isoprenyl adenine, dolichol, coenzyme Q, heme A, and prenylated proteins are produced from mevalonate (Goldstein and Brown, 1990) and some of these products are involved in a multivalent feedback mechanism of HMG-CoA reductase itself (Brown and Goldstein, 1980). While cholesterol mediates HMG-CoA reductase transcriptional repression (Osborne et al., 1988), non-sterol mevalonate metabolites mediate HMG-CoA reductase translational repression (Peffley and Sinensky, 1985; Cohen and Griffioen, 1988; Nakanishi et al., 1988) and cholesterol and non-sterol metabolites, acting together, enhance HMG-CoA reductase degradation (Nakanishi et al., 1988; Roitelman and Simoni, 1992; Correll and Edwards, 1994).

Squalene epoxidase (EC 1.14.99.7) is a microsomal enzyme that catalyzes the oxidation of squalene to 2,3-oxidosqualene, the last reaction of non-sterol metabolites in the cholesterol biosynthesis pathway (Hidaka et al., 1990). Therefore, inhibitors of this enzyme are unlikely to block the production of the above-mentioned non-sterol regulators, in contrast with HMG-CoA reductase inhibitors, which inhibit their production (Ghirlanda et al., 1993).

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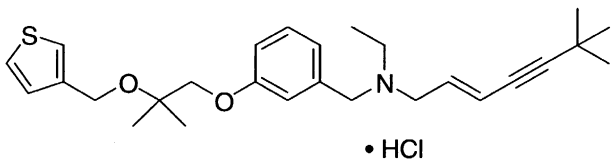

Fig. 1. Chemical structure of FR194738.

(*E*)-*N*-ethyl-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-3-[2-methyl-2-(3-thienylmethoxy)propyloxy]benzylamine hydrochloride (FR194738) (Fig. 1) is a potent inhibitor of hepatic squalene epoxidase that was found in our laboratories through screening for a novel hypolipidemic drug. We have recently reported that FR194738 lowered serum cholesterol and triglyceride levels in dogs and hamsters (Sawada et al., 1997). In the present study, we examined the effects of FR194738 on lipid synthesis and HMG-CoA reductase activity using a human hepatoma cell line, HepG2, which is thought to be a suitable model for investigating lipid metabolism in human liver (Cohen et al., 1984; Erickson and Fielding, 1986), and compared the results with those of the HMG-CoA reductase inhibitor, simvastatin.

2. Materials and methods

2.1. Materials

FR194738 was synthesized in our laboratories. Simvastatin was extracted from commercial tablets (Merck, US). The β-hydroxy acid form of simvastatin, L-654,969, was prepared from simvastatin in our laboratories. [4,8,12,13,17,21,-^{3}H]squalene (865.8 GBq/mmol, NET-645) and 3-hydroxy-3-methyl [3-^{14}C]glutaryl coenzyme A (HMG-CoA, 2.1083 GBq/mmol, NEC-642) were obtained from DuPont-New England Nuclear (Boston, MA, US). [^{14}C] acetate (2.07 GBq/mmol, CFA.13) was obtained from Amersham Life Science (Buckinghamshire, UK). Human lipoprotein deficient serum was purchased from Sigma (St. Louis, MO, USA).

2.2. Cells and cell culture

The established HepG2 cell line, derived from human hepatoma, was obtained from the American Type Culture Collection (Rockville, MD, US). Cells were cultured routinely in medium A (Eagle's modified minimum essential medium supplemented with 1 mM pyruvate and non-essential amino acids) with 10% heat-inactivated fetal calf serum at 37 °C in a humidified 5% CO_2, 95% air atmosphere.

