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N-[2-[*N'*-PENTYL-(6,6-DIMETHYL-2,4-HEPTADIYNYL)AMINO]ETHYL]-(2-METHYL-1-NAPHTHYLTHIO)ACETAMIDE (FY-087)

A NEW ACYL COENZYME A:CHOLESTEROL ACYLTRANSFERASE (ACAT) INHIBITOR OF DIET-INDUCED ATHEROSCLEROSIS FORMATION IN MICE

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Abstract—FY-087 (*N*-[2-[*N'*-pentyl-(6,6-dimethyl-2,4-heptadiynyl)amino]ethyl]-(2-methyl-1-naphthylthio)acetamide) was found to be a competitive inhibitor of human microsomal acyl coenzyme A:cholesterol acyltransferase (ACAT) with an IC_{50} value of 0.11 μM. Under our assay conditions, other ACAT inhibitors tested, specifically YM-750, E-5324, and melinamide, all of which are now in phase I clinical trials or in clinical use in Japan, inhibited this enzyme with IC_{50} values of 0.18, 0.14, and 3.2 μM, respectively. FY-087 also inhibited ACAT in acetyl-low density lipoprotein loaded human macrophages (THP-1 cells) with an IC_{50} of 0.17 μM. Following the oral administration of FY-087 (30 mg/kg) to rats, the plasma concentration of FY-087 reached 0.42 μg/mL after 2 hr. This concentration of FY-087 was enough to inhibit blood vessel ACAT activity. Cholesterol-lowering and anti-atherogenic effects of FY-087 were examined using C57BL/6J mice fed an atherogenic diet. In this mouse model, treatment with FY-087 (28 mg/kg) inhibited the increase in plasma cholesterol levels by about 20% and decreased the hepatic accumulation of free and esterified cholesterol by 61 and 67%, respectively. FY-087 also significantly inhibited the atherogenic diet-induced increase in the fatty-streak lesion area of the proximal aorta by 57% in C57BL/6J mice. These results indicate that FY-087 is not only a therapeutically bioavailable ACAT inhibitor that lowers plasma cholesterol levels, but also an effective anti-atherogenic agent in mice fed an atherogenic diet.

Key words: acyl coenzyme A:cholesterol acyltransferase (ACAT); ACAT inhibitor; FY-087; atherosclerosis; C57BL/6J mice

The esterification of cholesterol by the enzyme ACAT† (EC 2.3.1.26) is thought to play important roles in the intestinal absorption of exogenous cholesterol [1], in hepatic VLDL-cholesterol secretion [2, 3], and in the process of cholesterol ester accumulation in blood vessels undergoing atherogenic change [4]. The accumulation of lipid-laden foam cells originating from monocyte-macrophages in the aortic intima is thought to be an early event in the development of atherosclerosis [5–9]. ACAT is responsible for foam cell formation via esterification and storage of cholesterol in monocyte-macrophages. Therefore, theoretically, a bioavailable ACAT inhibitor would be not only a cholesterol-lowering agent by inhibiting cholesterol absorption and hepatic VLDL-cholesterol secretion, but also a direct anti-atherosclerosis drug [10]. For therapeutic purposes, a number of ACAT inhibitors, which have widely diverse structures, have been developed [10–12]. Many of these inhibitors are extremely effective in preventing the absorption of cholesterol in cholesterol-fed animals. Additionally, some systemically available ACAT inhibitors have been found to reduce fatty-streak lesion formation in hypercholesterolemic rabbits [13, 14]. Nevertheless, appropriate ACAT inhibitors that have more potent hypocholesterolemic and anti-atherogenic effects are still being sought. The present report introduces FY-087, a new ACAT inhibitor that exhibits potent anti-atherogenic properties. FY-087 was discovered during our search for ACAT inhibitors among synthetic compounds; we discovered an entire series of arylthioacetamide derivatives that are all potent mammalian ACAT inhibitors.‡§ Of these arylthioacetamide derivatives, FY-087 was found to be one of the most potent inhibitors of rabbit liver microsomal ACAT with an IC_{50} value of 28 nM.

Rabbits and non-human primates are known to be susceptible to atherosclerosis due to an athero-

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†Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; FBS, fetal bovine serum; FY-087, *N*-[2-[*N'*-pentyl-(6,6-dimethyl-2,4-heptadiynyl)amino]ethyl]-(2-methyl-1-naphthylthio)acetamide; HDL, high density lipoprotein; LDL, low density lipoprotein; and VLDL, very low density lipoprotein.

‡Eur. Pat. Appl. EP-587430-A, 16 March 1994.

