Studies on Acyl-CoA:Cholesterol Aeryltransferase (ACAT) Inhibitory Effects and Enzyme Selectivity of F-1394, a Pantotheic Acid Derivative

Jun Kusunoki, Katsumi Aragane, Tetsuaki Yamaura and Haruo Ohnishi
Pharmaceuticals Research Laboratories, Fujirebio Inc., 51 Komiya-cho, Hachioji, Tokyo 192, Japan
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ABSTRACT—(1s,2s)-2-[3-(2,2-Dimethylpropyl)-3-nonylureido]aminocyclohexane-1-yl 3-[N-(2,2,5,5-tetramethyl-1,3-dioxane-4-carbonyl)amino]propionate (F-1394), a pantotheic acid derivative, is a newly synthesized inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT). In the present study, we investigated the inhibitory effects of F-1394 on the activities of ACAT. F-1394 reduced the ACAT activities in rat liver microsomes, homogenate of rabbit small intestinal mucosa and lysate of J774 macrophages with IC₅₀ values of 6.4 nM, 10.7 nM and 32 nM, respectively. The kinetic studies showed that F-1394 exerted competitive-type inhibition, and the Kᵢ values in liver and small intestinal ACAT were 4.0 nM and 9.9 nM, respectively. The inhibitory effects of F-1394 on the activity of ACAT were more potent than that of other ACAT inhibitors or hypolipidemic agents. The study on enzyme selectivity indicated that F-1394 did not affect 3-hydroxy-3-methylglutaryl CoA reductase, acyl-CoA synthetase and cholesterol esterase. F-1394 weakly inhibited the activity of lecithine:cholesterol acyltransferase (LCAT) originating from rat plasma. The inhibitory potency of F-1394 for the activity of liver microsomal ACAT was 4,690-fold stronger than that for the activity of LCAT. These findings indicate that F-1394 is a potent and selective inhibitor of ACAT, and its inhibition manner is the competitive type.

Keywords: F-1394, Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Pantotheic acid derivative, Competitive inhibition, Enzyme selectivity
Pharmaceutical Co., Ltd., Tokyo), respectively, in our laboratories. Clofibrate was purchased from Wako Pure Chemical Industries (Osaka).

Other chemicals

\[ ^{1-14}\text{C}]\text{Oleoyl-CoA (2.2 GBq/nmol), }^{[1-14}\text{C}]\text{cholesteryl oleate (2.0 GBq/nmol), }^{[1-14}\text{C}]\text{palmitic acid (2.1 GBq/nmol), }^{[4-14}\text{C}]\text{cholesterol (2.1 GBq/nmol) and }^{[3-14}\text{C}]\text{HMG-CoA (1.8 GBq/nmol) were obtained from New England Nuclear Corp. (Boston, MA, USA). All other chemicals and ware used were standard commercial high purity materials.}

**Fig. 1.** Chemical structures of oleoyl-coenzyme A, F-1394, CL-277,082 and DL-melinamide.

**Cells and cell culture**

The murine macrophage cell line J774, which was a generous gift from Prof. Y. Saito (Department of Laboratory Medicine, School of Medicine, Yamagata University, Yamagata), was grown in a 75-cm\(^2\) culture flask (Corning, Corning, NY, USA) and maintained in Dulbecco's modified Eagle's medium (DME; Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) under a humidified atmosphere of 95% air, 5% CO\(_2\) before use.

**ACAT assay**

Livers were removed from male Sprague-Dawley (SD)
rats (160–180 g; Charles River Japan, Yokohama) fed a commercial chow (F-2; Funabashi Farms, Funabashi) containing 1% cholesterol, 2.5% olive oil and 0.2% cholic acid for 2 weeks and perfused with ice-cold saline. The livers were cut into small pieces and homogenized in 0.154 M potassium phosphate buffer (pH 6.2). After that, the homogenate was centrifuged at 1,000 × g for 15 min to discard nuclei and cell debris. The resulting supernatant was centrifuged at 12,000 × g for 15 min to remove mitochondria. Then the supernatant was centrifuged at 107,000 × g for 30 min to obtain microsomes. The sedimented microsomes were suspended in the buffer for use in the experiments.