2.3. Squalene epoxidase assay

HepG2 cells were grown in 225-cm^2 culture flasks, and incubated for 18 h in medium A containing 10% human lipoprotein deficient serum and 1 μM L-654,969 to increase their squalene epoxidase activity (Hidaka et al., 1990). The HepG2 cells were washed and harvested by trypsin treatment. After centrifugation (1000 × *g*, 5 min at 4 °C), the supernatant fraction was removed by aspiration. The cell pellet was frozen and kept at −80 °C until use. On the day of the experiment, the stocked cell pellet was thawed, ruptured by sonication (5 s at 4 °C) in 0.1 M Tris–HCl, pH 7.5 containing 1 mM EDTA, mixed with one-fourth volume of 2% Triton X-100, stood at 4 °C for 30 min, and assayed for squalene epoxidase activity according to the method of Tai and Bloch (1972) with some modifications. Aliquots of the mixture described above were incubated for 90 min at 37 °C with or without test compound dissolved in dimethyl sulfoxide (DMSO, final 1%) in a final volume of 0.3 ml containing 0.1 M Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM NADPH, 0.1 mM FAD, 0.3 mM AMO1618, an inhibitor of 2,3-oxidosqualene cyclase, 0.17% Triton X-100, and 8 μM [^{3}H]squalene (3.7 kBq) dispersed in 0.075% Tween 80. The reaction was stopped by the addition of 0.3 ml of 10% ethanolic KOH. After incubation for 90 min at 75 °C, non-saponifiable materials were extracted with 2 ml of petroleum ether. The extracts were evaporated under a nitrogen stream. The residue was taken up in a small volume of diethylether, spotted on a silica gel thin layer chromatography (TLC) plate and developed in benzene/ethyl acetate (99.5:0.5, v/v). The radioactivity of the band corresponding to authentic 2,3-oxidosqualene was counted with digital autoradiography (Berthold, Japan).

2.4. HMG-CoA reductase assay

2.4.1. Direct inhibition

HepG2 cells grown in a 21-cm^2 petri dish were incubated for 18 h in medium A containing 10% human lipoprotein deficient serum. The dishes were then washed with phosphate-buffered saline (PBS) and scraped for cells with a cell-scraper. After centrifugation (1000 × *g*, 5 min at 4 °C), the cell pellet was frozen and kept at −80 °C until use. Cell extract was prepared by suspending the thawed pellet in 200 μl of buffer containing 0.1 M potassium phosphate (pH 7.5), 5 mM EDTA, 0.2 M KCl and 0.25% Brij 97. After incubation for 15 min at 37 °C, the suspension was centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant was used for protein determination and measuring HMG-CoA reductase activity. HMG-CoA reductase activity was determined according to the method of Brown et al. (1974) with some modifications. Aliquots of the cell extract were incubated for 60 min at 37 °C with

or without test compound dissolved in DMSO (final 0.1%) in a final volume of 50 μl containing 0.1 M potassium phosphate, pH 7.5, 20 mM glucose-6-phosphate, 2.5 mM NADPH, 20 U/ml glucose-6-phosphate dehydrogenase, 4 mM dithiothreitol, and 35 μM [^{14}C]HMG-CoA (3.7 kBq). The enzyme reaction was terminated by the addition of 20 μl of 2 N HCl. After standing for 15 min at 37 °C for lactonization of mevalonic acid, [^{14}C]mevalonolactone was isolated by TLC on silica gel using acetone/benzene (1:1, v/v) and determined with an imaging analyzer (BAS2000, Fuji Film, Japan). Enzyme activity is expressed in picomoles of mevalonic acid formed per milligram of cell protein per minute.

2.4.2. Regulation

HepG2 cells in 21-cm^2 Petri dishes were incubated with or without test compound dissolved in DMSO (final 0.1%) for 18 h in medium A containing 10% human lipoprotein deficient serum. Thereafter, the cells were washed with medium A and incubated in medium A for another 15 min at 37 °C to remove intracellular compounds. The cells were washed with cold PBS and were scraped with a cell-scraper. After centrifugation ($1000 \times g$, 5 min at 4 °C), the supernatant fraction was removed by aspiration. The cell pellet was frozen and kept at −80 °C until use. Cell extract was prepared and the HMG-CoA reductase activity in cell free extract was determined as described above. Enzyme activity was determined under conditions in which the inhibitory effects of compounds were overcome by dilution.