§Iwasawa *et al.*, *Third Annual Meeting of the Division of Medicinal Chemistry*, Shizuoka, Japan, p. 47, 1993.

genic diet and, therefore, they are thought to be appropriate models for human atherosclerosis [5–7]. Recently, the anti-atherosclerotic activity of E-5324 was shown in cholesterol-fed rabbits [14]. However, when evaluating the anti-atherogenic effects of ACAT inhibitors using these animals, a considerable amount of compound is needed for long-term administration. In the case of non-human primates, quantitative evaluation of the anti-atherogenic effects of ACAT inhibitors is difficult because of the limited availability of the primates. Therefore, little information is available concerning the anti-atherogenic effects of ACAT inhibitors *in vivo*. An inbred strain of mice, C57BL/6J, has been reported to be susceptible to atherosclerosis related to an atherogenic diet [15]. Formation of atherosclerotic lesions have been shown to be reproducible in C57BL/6J mice [15]. The great advantage of this atherosclerosis model is that the mouse is the most commonly used experimental animal, and a minimum amount of drug is needed for evaluation. Therefore, we describe the discovery of FY-087, a new, structurally unique ACAT inhibitor, and its effect on atherosclerosis in C57BL/6J mice.

MATERIALS AND METHODS

Materials

FY-087 was synthesized in our laboratories. YM-750 and E-5324 were synthesized following methods previously described [16,*]. Melinamide (Artes®) was obtained from the Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan) and was purified in our laboratory. [9,10-^{3}H(N)]Oleic acid (7.4 Ci/mmol), [1-^{14}C]oleoyl-CoA (57.8 mCi/mmol) and [1,2-^{3}H(N)] cholesterol (41.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals used were standard commercial high-purity materials.

In vitro *study*

Preparation of microsomes from Hep G2 cells. Microsomes were prepared from cultured Hep G2 cells (ATCC No. HB8065; human hepatocarcinoma cell line) according to the method of Field and Salome [17]. Cells were homogenized in cold buffer A [0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4 and 0.03 M EDTA (pH 7.2)]. After centrifugation (10,000 *g*, 20 min), the supernatant was recentrifuged at 105,000 *g* for 60 min. The sediment was washed and resuspended in buffer A. The washed microsomes were used for the ACAT assay.

Assay of microsomal ACAT. Microsomal ACAT activity was determined according to the method described by Smith *et al.* [18] with some modifications. The reaction mixture (200 μL) consisted of 90 μg of microsomal protein, 100 mM Tris–HCl (pH 8.4), 120 μM BSA, 125 μM dithiothreitol, 125 μM EDTA, 0.1% Tween 80, 0.004% cholesterol, and 60 μM [^{14}C]-oleoyl-CoA (120,000 dpm) in final concentrations. Reaction mixtures without [^{14}C]oleoyl-CoA were preincubated for 75 min, and then test compounds dissolved in DMSO (2 μL) were added. After 15 min of preincubation, the enzyme reaction was started by the addition of [^{14}C]oleoyl-CoA. The reaction was terminated by adding 4 mL of chloroform:methanol (2:1, v/v) after 5 min of incubation. [^{14}C]Cholesterol oleate was separated by TLC. The area corresponding to authentic cholesterol oleate was scraped into a vial, and the radioactivity was counted in a scintillation mixture by a scintillation counter (TRI-CARB 2000CA, Packard Instrument, Meriden, CT, U.S.A.)

Cell cultures. Hep G2 cells were cultured and maintained using a method previously described [19]. THP-1 cells (ATCC No. TIB202; human monocytic leukemia cell line) were maintained as described by Banka *et al.* [20]. The cells (6×10^5) were plated onto cell wells (12-well plate, Corning) and cultured in RPMI-1640 supplemented with 10% (v/v) FBS, penicillin G (50 μg/mL), streptomycin (50 μg/mL), 2-mercaptoethanol (50 μM), and phorbol myristate acetate (400 ng/mL) for 3 days. On day 4, the culture medium was replaced with assay medium that contained 10% (v/v) lipoprotein-deficient FBS in place of FBS and acetyl-LDL (50 μg protein/mL). The cells were cultured for another 18 hr and then used for radiolabeled oleic acid incorporation.

Assay of ACAT activity in cultured cells. Cellular ACAT activity was estimated by the incorporation of [^{3}H]oleic acid. ACAT inhibitors were dissolved in DMSO. The final concentration of DMSO in the medium was 0.1%. Transformed THP-1 cells were preincubated with ACAT inhibitors for 0.5 hr, [^{3}H]oleic acid (3 μCi) bound to albumin (final concentration; 50 μM oleic acid, 12.5 μM albumin) was then added, and the cells were incubated for another 2 hr. After separating the medium, the cells were washed twice with PBS containing 0.2% BSA and then washed once with PBS. Cellular cholesterol [^{3}H]oleate was extracted and quantified according to the methods described in the microsomal ACAT assay.

In vivo *study*

Animals. Male Sprague–Dawley (SD) rats were obtained from Charles River Japan (Atsugi, Japan). Female C57BL/6J mice were purchased from Crea Japan (Tokyo, Japan). All animals were housed with a 12-hr light/dark cycle (light period 6:00 a.m. to 6:00 p.m.).