Small intestines were removed from male Japanese white rabbits (1.8–2.0 kg; Nihon SLC, Hamamatsu) fed a 1% cholesterol-supplemented rabbit chow (RC-4; Oriental Yeast, Tokyo) for 6 weeks and placed on ice. After being opened longitudinally, the intestinal wall was washed with ice-cold saline. The mucosa was scraped with a microscopic slide and suspended in 0.25 M sucrose solution. The suspension was centrifuged at 900 × g for 10 min. After that, the residue was homogenized in 0.154 M potassium phosphate buffer (pH 6.2). The resulting homogenate was centrifuged at 1,000 × g for 15 min, and the resulting supernatant was used for the ACAT assay.

J774 macrophages were cultured for 18 hr in DME containing 1% of serum, which was obtained from a rabbit fed a 1%-cholesterol diet for a month, in order to load cholesterol in the macrophages. Then the macrophages were homogenized, and the lysate was used for ACAT assay.

ACAT activities in various sources were determined by incorporation of 14C-oleyl-CoA into cholesteryl olate according to the method of Heider et al. (6). The incubation mixture (total volume of 0.5 ml) consisting of 0.154 M of potassium phosphate buffer (pH 7.4), 2 μM of bovine serum albumin (BSA, Oriental Yeast) and 14C-oleoyl-CoA was incubated at 37°C for 5 min. After that, ACAT protein and 5 μl of dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto) with the dissolved test compound were added to the incubation mixture, and the incubation was carried out in triplicate assays for 5 min at 37°C. Then 1 ml of Dole’s reagent (isopropanol : n-heptane : 1 N H2SO4 = 40:10:1) was added to the mixture to stop the enzyme reaction. One milliliter of n-heptane and 0.35 ml of water were added to the mixture. The resulting supernatant was passed through a nylon mesh. The filtrate was used for the acyl-CoA synthetase assay.

Acyl-CoA synthetase assay

Small intestines were removed from male SD rats (160–180 g, Charles River Japan) fasted for one night and placed on ice. The intestinal mucosa was scraped with a microscopic slide and suspended in ice-cold 5 mM Tris-HCl buffer containing 0.25 M sucrose. The suspension was centrifuged at 1,000 × g for 10 min and the resulting supernatant was passed through a nylon mesh. The filtrate was used for the acyl-CoA synthetase assay.

Acyl-CoA synthetase activity in the homogenate was determined by incorporation of 14C-palmitic acid into palmitoyl-CoA according to the method of Berl-Tana et al. (9). The incubation mixture (total volume of 0.2 ml) consisted of 0.15 M of Tris-HCl buffer (pH 7.2), 20 mM of ATP, 0.2 mM of coenzyme A, 2.25 mM of glutathione, 6.6 mM of MgCl2, 0.1% of Triton X-100 and 3.7 kBq of 14C-palmitic acid. Twenty micrograms of the enzyme protein and 2.5 μl of DMSO solution containing the dissolved test compound were added to the incubation mixture, and the incubation was performed in triplicate assays for 5 min at 37°C. Then 1 ml of Dole’s reagent (isopropanol : n-heptane : 1 N H2SO4 = 40:10:1) was added to the mixture to stop the enzyme reaction. One milliliter of n-heptane and 0.35 ml of water were added to the mixture. The resulting mixture was shaken vigorously, and the heptane layer was discarded. The radioactivity of 14C-palmitoyl-CoA in the aqueous phase was measured by LSC.

HMG-CoA reductase assay

Livers were removed at midnight (1:00 a.m.) from male SD rats (160–180 g, Charles River Japan) fed a commercial chow (F-2) containing 2% cholestyramine for 2 weeks, and the microsomal fraction was prepared as described above. All procedures of this subfractionation were carried out in 0.1 M potassium phosphate buffer containing 15 mM of nicotinamide and 2 mM of MgCl2 at 0–4°C.