2.5. Lipid synthesis in HepG2 cells

Lipid synthesis from [^{14}C]acetate was examined according to the method of Brown et al. (1978) with some modifications. HepG2 cells in a 6-well plate were incubated for 18 h in medium A containing 10% human lipoprotein deficient serum. After the incubation, the cells were pre-incubated with or without test compound in fresh medium A with 10% human lipoprotein deficient serum for 1 h and then labeled with 1 mM [^{14}C] acetate (74 kBq) for 2 h. The cells were then washed with PBS and were scraped with a cell-scraper. After disruption by sonication, lipids in the homogenate were extracted with 8 volumes of chloroform/methanol (2:1, v/v). Lipid classes were separated on a silica gel TLC plate using hexane/diethylether/acetic acid/methanol (85:15:1:1, v/v) as a solvent system for triglycerides and free cholesterol. Another solvent system (petroleum ether/diethylether/acetic acid = 95:5:0.5, v/v) was used for squalene and cholesteryl ester separation. Lipids on TLC were visualized by exposure to I_2 vapor. The radioactivity in the area corresponding to an authentic standard was determined using an imaging analyzer (BAS2000, Fuji Film). Cholesterol, cholesterol oleate, triolein and squalene were used as authentic standards.

2.6. Determination of protein concentration

Protein concentration was measured using the Bio-Rad Protein Assay Reagent and bovine γ-globulin as standard.

2.7. Data analysis

The results are expressed as means ± S.D. The results were evaluated by repeated measures analysis of variance, followed by Dunnett's multiple comparisons, and differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of FR194738, simvastatin and L-654,969 on squalene epoxidase and HMG-CoA reductase from HepG2 cells

FR194738 inhibited squalene epoxidase activity in HepG2 cell homogenate in a concentration-dependent manner (Fig. 2), with an IC_{50} value of 9.8 nM. Simvastatin and L-654,969 hardly affected the enzyme activity even at a concentration of 10 μM. The enzyme activity following treatment with these drugs was 110% and 93% of control, respectively.

FR194738 hardly affected HMG-CoA reductase activity in HepG2 cell extracts (103% of control at 10 μM). In contrast, simvastatin and L-654,969 dose-dependently inhibited the enzyme activity (Fig. 3). Although simvastatin

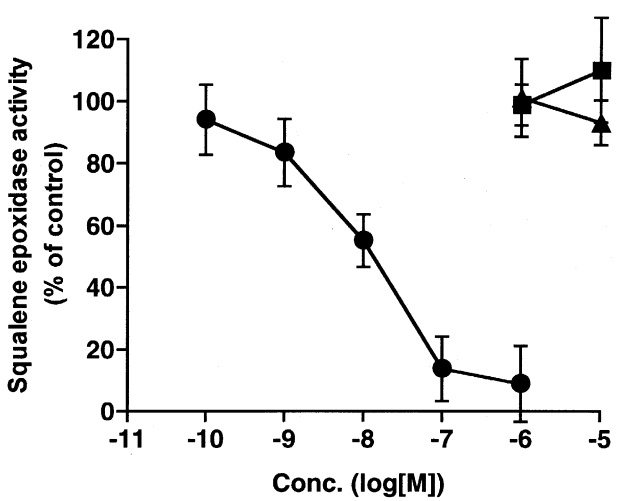

Fig. 2. Inhibition of squalene epoxidase activity from HepG2 cells by FR194738, simvastatin and L-654,969. HepG2 cell homogenate was incubated with FR194738 (●), simvastatin (■) or L-654,969 (▲) dissolved in DMSO (final 1%) together with [^{3}H]squalene. After 90-min incubation, the reaction was stopped by addition of 10% ethanolic KOH. Lipids were extracted with petroleum ether and separated by TLC. Radioactivity of [^{3}H]2,3-oxidosqualene was measured by autoanalyzer. Results are the mean ± S.D. of three experiments.