Rat study. Male SD rats (200–300 g) were used for *in vivo* experiments. Oral [^{3}H]cholesterol administration was performed according to the methods of Fukushima *et al.* [21]. [^{3}H]Cholesterol (12 μCi, 6.96 mg) was dissolved in 177 mg of triolein. This mixture was suspended by sonication in 0.8 mL of aqueous 6.8% skim milk powder [22]. FY-087 was dispersed with the aid of 5% aqueous arabic gum. [^{3}H]Cholesterol and FY-087 were administered orally to rats simultaneously at 9:00 a.m. Control rats received [^{3}H]cholesterol and 5% arabic gum. At 1, 2, 4, 8, and 24 hr post-dosing, heparinized plasma was taken from the carotid artery of each animal by cannulation, and radioactivity and drug levels in the plasma samples were determined. Fifty microliters of plasma was mixed directly with 5 mL of Clear-Sol I (Nacalai Tesque, Kyoto, Japan), and the radioactivity was determined with a liquid

*Yamanouchi Pharm, WO9113871.

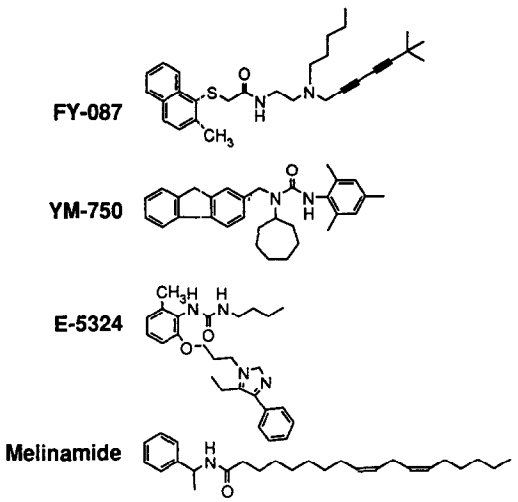

Fig. 1. Structures of ACAT inhibitors.

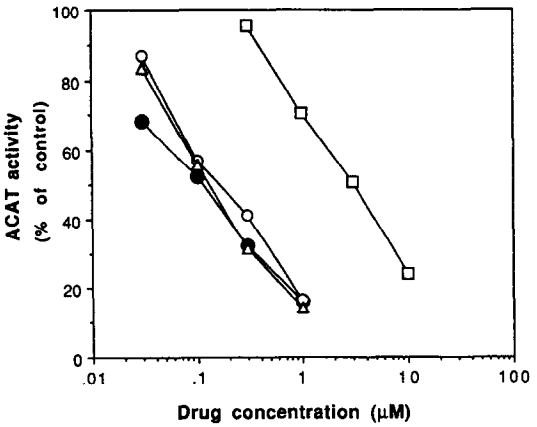

Fig. 2. Effects of ACAT inhibitors on Hep G2 microsomal ACAT activity. Cholesterol-loaded microsomes and each concentration of FY-087 (●), YM-750 (○), E-5324 (△), and melinamide (□) were preincubated for 15 min, and then [^{14}C]oleoyl-CoA was added and incubation was continued for 5 more minutes. The cholesteryl [^{14}C]oleate that was produced was determined as described in Materials and Methods. Each value is the mean of duplicate determinations. In the control incubation, 322 pmol cholesteryl [^{14}C]oleate/mg microsomal protein/min was formed.

scintillation counter as described above. For the determination of FY-087 levels, 30 μL of plasma was mixed with 120 μL of ethanol, and denatured protein was removed by centrifugation. The supernatant was analyzed directly by measuring the absorbance at 250 nm using HPLC (Gilson model 305 series; WI, U.S.A.) with a C18 column (Capcell Pak C18; Shiseido Co. Ltd., Tokyo, Japan) and 1 mM ammonium acetate-aqueous 90% ethanol as the mobile phase.

Effect of drugs on atherosclerotic lesion formation in C57BL/6J mice. Female C57BL/6J mice (14 weeks old) were used for this experiment. The atherosclerotic lesions were formed according to the method described by Paigen *et al.* [15] with some minor modifications. Before starting the study, mice were randomized into three groups according to plasma cholesterol levels and body weight. The atherogenic diet was made as described by Nishina *et al.* [23]. Normal diet (CA-1, Crea, Japan) was given to non-treated-control mice (N = 7). Atherogenic-diet-treated mice (treated-control, N = 9) and the FY-087-treated mice (N = 9) were given the atherogenic diet and 0.02% FY-087 plus the atherogenic diet, respectively, for 14 weeks. Heparinized plasma was collected from the eye vein of each mouse. At the end of the study, the mice were anesthetized, whole plasma was collected by heart puncture, and their livers and hearts were removed. For the determination of free and esterified cholesterol content in the liver, lipids were extracted with chloroform:methanol (2:1, v/v) and measured using a standard cholesterol assay kit (Kyowa Medex). Hearts were fixed (4% phosphate-buffered formaldehyde), embedded (25% gelatin), and sectioned exactly as described in the methods of Paigen *et al.* [15]. Briefly, a total of forty 10 μm cross-sections were taken sequentially, starting just above the aortic valve and moving along the aorta in the direction of blood flow. The oil red O-positive lesion area of every eighth section was quantified as described [15]. An average of the lesion area found in five sections for a given mouse was taken as the numerical value as described [15].