HMG-CoA reductase activity in the microsomes was determined by converting 14C-HMG-CoA into mevalonolactone according to the method of Kuroda and Endo (8). The 40-μl incubation mixture consisted of 0.1 M potassium phosphate buffer (pH 7.4), 12.5 mM of ethylenediaminetetraacetic acid (EDTA), 12.5 mM of dithiothreitol, 1.25 mM of β-nicotinamide adenine dinucleotide phosphate (β-NADPH, type I; Sigma, St. Louis, MO, USA) and 14C-HMG-CoA. The microsomal protein and 2 μl of DMSO containing the dissolved test compound were added to the mixture, and the incubation was carried out in triplicate assays for 15 min at 37°C. After that, 0.01 ml of 2 M HCl was added to the mixture, and the incubation was carried out at 37°C for another 15 min to convert mevalonic acid to mevalonolactone. The radioactive mevalonolactone was isolated by TLC, and the radioactivity was detected by LSC.

Acyl-CoA synthetase assay

Small intestines were removed from male SD rats (160–180 g, Charles River Japan) fasted for one night and placed on ice. The intestinal mucosa was scraped with a microscopic slide and suspended in ice-cold 5 mM Tris-HCl buffer containing 0.25 M sucrose. The suspension was centrifuged at 1,000 × g for 10 min and the resulting supernatant was passed through a nylon mesh. The filtrate was used for the acyl-CoA synthetase assay.

Acyl-CoA synthetase activity in the homogenate was determined by incorporation of 14C-palmitic acid into palmitoyl-CoA according to the method of Berl-Tana et al. (9). The incubation mixture (total volume of 0.2 ml) consisted of 0.15 M of Tris-HCl buffer (pH 7.2), 20 mM of ATP, 0.2 mM of coenzyme A, 2.25 mM of glutathione, 6.6 mM of MgCl2, 0.1% of Triton X-100 and 3.7 kBq of 14C-palmitic acid. Twenty micrograms of the enzyme protein and 2.5 μl of DMSO solution containing the dissolved test compound were added to the incubation mixture, and the incubation was performed in triplicate assays for 5 min at 37°C. Then 1 ml of Dole’s reagent (isopropanol : n-heptane : 1 N H2SO4 = 40:10:1) was added to the mixture to stop the enzyme reaction. One milliliter of n-heptane and 0.35 ml of water were added to the mixture. The resulting mixture was shaken vigorously, and the heptane layer was discarded. The radioactivity of 14C-palmitoyl-CoA in the aqueous phase was measured by LSC.
buffer (pH 7.4) containing 0.25 M sucrose, and the lysate was used for the aCEase assay or nCEase assay. Porcine pCEase was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA) and dissolved in the same Tris buffer.

CEase activities in various sources were determined by hydrolysis of cholesteryl-14C-oleate to cholesterol and 14C-oleic acid (10-12). The reaction mixture (total volume of 0.2 ml) consisting of 1-α-lyso phosphatidylcholine, cholesteryl-14C-oleate, enzyme protein, DMSO containing the dissolved test compound, 0.1 M Tris-HCl buffer (pH 7.5, nCEase; pH 7.0, pCEase) or 0.1 M acetic buffer (pH 4.5, aCEase) and saline was incubated for 1 hr at 37°C (duplicate or triplicate assays). The incubation was stopped by addition of 3.25 ml of Belfrage's solution (methanol : chloroform : n-heptane=1.42:1.25:1.00) and 1 ml of 0.1 N NaOH, and the mixture was shaken vigorously. After that, the mixture was centrifuged, and the radioactivity in the resulting aqueous phase was counted by LSC.

**LCAT assay**

Blood was drawn from male SD rats (160-180 g, Charles River Japan) by using syringes containing 5 mM EDTA solution. The plasma was separated immediately by centrifugation at 4°C and used for the LCAT assay.

LCAT activity in the plasma was determined by incorporation of 14C-cholesterol into cholesteryl ester according to the method of Bell and Hubert (13). The substrate solution (total volume of 0.6 ml) containing 0.154 M phosphate buffer (pH 7.4), 1.1 mM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), 3% BSA, 7.4 kBq of 14C-cholesterol and 0.2 ml of the plasma was incubated for 4 hr at 37°C. After that, 20 μl of the phosphate buffer containing 9.4 mM 2-mercaptoethanol and 5 μl of F-1394 dissolved in DMSO were added to the substrate mixture, and the incubation was carried out in triplicate assays at 37°C for another 40 min. The isolation and measurement of radioactive esterified cholesterol was performed as described in the ACAT assay.