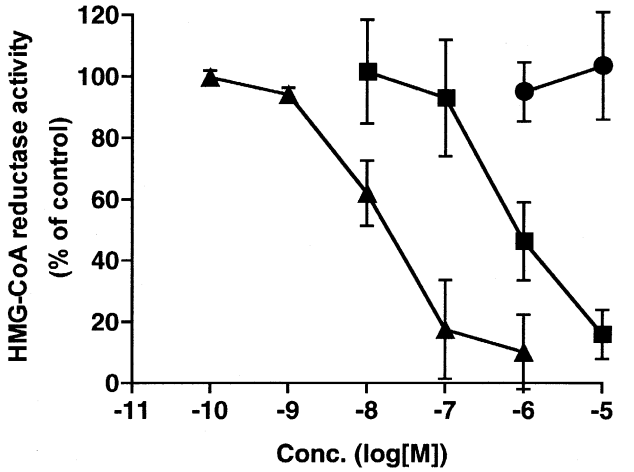

Fig. 3. Inhibition of HMG-CoA reductase activity from HepG2 cells by FR194738, simvastatin and L-654,969. HepG2 cell extract was incubated with FR194738 (●), simvastatin (■) or L-654,969 (▲) dissolved in DMSO (final 0.1%) together with [^{14}C]HMG-CoA. After 60-min incubation, the enzyme reaction was stopped by addition of 2 N HCl. After 15-min incubation, [^{14}C]mevalonolactone was separated by TLC and the radioactivity was measured by image analyzer. Results are the mean ± S.D. of three experiments.

itself was a weak inhibitor of the enzyme (IC_{50} value: 1,100 nM), the active β-hydroxy acid form of simvastatin, L-654,969 (Hoffman et al., 1986), was about 50 times more potent (IC_{50} value: 23 nM).

3.2. Effects of FR194738 and simvastatin on the lipid synthesis in HepG2 cells

To investigate the effects of FR194738 and simvastatin on lipid synthesis, HepG2 cells were preincubated with each compound for 1 h, and the incorporation of [^{14}C]acetate into free cholesterol, cholesteryl ester, squalene and triglyceride in the following 2 h was determined (Table 1). FR194738 concentration-dependently inhibited the incorporation of [^{14}C]acetate into free cholesterol and cholesteryl ester, with IC_{50} values of 4.9 and 8.0 nM, respectively. Simvastatin also inhibited the incorporation of [^{14}C]acetate into free cholesterol and cholesteryl ester in a concentration-dependent manner, the IC_{50} values being 68 and 140 nM, respectively. FR194738 increased the incorporation of [^{14}C]acetate into squalene, an intermediate of cholesterol synthesis, in HepG2 cells. In contrast, simvastatin inhibited the incorporation of [^{14}C]acetate into squalene in parallel with the inhibition of cholesterol synthesis. Neither compound affected triglyceride synthesis in HepG2 cells.

3.3. The relationship between cholesterol synthesis and HMG-CoA reductase activity

Fig. 4 shows the relationship between cholesterol synthesis and HMG-CoA reductase activity in HepG2 cells treated with FR194738 and simvastatin. FR194738 did not increase HMG-CoA reductase activity (the same as that of control cells) at 1 and 10 nM, concentrations that produced 24% and 69% inhibition of cholesterol synthesis, respectively. Moderate increases (2.6- and 4.6-fold) in enzyme activity were observed at 100 and 1000 nM, concentrations which produced 85% and 90% inhibition of cholesterol synthesis, respectively. The increase at 1000 nM was statistically significant. In contrast, a mirror-image-like change in HMG-CoA reductase activity and cholesterol synthesis was observed in response to treatment with simvastatin. Simvastatin dose-dependently increased HMG-CoA reductase activity, and the increase was related to the degree of reduction in cholesterol synthesis. The HMG-CoA reductase activity was 1.7-, 4.2-, 12.9- and 18.8-fold of control cells at the concentrations (1, 10, 100, and 1000 nM) of simvastatin that produced 9%, 21%, 65% and 82% inhibition of cholesterol synthesis, respectively.