Gel filtration chromatography. Lipoprotein cholesterol levels were determined by gel filtration chromatography. Plasma samples (10 μL) were injected into a combined two-column system composed of Lipopropak® (Toso, Tokyo, Japan) and eluted with TSKeluent LP-1® (Toso) at a rate of 0.5 mL/min. According to the supplier's protocol (Toso), the eluent was monitored at 280 nm and was then combined with the cholesterol assay reagent (75% LTC-R-1®, 25% LTC-R-2®, Kyowa Medex, Tokyo, Japan) for measurement of cholesterol at a rate of 0.25 mL/min. The enzymatic reaction for the determination of cholesterol was performed in a Teflon tube in a temperature-controlled bath (45°). The final eluent was monitored at 550 nm. For plasma samples of normal diet-fed mice, this high performance liquid chromatography system showed elution patterns with three distinct elution positions of the major lipoprotein classes: VLDL, LDL, and HDL (see Fig. 8). However, for plasma samples of mice fed the atherogenic diet, separation of VLDL and LDL was found to be difficult. Therefore, we represented the sum of VLDL and LDL cholesterol as non-HDL cholesterol instead of the individual values (see Table 1).

Determination of protein. Protein concentrations were determined according to the method of Lowry *et al.* [24], using BSA as a standard.

Data analysis. The data from these studies were statistically analyzed using the Mann–Whitney U-test.

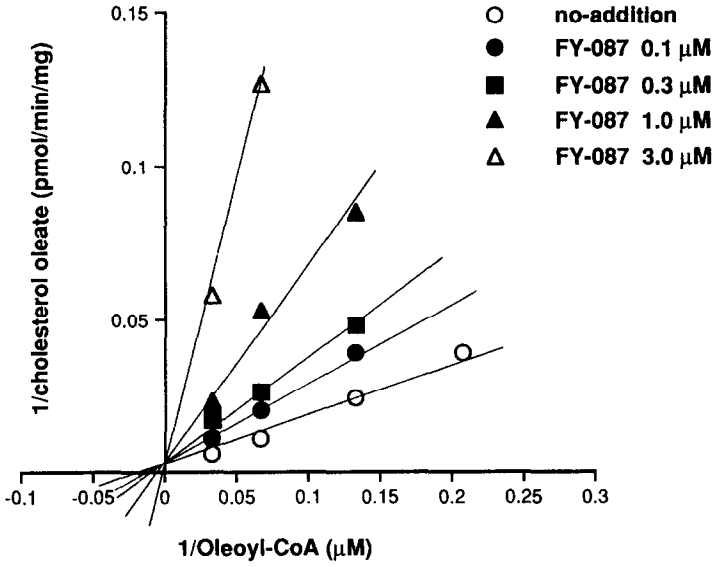

Fig. 3. Lineweaver–Burk plot of the inhibition of Hep G2 microsomal ACAT by FY-087. ACAT activity was determined as described in the legend of Fig. 2. Each value is the mean of duplicate determinations.

RESULTS

In vitro *profile of FY-087*

FY-087 has a unique structure in its tertiary butyl and diyne moieties (Fig. 1). The ACAT-inhibiting activity of FY-087 was evaluated using Hep G2 microsomes. The inhibitory potency for ACAT is shown in Fig. 2. FY-087 is compared with the other known ACAT inhibitors YM-750, E-5324, and melinamide, which are now in phase I clinical studies or in clinical use in Japan [14, 25, *]. Under the assay conditions used, FY-087 had a higher potency than the other ACAT inhibitors tested. The IC_{50} value of FY-087 was 0.11 μM. The IC_{50} values of YM-750, E-5324, and melinamide were 0.18, 0.14, and 3.2 μM, respectively. Figure 3 shows the Lineweaver–Burk analysis of Hep G2 microsomal ACAT inhibition by FY-087 over a range of oleoyl-CoA concentrations. FY-087 inhibited ACAT in a competitive manner with a K_i value of 0.29 μM.

To demonstrate the inhibitory activity of FY-087 in cells, FY-087 was added to acetyl-LDL-loaded THP-1-derived macrophages and incubated with radiolabeled oleic acid. In this established *in vitro* model of monocyte-macrophage function, FY-087 inhibited ACAT activity with an IC_{50} value of 0.17 μM (Fig. 4). The IC_{50} values of YM-750, E-5324, and melinamide were 0.24, 0.32, and 1.69 μM, respectively.