**RESULTS**

*Effects on activities of ACATs*

The ACAT activity in rat liver microsome was 129.1 ± 6.5 pmol/mg microsome/min. F-1394, CL-277,082 and DL-MA inhibited the ACAT activity in rat liver (Fig. 2a) in a dose-dependent fashion with the estimated IC50 values of 6.4 nM, 23 nM and 130 nM, respectively (Table 1). The ACAT activity in the homogenate of rabbit small intestinal mucosa was 28.8 ± 1.4 pmol/mg protein/min. F-1394, CL-277,082 and DL-MA inhibited the activity in rabbit small intestinal mucosa (Fig. 2b) in a
Table 1. Inhibitory effects of test compounds on the activity of ACAT in rat liver microsomes

| Test compounds | IC50 values (M) | K, values (M) | Inhibition type |
|----------------|----------------|--------------|----------------|
| F-1394         | 6.4 x 10^-9    | 4.0 x 10^-9  | Competitive    |
| CL-277,082     | 2.3 x 10^-8    | 5.6 x 10^-8  | Non-competitive|
| du-Melinamide  | 1.3 x 10^-7    | —            | —              |
| Pravastatin    | >1.0 x 10^-6   | —            | —              |
| Probuloc       | >1.0 x 10^-6   | —            | —              |
| Clofibrate     | >1.0 x 10^-6   | —            | —              |

ACAT activity in rat liver microsomes was determined by incorporation of 14C-oleoyl-CoA into cholesteryl oleate. Liver microsomes were isolated from SD rats fed a high cholesterol diet for 2 weeks. The reaction mixture (total volume of 0.5 ml) consisted of 0.154 M of potassium phosphate buffer (pH 7.4), 2 nM of BSA, 185 MBq of 14C-oleoyl-CoA, 0.2 mg of the microsomal protein and 5 µl of test compound dissolved in DMSO. The incubation was carried out in triplicate assays for 5 min at 37°C. Lipids in the mixture were extracted by chloroform : methanol (2:1), and the radioactive product was isolated by TLC. The radioactivity was detected by a liquid scintillation counter. —, not tested.

Table 2. Inhibitory effects of test compounds on the activity of ACAT in rabbit small intestinal homogenates

| Test compounds | IC50 values (M) | K, values (M) | Inhibition type |
|----------------|----------------|--------------|----------------|
| F-1394         | 1.1 x 10^-8    | 9.9 x 10^-9  | Competitive    |
| CL-277,082     | 5.8 x 10^-9    | 4.3 x 10^-8  | Non-competitive|
| du-Melinamide  | 3.5 x 10^-4    | —            | —              |
| Pravastatin    | >1.0 x 10^-6   | —            | —              |
| Probuloc       | >1.0 x 10^-6   | —            | —              |
| Clofibrate     | >1.0 x 10^-6   | —            | —              |

ACAT activity in the homogenate of small intestinal mucosa was determined by incorporation of 14C-oleoyl-CoA into cholesteryl oleate. The homogenate of small intestinal mucosa was isolated from Japanese white rabbits fed a high cholesterol diet for 6 weeks. The reaction mixture (total volume of 0.5 ml) consisted of 0.154 M of potassium phosphate buffer (pH 7.4), 2 nM of BSA, 2.78 kBq of the substrate, 80 µg of cell protein and 5 µl of test compound dissolved in DMSO. The incubation was carried out in triplicate assays for 5 min at 37°C. Lipids in the mixture were extracted by chloroform : methanol (2:1), and the radioactive product was isolated by TLC. The radioactivity was detected by a liquid scintillation counter. —, not tested.