Table 1
Effects of FR194738 and simvastatin on lipid synthesis in HepG2 cells

	Concentration (M)	Incorporation of [^{14}C]acetate (nmol/mg/2 h)			
		Free cholesterol	Cholesteryl ester	Squalene	Triglyceride
Control		21.77 (100)	0.488 (100)	0.378 (100)	62.1 (100)
FR194738	10^{-9}	16.18 (74)	0.395 (81)	1.542 (408)	43.8 (70)
	10^{-8}	7.89 (36)	0.223 (46)	13.685 (3624)	55.0 (89)
	10^{-7}	3.89 (18)	0.146 (30)	22.836 (6048)	60.4 (97)
	10^{-6}	2.98 (14)	0.168 (34)	24.978 (6615)	62.1 (100)
	10^{-5}	2.80 (13)	0.129 (26)	20.398 (5402)	57.2 (92)
Control		18.94 (100)	0.349 (100)	0.522 (100)	60.3 (100)
Simvastatin	10^{-9}	18.78 (99)	0.330 (95)	0.486 (93)	59.5 (99)
	10^{-8}	12.92 (68)	0.262 (75)	0.226 (43)	58.9 (98)
	10^{-7}	6.88 (36)	0.161 (46)	0.093 (18)	55.4 (92)
	10^{-6}	4.23 (22)	0.123 (35)	0.032 (6)	74.0 (123)
	10^{-5}	3.92 (21)	0.094 (27)	0.012 (2)	89.5 (148)

Results are representative of two experiments. Figures in parentheses indicate percent of control value.

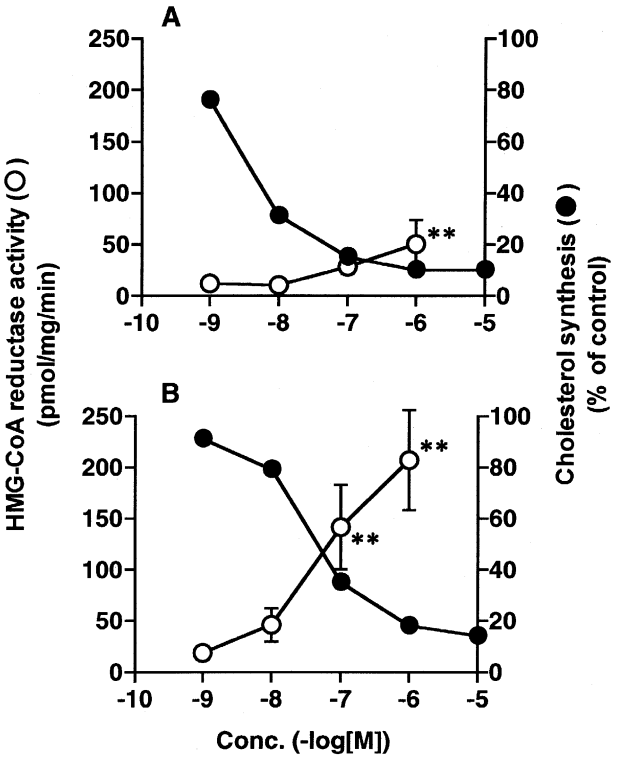

Fig. 4. Cholesterol synthesis inhibition and compensatory increases in HMG-CoA reductase activity by FR194738 (A) and simvastatin (B). For the determination of cholesterol synthesis, HepG2 cells were pre-incubated for 1 h at 37 °C with FR194738 or simvastatin and then labeled with 1 mM [^{14}C]acetate for 2 h. After incubation, lipids were extracted and separated, and radioactivity of [^{14}C]cholesterol were measured. Results (●) are the mean of two experiments. For the determination of HMG-CoA reductase activity, HepG2 cells were incubated for 18 h at 37 °C with FR194738 or simvastatin. Cells were then thoroughly washed to remove all substances that could interfere with HMG-CoA reductase. Determination of enzyme activity was performed as described in Fig. 3. HMG-CoA reductase activity without test compound was 11.0 ± 4.4 pmol/mg/min. Results (○) are the mean ± S.D. of three experiments. $^{**}P < 0.01$, compared to the value without test compound.

The increases at 100 and 1000 nM were statistically significant.