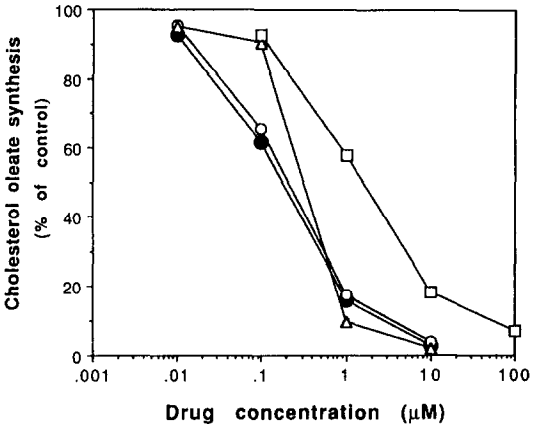

Fig. 4. Effect of ACAT inhibitors on cholesterol oleate synthesis in PMA-treated THP-1 cells. THP-1 cells were transformed to macrophages by PMA treatment (400 ng/mL) for 3 days. Then acetyl-LDL (50 μg protein/mL) was added, and the cells were incubated for 18 hr. Then the cells were preincubated with each concentration of FY-087 (●), YM-750 (○), E-5324 (△), and melinamide (□) for 0.5 hr, and cellular ACAT activity was estimated by the incorporation of [^{3}H]oleic acid bound to albumin (3 μCi, final concentration 50 μM), as described in Materials and Methods. Each value is the mean of duplicate determinations. In the control cells, 89,430 dpm cholesteryl [^{3}H]oleate/mg cell protein/hr was formed.

In vivo *activity of FY-087 in rats*

To determine biological activity of FY-087, [^{3}H]-cholesterol emulsion and a single oral dose of FY-087 (30 mg/kg) were given to rats, simultaneously. The plasma radioactivity and FY-087 levels were measured for up to 24 hr. Compared with the

*Matuda K, Ito N, Iwaoka K, Miyauchi H and Iizumi Y, Synthesis and hypolipidemic activity of tricyclic-arylmethyl substituted ureas: potent ACAT inhibitors. *XIIth International Symposium on Medicinal Chemistry, Basel, Switzerland, 13–17 September, 1992*, p. 093.

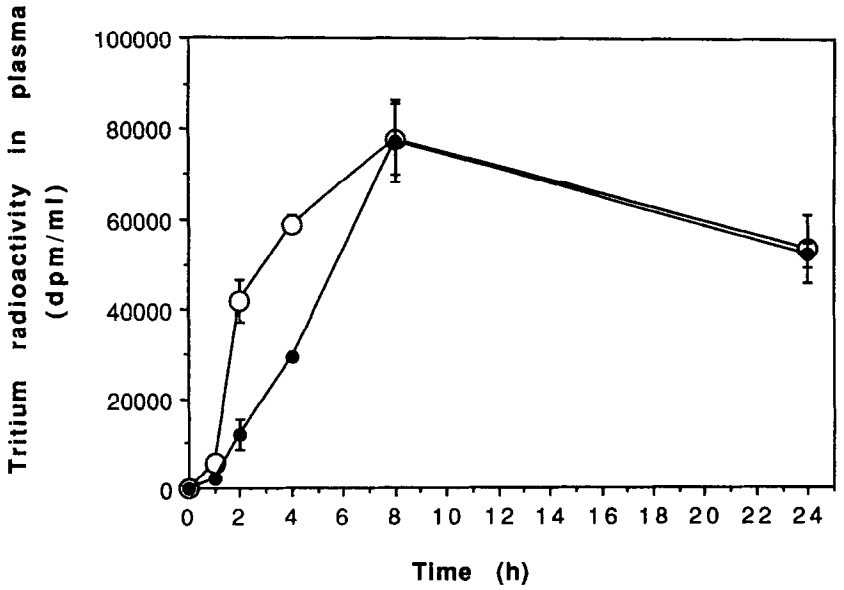

Fig. 5. Biological activity of FY-087 in rats. FY-087-treated rats (●, N = 3) were given orally [^{3}H]-cholesterol emulsion and FY-087 (30 mg/kg) dispersed in 5% arabic gum simultaneously, as described in Materials and Methods. Control rats (○, N = 3) were given [^{3}H]cholesterol emulsion (12 μCi) and vehicle. Heparinized plasma samples were taken from each rat from 1 to 24 hr after drug administration. Plasma radioactivity was determined as described in Materials and Methods. Each value is the mean ± SEM of triplicate determinations.

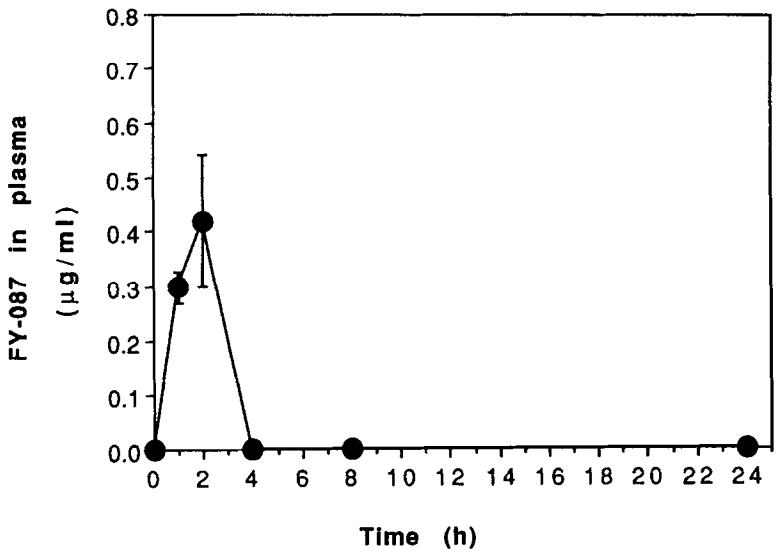

Fig. 6. Systemic activity of FY-087 in rats. Plasma FY-087 concentrations were determined as described in Materials and Methods. The closed circles indicate the plasma FY-087 concentration found in FY-087-treated rats. Each value is the mean ± SEM of triplicate determinations.