Table 3. Inhibitory effects of test compounds on the activity of ACAT in J774 macrophage homogenates

| Test compounds | IC50 values (M) | Inhibition at 10^-4 M (%) |
|----------------|----------------|--------------------------|
| F-1394         | 3.2 x 10^-8    | —                        |
| CL-277,082     | 2.3 x 10^-7    | —                        |
| du-Melinamide  | 4.5 x 10^-7    | —                        |
| Pravastatin    | >1.0 x 10^-4   | 7.7                      |
| Probuloc       | >1.0 x 10^-4   | 15.4                     |
| Clofibrate     | >1.0 x 10^-4   | 86.9                     |

F-1394 was a competitive inhibitor (Fig. 3a), and the K, values of F-1394 in the liver and small intestinal mucosa were 4.0 nM and 9.9 nM, respectively. In contrast, CL-277,082 was a non-competitive inhibitor (Fig. 3b), and the K, values of CL-277,082 in the liver and small intestinal mucosa were 56 nM and 43 nM, respectively (Tables 1 and 2). The IC50 values of CI-976 on ACAT activities in rat liver and J774 macrophages were 2.1- and 1.3-times that of F-1394, respectively. In rabbit small intestine, the IC50 value of CI-976 on ACAT activity was the same as that of F-1394 (data not shown). The inhibition manner of CI-976 was the non-competitive type in rat liver microsomes and rabbit small intestinal mucosa (data not shown).

Pravastatin, probuloc and clofibrate had less effect on ACAT activities in the liver, small intestinal mucosa and J774 macrophages, except that 0.1 mM of clofibrate reduced the activity of ACAT in J774 macrophages by 86% (Tables 1-3).

Effect on activities of other enzymes

F-1394 at a concentration of 0.1 mM weakly inhibited the activity of HMG-CoA reductase in rat liver microsome by 8.1%. In contrast, 0.1 µM of pravastatin, a HMG-CoA reductase inhibitor, inhibited the activity of HMG-CoA reductase by 81.4% (Table 4).

F-1394 did not affect the activities of acyl-CoA synthetase in rat small intestinal mucosa (Table 5) and acid cholesterol esterase in J774 macrophages (Table 6).
Table 4. Effects of F-1394 and pravastatin on the activity of HMG-CoA reductase in rat liver microsomes

| Test compounds | HMG-CoA reductase activity (nmol/mg microsomes/min) | Inhibition (%) |
|----------------|-----------------------------------------------|----------------|
| None           | 2.10±0.06                                      | 0.0            |
| F-1394, 10⁻⁴ M | 1.93±0.13                                      | 8.1            |
| Pravastatin, 10⁻⁷ M | 0.39±0.02                                    | 81.4           |

HMG-CoA reductase activity was determined by conversion of [¹⁴C]-HMG-CoA into mevalonolactone. Liver microsomes were isolated at midnight from SD rats fed a 2% cholesterol diet for 2 weeks. The incubation mixture (total volume of 50 µl) consisted of 0.1 M potassium phosphate buffer (pH 7.4), 12.5 mM of EDTA, 12.5 mM of dithiothreitol, 1.25 mM of p-NADPH, 18.5 kBq of the substrate, microsomal protein and 2 µl of test compound dissolved in DMSO. The incubation was carried out in triplicate assays for 15 min at 37°C. After that, 0.01 ml of 2 M HCl was added to the mixture, and the incubation was perfomed at 37°C for another 15 min to convert mevalonic acid into mevalonolactone. Mevalonolactone in the reaction mixture was isolated by TLC, and the radioactivity was detected by liquid scintillation counter. The enzyme activities are given as means±S.E.

Table 5. Effect of F-1394 on the activity of acyl-CoA synthetase in rat small intestinal homogenates

| Drug concentration | Acyl-CoA synthetase activity (nmol/mg protein/min) |
|--------------------|-----------------------------------------------|
| None               | 2.13±0.02                                      |
| F-1394 10⁻⁴ M      | 2.22±0.14                                      |
| 10⁻⁷ M             | 2.21±0.18                                      |
| 10⁻⁴ M             | 2.12±0.02                                      |

Acyl-CoA synthetase activity in the homogenate of rat small intestinal mucosa was determined by incorporation of [¹⁴C]-oleoyl-CoA into cholesterol oleate. The homogenate of small intestinal mucosa was isolated from SD rats fasted for one night. The reaction mixture (total volume of 0.2 ml) consisted of 0.15 M of Tris/HCl buffer (pH 7.2), 20 mM of ATP, 0.2 mM of coenzyme A, 2.25 mM of glutathione, 6.6 mM of MgCl₂, 0.1% of triton X-100, 20 µg of the mucosal protein, 3.7 kBq of the substrate and 2.5 µl of F-1394 dissolved in DMSO. The incubation was carried out in triplicate assays for 5 min at 37°C. After that, 1 ml of Dole's reagent (isopropanol : n-heptane : 1 N H₂SO₄=40:10:1), 0.35 ml of water and 1 ml of n-heptane were added to the mixture. The radioactivity of the product in the aqueous phase was measured by a liquid scintillation counter. The enzyme activities are given as means±S.E.