4. Discussion

The present study clearly demonstrated that FR194738 dose-dependently inhibited squalene epoxidase activity but not HMG-CoA reductase activity prepared from HepG2 cells. Consistent with its direct inhibitory effect on squalene epoxidase activity, FR194738 caused an accumulation of squalene and reduced cholesterol synthesis from [^{14}C]acetate in HepG2 cells. The sum total of [^{14}C]acetate incorporated into free and cholesteryl ester plus squalene with FR194738 treatment was similar to that of control. Furthermore, the amount of accumulated [^{14}C]squalene following FR194738 treatment was almost the same as that of reduced [^{14}C]cholesterol. These results suggest that FR194738 is a specific inhibitor of squalene epoxidase and has little effect either on the upper or on the lower stream of cholesterol synthesis pathway. On the other hand, simvastatin caused parallel decreases in cholesterol and squalene synthesis from [^{14}C]acetate, suggesting that it inhibits the production of mevalonate-derived molecules including cholesterol and squalene. Although simvastatin itself was a weak inhibitor of the HMG-CoA reductase activity in cell extract, the β-hydroxy acid form of simvastatin, L-654,969, was about 50 times more potent. These results are consistent with the previous reports that simvastatin is a prodrug for the HMG-CoA reductase inhibitor, L-654,969 (Hoffman et al., 1986) and easily converts to L-654,969 in the cells and/or medium (Nagata et al., 1990).

In an experiment to study the long-term effect of drugs on HMG-CoA reductase activity in HepG2 cells, simvastatin caused increases that were related to the degree of reduction in cholesterol synthesis. It has been speculated that HMG-CoA reductase is controlled through multivalent feedback regulation mediated by sterols and non-sterol metabolites of mevalonate (Brown and Goldstein, 1980), and that inhibition of mevalonate production by HMG-CoA reductase inhibition limits production not only of regulatory sterols but also of non-sterol regulators and induces compensatory increases in HMG-CoA reductase activity (Chun et al., 1990; Ness et al., 1994; Edwards et al., 1983).

FR194738 slightly increased HMG-CoA reductase activity at concentrations that inhibit cholesterol synthesis by more than 85%, but did not increase HMG-CoA reductase activity at all at concentrations that inhibited cholesterol synthesis by 24% and 69%. These observations suggest that HMG-CoA reductase regulation mediated by non-sterols is maintained after squalene epoxidase inhibition presumably through continued formation of non-sterol regulators derived from mevalonate, and that formation of non-sterol regulators derived from mevalonate occurs prior to the action of squalene epoxidase. This is consistent with the recent reports that farnesol, derived from farnesyl pyrophosphate hydrolysis, or a farnesol metabolite are candidates for non-sterol regulatory molecules (Correll et al., 1994; Bradfute and Simoni, 1994; Petras et al., 1999), since squalene epoxidase is located downstream of farnesyl pyrophosphate and FR194738 is not thought to inhibit the production of farnesyl pyrophosphate as mentioned above.

Furthermore, it has been demonstrated that sterols suppress HMG-CoA reductase at the transcriptional level while non-sterols suppress at the post-transcriptional level (Ness et al., 1994). We speculate that the increase in HMG-CoA reductase mRNA at the transcriptional levels is induced in response to inhibition of cholesterol production by FR194738 but it results in a slight increase in enzyme activity because suppression at post-transcriptional level by non-sterol regulator is intact. This speculation is sup-

ported by the observation that the increase in HMG-CoA reductase activity induced by NB-598, another squalene epoxidase inhibitor, which potently inhibits cholesterol synthesis, is lower than that induced by L-654,969, whereas HMG-CoA reductase mRNA is increased to the same extent by both compounds (Hidaka et al., 1991). On the contrary, depletion of both sterol and non-sterol regulators by simvastatin is thought to increase HMG-CoA reductase activity at the transcriptional and post-transcriptional levels, synergistically.

In conclusion, FR194738 is a specific squalene epoxidase inhibitor and potently inhibits cholesterol synthesis without considerable up-regulation of HMG-CoA reductase activity, unlike simvastatin. This may relate to the potent lipid-lowering activity of FR194738 in vivo (Sawada et al., 1997) because diminished induction of HMG-CoA reductase activity could result in efficient reduction of cholesterol synthesis and cholesterol content in the liver cells. Further in vivo studies are ongoing to substantiate this potency of FR194738 as a new class of lipid lowering drug.

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