radioactivity of plasma samples from the control rats, FY-087 reduced plasma radioactivity by 72% at 2 hr and 50% at 4 hr post-dosing (Fig. 5). The maximum level of FY-087 (0.42 μg/mL) was found in plasma at 2 hr post-dosing; the AUC (area under the curve) value from 0 to 8 hr was 0.9 (μg·hr)/mL (Fig. 6). After 8 hr post-dosing, no detectable FY-087 was observed, and plasma radioactivity returned to control levels (Figs. 5 and 6).

Anti-atherogenic effect of FY-087 in C57BL/6J mice fed atherogenic diet

The cholesterol-lowering and anti-atherogenic

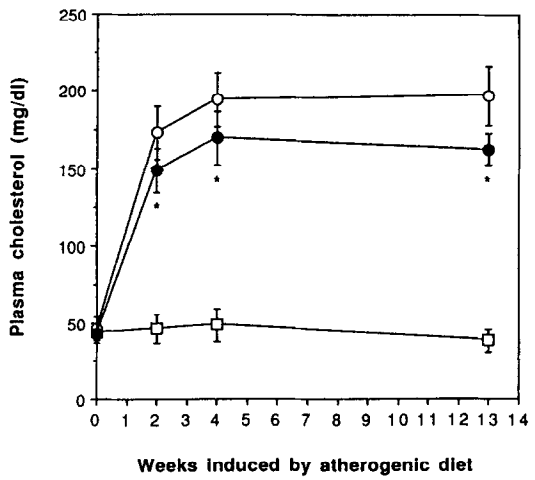

Fig. 7. Effects of FY-087 on plasma cholesterol levels induced by an atherogenic diet in C57BL/6J mice. Non-treated-control mice (□, N = 7) received a normal diet for 14 weeks. Treated-control mice (○, N = 9) and FY-087-treated mice (○, N = 9) received an atherogenic diet and an atherogenic diet containing 0.02% FY-087, respectively, for 14 weeks as described in Materials and Methods. Each value is the mean ± SEM. Key: (*) significantly different from the value of the treated-control group, $P < 0.05$.

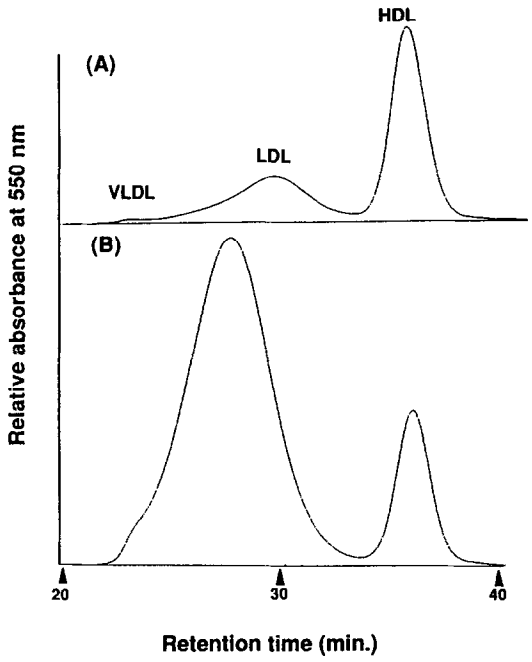

Fig. 8. Profile of plasma lipoprotein cholesterol in non-treated and treated-control mice. A portion of pooled plasma (10 μL) from the mice was applied to a high performance liquid chromatography system composed of two columns of Lipopropak® (Toso), as described in Materials and Methods. The peak positions of VLDL, LDL, and HDL are indicated. The typical patterns of plasma lipoprotein cholesterol found in non-treated-control mice (A) and treated-control mice (B) are shown.