DISCUSSION

The present enzymological studies were designed to investigate the ACAT inhibitory potency of F-1394, a pantotetheic acid derivative, its manner of inhibition and its enzyme selectivity.

The inhibitory potencies of F-1394 on ACAT activities...
Table 6. Effects of F-1394 on the activities of acid cholesteresterase (aCEase), neutral cholesteresterase (nCEase) or pancreatic cholesteresterase (pCEase)

| Drug concentration | aCEase (nmol/mg protein/hr) | nCEase (nmol/mg protein/hr) | pCEase (nmol/mg protein/hr) |
|--------------------|----------------------------|----------------------------|----------------------------|
| None               | 1.87 ± 0.05                | 130 ± 3.9                  | 4.87 ± 0.13                |
| F-1394 10⁻⁶ M     | 1.79 ± 0.05                | 112 ± 3.0                  | 4.98 ± 0.15                |
| 10⁻³ M            | 1.91 ± 0.15                | 95 ± 2.3                   | 5.12 ± 0.15                |
| 10⁻⁴ M            | 1.46 ± 0.05                | 181 ± 8.2                  | 5.46 ± 0.17                |
| DFP 10⁻⁴ M        | -                          | -                          | 0.44 ± 0.18                |

Acid cholesteresterase, neutral cholesteresterase and pancreatic cholesteresterase activities were determined by hydrolysis of cholesteryl-[¹⁴C]-oleate to cholesterol and oleic acid. The experimental conditions were described in Materials and Methods. The enzyme activities are given as means ± S.E. (duplicate or triplicate assays). -, not tested.

Table 7. Effect of F-1394 on the activity of lecithine:cholesterol acyltransferase (LCAT) in rat plasma

| Drug concentration | LCAT activity (nmol/ml plasma/hr) | LCAT activity (%) |
|--------------------|----------------------------------|------------------|
| None               | 444.2 ± 33.5                    | 100.0            |
| F-1394 10⁻⁷ M     | 421.2 ± 9.7                     | 94.8             |
| 10⁻⁴ M            | 373.8 ± 21.5                    | 85.3             |
| 10⁻³ M            | 319.0 ± 25.2                    | 71.8             |
| 10⁻⁴ M            | 135.8 ± 3.9                     | 39.6             |

LCAT activity in rat plasma was determined by incorporation of [¹⁴C]-cholesterol into cholesteryl ester. The substrate solution (total volume of 0.6 ml) containing 0.154 M of phosphate buffer (pH 7.4), 1.1 mM of DTNB, 3% of BSA, 0.2 ml of the plasma and 7.4 kBq of [¹⁴C]-cholesterol was incubated for 4 hr at 37°C. After that, 20 nl of phosphate buffer containing 9.4 mM of 2-mercaptoethanol and 5 pl of F-1394 dissolved in DMSO were added to the substrate solution, and the incubation was carried out in triplicate assays at 37°C for another 40 min. Lipid in the mixture was extracted by chloroform : methanol (2:1), and the radioactive product was isolated by TLC. The radioactivity was detected by a liquid scintillation counter. The enzyme activities are given as means ± S.E.