effects of FY-087 were examined in C57BL/6J mice fed the atherogenic diet. FY-087 (0.02%) in an atherogenic diet was given to female C57BL/6J mice for 14 weeks. Throughout the experimental period, no significant differences were observed in food consumption and body weight gain between the treated-control and FY-087-treated mice. Based on calculations from food consumption, the mice ingested an average 28.1 mg/kg/day of FY-087. As shown in Fig. 7, plasma cholesterol levels in treated-control mice were increased dramatically during the first 2 weeks. The total plasma cholesterol levels in treated-control mice were elevated consistently throughout 13 weeks. At 13 weeks, the total cholesterol levels in treated-control mice were roughly five times higher than those in non-treated-control mice. FY-087 treatment significantly inhibited this increase in plasma cholesterol levels. At 2, 4, and 13 weeks, FY-087 inhibited this increase in cholesterol levels by 19, 17, and 22%, respectively, compared with cholesterol levels in treated-control mice. At 14 weeks, the mice were killed, and their plasma, livers, and hearts were taken for analysis of lipoprotein cholesterol, hepatic cholesterol accumulation, and fatty-streak lesions, respectively. Figure 8 shows the distribution of plasma cholesterol in each lipoprotein fraction in non-treated-control mice and treated-control mice. A large amount of cholesterol was associated with the VLDL/LDL fraction in treated-control mice. FY-087 significantly reduced VLDL/LDL-cholesterol by 23% compared with treated-control mice (Table 1). There was no change in HDL-cholesterol levels between treated-control mice and FY-087-treated mice. Table 2 shows hepatic cholesterol levels in mice. In the treated-controls, hepatic free and esterified cholesterol were 3.3- and 74-fold higher, respectively, than those in the non-treated-controls. Compared with the treated-controls, FY-087 significantly inhibited the increase

Table 1. Distribution of lipoprotein cholesterol

Treatment	Cholesterol (mg/dL) non-HDL	Cholesterol (mg/dL) HDL	Number of mice
Treated-control	186 ± 8.9	46.4 ± 2.6	9
FY-087 (0.02%)	142 ± 10.2*	44.2 ± 1.9	9

At the end of feeding for 14 weeks, non-treated-control, treated-control, and FY-087-treated mice were killed, and whole plasma samples were obtained by heart puncture as described in Materials and Methods. Plasma lipoprotein cholesterol in treated-control and FY-087-treated mice was analyzed as described by the methods in the legend of Fig. 8. The cholesterol levels in non-HDL (VLDL + LDL) and HDL fraction were calculated from the plasma total cholesterol and from the curve area in the elution pattern from the HPLC determinations. Each value represents the mean ± SEM.

*Significantly different from the value of the treated-control group, $P < 0.01$.

Table 2. Effect of FY-087 on liver cholesterol content induced by an atherogenic diet in C57BL/6J mice

Treatment	Cholesterol (μg/mg liver protein)			Ester/ Free ratio	Number of mice
	Total	Free	Ester		
Non-treated-control	12.1 ± 0.51	8.26 ± 0.3	3.86 ± 0.41	0.47	7
Treated-control	315 ± 11.3	27.6 ± 0.8	287 ± 10.9	10.4	9
FY-087 (0.02%)	114 ± 13.5*	15.8 ± 1.28*	98.5 ± 12.3*	6.23	9

At the end of feeding for 14 weeks, non-treated-control, treated-control, and FY-087-treated mice were killed and their livers were obtained as described in Materials and Methods. Liver free and esterified cholesterol levels were determined using a standard enzyme assay kit as described in Materials and Methods. Each value represents the mean ± SEM.

*Significantly different from the value of the treated-control group, $P < 0.01$.

Table 3. Effect of FY-087 on atherogenic diet-induced fatty-streak lesions in C57BL/6J mice

Treatment	Lesion/section (μm^2)	Number of mice
Non-treated-control	71 ± 26	7
Treated-control	5395 ± 997	9
FY-087 (0.02%)	2385 ± 676*	9

At the end of feeding for 14 weeks, non-treated-control, treated-control, and FY-087-treated mice were killed, and their hearts were obtained as described in Materials and Methods. Hearts were fixed, and sections from the proximal aorta were stained with oil-red O. The mean atherosclerotic lesion area in the proximal aorta was quantified as described in Materials and Methods. Each value represents the mean ± SEM.

*Significantly different from the value of the treated-control group, $P < 0.05$.

of hepatic free and esterified cholesterol by 61 and 67%, respectively. For the evaluation of fatty-streak lesions, the heart was fixed, and oil red O-positive sections from the juncture of the aorta and the heart to the aortic arch were analyzed. In the treated-controls, 5395 ± 997 μm^2 of lesion area was observed (Table 3). FY-087 significantly inhibited the increase in the lesion area by 57%.

DISCUSSION

FY-087 was one of the potent inhibitors of Hep G2 ACAT among the ACAT inhibitors tested. The ACAT assay was performed using Hep G2 microsomes in the presence of a saturating level of exogenously added cholesterol, because it has been reported that microsomal cholesterol content influences ACAT activity [18]. Using kinetic analysis, FY-087 was found to be a competitive inhibitor of oleoyl-CoA under the condition of saturation of the microsomal cholesterol level. Presumably, FY-087 may bind to the same catalytic site of ACAT in competition with acyl-CoA. Recently, a candidate for human ACAT cDNA was cloned by Chang *et al.* [26]. More accurate evaluation of Michaelis–Menten analysis using purified ACAT will be achieved in the near future. For evaluation of the therapeutic effect of ACAT inhibitors as anti-atherosclerosis agents, it has been theoretically important to examine the effects at the level of arterial monocyte-macrophages [10–12]. Therefore, the effects of FY-087 were examined using human target cells. FY-087 was one of the potent inhibitors of human macrophage ACAT among the ACAT inhibitors tested. These results indicate that appropriate distribution of FY-087 would prevent arterial foam cell formation in humans.