The kinetic studies showed that F-1394 is a competitive inhibitor, whereas CL-277,082 was a non-competitive inhibitor (Fig. 3). Our results on CL-277,082 agree with the previously report of Largis et al. (15). Natori et al. reported that DL-MA showed un-competitive inhibition on ACAT activity from rabbit small intestinal mucosa (5). Since F-1394 was found as a result of a structure-activity relationship study on the moiety of acyl-CoA, which is a substrate of ACAT, the chemical structure of F-1394, which has pantothenate, is more similar to that of acyl-CoA as compared with the structures of CL-277,082 and DL-MA (Fig. 1). Therefore, F-1394 may exert competitive inhibition on ACAT activity, unlike CL-277,082 or DL-MA. Field et al. reported that CI-976 was a competitive inhibitor of ACAT in the lysate from CaCo2 cells (14). However, our result indicates that the inhibition manner of CI-976 is not a competitive type in rat liver and rabbit small intestinal mucosa. Thus, there are two possible explanations for the apparent discrepancy of the data between our group and Field et al.: First, we used isolated tissue preparations from the whole animal for the ACAT assays, while Field and coworkers used the lysate from human intestinal cell line CaCo2; Secondly, the experimental condition in our assay system was different from that used by Field and coworkers, especially with respect to incubation time for the enzyme reaction. Thus, CI-976 (PD128042, fatty acid trimethoxyanilide) (14) has
no substitute corresponding to pantothenate like DL-MA and CL-277,082 in contrast to F-1394. In the enzyme selectivity study, F-1394 did not affect HMG-CoA reductase from rat liver (Table 4), acyl-CoA synthetase from rat small intestinal mucosa (Table 5), aCEase from 3774 macrophages, nCEase from 3774 macrophages and pCEase from swine (Table 6). F-1394 showed weak inhibition on the activity of LCAT originating from rat plasma (Table 7). However, the inhibitory potency of F-1394 against the activity of liver microsomal ACAT activity was 4, 690-fold stronger than that against the activity of LCAT. Moreover, the affinity of F-1394 to the catalytic site of ACAT in rat liver was 16,000-fold greater than that to the catalytic site of LCAT in rat plasma. Therefore, we conclude that the effect of F-1394 on LCAT activity is negligible. These results suggest that F-1394 does not affect a key step of cholesterol biosynthesis in the liver; the enzyme involved in cholesterol absorption via the gut, such as a pCEase (16); and the enzymes implicated in reversed cholesterol transport in the body, such as nCEase (17) and LCAT (18, 19); and this drug only affects ACAT. Therefore, our observations indicate that F-1394 does not inhibit the enzymes involved in the utilization of both cholesteryl ester in peripheral cells, such as an aCEase (20), and fatty acid in the body, such as an acyl-CoA synthetase (21).

Non-esterified cholesterol, derived from the diet, is esterified by ACAT in mucosal cells, prior to incorporation into chylomicron particles in the gut, and re-esterification of cholesterol occurs under the influence of hepatic ACAT, prior to incorporation into VLDL particles and release into the circulation (2). Under the atherosclerotic condition including lipoprotein alteration, ACAT catalyzes the growth of lipid droplets in macrophages, and subsequently contributes much to the formation of foam cells that exist in atheroma (20). Thereby, ACAT inhibition may have potential for hypocholesterolemic activity by the prevention of absorption of cholesterol via the gut and secretion of VLDL from the liver, and it may have anti-atherosclerotic activity by impeding the formation of foam cells (3). Indeed, several investigators reported that ACAT inhibition increased the reduction of dietary-cholesterol absorption via the gut in vitro (14) and in vivo (6, 15), prevented the release into the circulation of VLDL-cholesterol from the liver in vitro (22) and in vivo (23) and inhibited foam cell formation (24).

In this study, we indicated that F-1394 strongly inhibited the activities of ACAT originating from the liver, small intestine and macrophages. Thus, F-1394, a potent ACAT inhibitor, may have therapeutic potential for the treatment of hypercholesterolemia and atherosclerosis. Our results indicated that the inhibitory potency of DL-MA on ACAT activities from various sources was much less than that of F-1394 (Tables 1 - 3). These results suggest that F-1394 may have beneficial anti-hyperlipidemic and anti-atherosclerotic effects in the clinical situation compared with those of DL-MA.

On the other hand, pravastatin, probucol and clofibrate did not inhibit the activities of ACAT in these preparations (Tables 1-3). Therefore, these hypolipidemic agents may not be expected to be drugs for the prevention of cholesterol absorption via the gut and may have no direct effect on atherosclerosis.

In conclusion, F-1394, a pantothenic acid derivative, is a potent and selective inhibitor of ACAT and its inhibition manner is the competitive type.

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