We found FY-087 to be systemically bioavailable in rats (Fig. 6). In fact, after administration of a single dose of FY-087 (30 mg/kg) and a [^{3}H]-cholesterol-emulsion to rats, the plasma concentration of FY-087 reached 0.43 μg/mL (1 μM) 2 hr post-dosing. This concentration was 5.9-fold higher than that of the IC_{50} value (0.17 μM) for ACAT inhibition in monocyte-macrophages. It thus seems that the plasma level of FY-087 would be high enough to affect foam cell formation. Furthermore, in the above experiments in rats, FY-087 reduced plasma tritium radioactivity originated from oral [^{3}H]cholesterol in the first 4 hr. We did not determine the increased excretion of radioactivity in feces. However, it is plausible that FY-087 may inhibit the [^{3}H]cholesterol absorption, as has been shown for other ACAT inhibitors [11, 27]. Mehrabian *et al.* [28] found that C57BL/6J mice fed an atherogenic diet exhibited inflammatory monocyte-macrophage inflation and apoB-containing lipoprotein accumulation in the intima of the aortic wall similar to that observed in the early atherosclerotic lesions of primates or humans. That is why this mouse model was used to evaluate the anti-atherogenic effect of FY-087. In C57BL/6J female mice maintained for 14 weeks on an atherogenic diet, fatty-streak lesions were found in the proximal aorta. In agreement with the results of Paigen and colleagues [15], about 5400 μm^2/section of lesion area was observed in treated-control mice. And in agreement with previous work, the total cholesterol level of C57BL/6J mice fed an atherogenic diet for 14 weeks was 230 mg/dL [29]. In this mouse model, FY-087 treatment significantly inhibited the increase in plasma total cholesterol levels by about 20%. FY-

087 also significantly reduced the accumulation of hepatic free and esterified cholesterol levels induced by an atherogenic-diet by 61 and 67%, respectively. FY-087 is thought to inhibit liver ACAT because it reduced esterified cholesterol content preferentially. In a separate experiment, we found by radiolabeled oleate incorporation that FY-087 inhibited ACAT in rat primary cultured hepatocytes with an IC_{50} value of 0.58 μM. Therefore, it is possible to consider that the plasma cholesterol-lowering effects of FY-087 are caused by the inhibition of cholesterol absorption or hepatic VLDL cholesterol ester secretion in mice fed an atherogenic diet. HDL-cholesterol is negatively correlated with coronary artery disease in humans. By lipoprotein analysis, FY-087 preferentially decreased VLDL/LDL-cholesterol levels without affecting HDL-cholesterol levels. Several ACAT inhibitors, such as CI-976 and CL-277, 082, have been reported to elevate HDL-cholesterol levels at various treatment doses in cholesterol-fed rats [30]. No mechanism has been proposed for why such ACAT inhibitors elevate HDL-cholesterol levels. A much higher dose of FY-087 may be needed to elevate HDL-cholesterol levels in mice, since some of the ACAT inhibitors needed to be given in relatively higher doses (100 mg/kg) for elevation of HDL-cholesterol [30]. In "susceptible" strains of mice including C57BL, HDL-cholesterol levels have been reported to correlate negatively with the lesion area [23, 31]. However, 28 mg/kg of FY-087 inhibited the development of the lesion area without affecting HDL-cholesterol levels. These results indicate that FY-087 reduced the lesion area by reducing plasma apo-B-containing lipoprotein levels. Possibly, some direct effects of FY-087 on arterial foam cell formation may be involved. We could not show any direct effect of FY-087 on arterial lesion formation because FY-087 also prevented hypercholesterolemia induced by an atherogenic diet in mice. Suzuki *et al.* recently investigated the effect of a well-known antioxidant, probucol, on an aortic lesion area using the same atherosclerosis model that we used. They found that 200 mg/kg/day of probucol for 13 weeks, despite causing a 55% reduction in the plasma cholesterol level, did not change markedly the oil-red O-positive aortic lesion area in mice.* Their results may indicate that a significant decrease in plasma cholesterol levels would not affect lesion area size in this mouse model. If so, FY-087 may have affected the aortic lesion area directly. In the present study, we evaluated the anti-atherogenic effects of ACAT inhibitors using the atherogenic mouse model for the first time. When FY-087 at a dose of 0.02% was included in the diet of ten mice for 14 weeks, about 1.2 g of the drug was needed to evaluate its anti-atherosclerosis effects. Therefore, because a minimum amount of drug is needed, this model may be useful for the comparative evaluation of anti-atherogenic drugs. Finally, the major implications of the present study are that FY-087 not only was a potent, therapeutically useful ACAT inhibitor for lipid lowering but also was effective in inhibiting atherosclerosis formation in this mouse atherosclerosis model. FY-087 is now undergoing further pharmacological evaluation.

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