Hypocholesterolemic Action and Prevention of Cholesterol Absorption via the Gut by F-1394, a Potent Acyl-CoA:Cholesterol Acyltransferase (ACAT) Inhibitor, in Cholesterol Diet-Fed Rats

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ABSTRACT—In the present study, we investigated the hypocholesterolemic effect of F-1394 ((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), a potent and selective inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT), and the effect on cholesterol absorption via the gut in rats fed a 1% cholesterol diet. Single administration of F-1394 to the cholesterol diet-fed rats at the doses of 3–30 mg/kg, p.o. decreased the serum cholesterol levels by 16–54% 3 hr after the administration. The ACAT activity in the small intestinal mucosa of the rats given orally F-1394 (30 mg/kg) was significantly inhibited 3 hr after the administration. The hypocholesterolemic action of F-1394 had a faster onset than that of DL-melinamide or CL-277,082. The study by the dual isotope ratio method showed that F-1394 (30 mg/kg, p.o.) significantly suppressed the dietary cholesterol absorption. Furthermore, in the determination of cholesterol absorption by using 14C-cholesterol as the oral tracer, the administration of F-1394 (30 mg/kg, p.o.) 1 or 2 hr before or immediately after the application of the oral tracer significantly prevented the appearance of the radioactivity in the circulation by around 90%. These results indicate that oral administration of F-1394 inhibits the ACAT activity in the small intestinal mucosa and subsequently contributes much to the prevention of cholesterol absorption via the gut, resulting in the decrease in serum cholesterol levels in the cholesterol diet-fed rats. Furthermore, the effect of F-1394 appears immediately after its administration in contrast to that of DL-melinamide or CL-277,082.

Keywords: F-1394, Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, Cholesterol absorption, Hypocholesterolemic action, Cholesterol-fed rat

(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 pantethic derivative, is a potent, selective and competitive inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT) (1).

ACAT (EC 2.3.1.26) is an enzyme that catalyzes the intracellular esterification of free cholesterol to cholesterol ester, and it appears to be localized to the rough endoplasmic reticulum (2). ACAT has been implicated as a key enzyme involved in dietary cholesterol absorption via the gut (3–6) and the release into the circulation of esterified cholesterol at the liver (7–9). Under the atherogenic condition, including lipoprotein alteration, ACAT catalyzes growth of the lipid droplet in macrophages and subsequently contributes much to the formation of the foam cells, which are responsible for the initiation and progression of atherosclerosis (10, 11). Therefore, ACAT inhibition by F-1394 may have potential hypocholesterolemic activity by preventing dietary cholesterol absorption and of secretion of very low density lipoprotein from the liver and may exert anti-atherosclerotic activity by impeding the formation of foam cells.

We previously reported that F-1394 inhibited the ACAT activity in rat liver microsomes, the homogenate of rabbit small intestinal mucosa and the lysate of J774 macrophages with IC50 values of 6.4 nM, 10.7 nM and 32 nM, respectively (1). The inhibitory potency of F-1394 on the activity of ACAT was stronger than those of other ACAT inhibitors and hypolipidemic agents (1).

The present study was conducted to clarify the effect of F-1394 on hypercholesterolemia in cholesterol diet-fed rats and to compare its effect with those of other ACAT...
inhibitors such as N-(α-methylbenzyl)-linoleamide (DL-melinamide: DL-MA) (12) and N-(2,4-difluorophenyl)-N-[4-(2,2-dimethylpropyl)phenyl]methyl]-N-heptylurea (CL-277,082) (13). In addition, the inhibitory effect of F-1394 on dietary cholesterol absorption via the gut was studied by using radiolabeled cholesterol as the tracer.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (Charles-River Japan, Atsugi), 6-weeks-old, were used in all experiments. They were maintained in a temperature- and humidity-regulated room (22 ± 2°C, 55 ± 15%) with controlled lighting (12-hr light dark cycle), and they had free access to tap water and commercial chow (CE-2; Clea Japan, Tokyo) for at least a week before the experiment.

Cholesterol diet
The cholesterol diet containing CE-2, 1% of cholesterol, 0.2% of cholic acid and 2.5% of olive oil was purchased from Clea Japan.

Chemicals
F-1394, DL-MA and CL-277,082 were synthesized by Pharmaceuticals Research Laboratories, Fujirebio, Inc. These drugs were suspended in 5% Arabic gum solution for the oral administration.

[Oleoyl-1-14C]-oleoyl coenzyme A (2.0 GBq/mmol), [1,2-3H]-cholesterol (1890.7 GBq/mmol) and [4-14C]-cholesterol (2.1 GBq/mmol) were obtained from New England Nuclear Corp. (Boston, MA, USA). All other chemicals were used as the standard commercial high purity materials.

Hypocholesterolemic action
The experimental protocol is shown in Fig. 1. Rats were fed with the cholesterol diet for 24 hr before the administration of test compounds and throughout the experiment. F-1394 was orally administered to the rats at a dose of 3, 10, 20 or 30 mg/kg (17:00). The blood was collected immediately before, 3, 6, 9 or 12 hr after the dosing with the test compounds. Rats in the recovery group were fed with normal commercial chow after the administration of its vehicle. In the comparative study of F-1394 with other ACAT inhibitors, CL-277,082 or DL-MA was orally administered to the rats at a dose of 30 mg/kg, and the blood was collected at the time described in the legend of Figs. 3 and 4. The serum cholesterol levels were measured by the enzymatic/colorimetric method (cholesterol E-test Wako; Wako Pure Chemical Industries, Ltd., Osaka).

![Blood collecting diagram](image)

**Fig. 1.** Experimental protocol for the hypocholesterolemic action by single oral administration of F-1394 in the cholesterol diet-fed rats. The rats were fed the high cholesterol diet for 24 hr before the administration of F-1394. F-1394 was orally administered to the rats at a dose of 3, 10, 20 or 30 mg/kg (17:00). Rats in the recovery group were fed normal chow after the application of its vehicle.
Ex vivo ACAT assay

Rats were fed with the cholesterol diet for 24 hr before the administration of F-1394 and throughout the experiment. F-1394 was orally administered to the rats at a dose of 30 mg/kg, and their small intestines were quickly excised 3 hr after the administration. After being opened longitudinally, the intestinal wall was washed out with ice-cold saline. The mucosa was scraped with the microscopic slide glass and suspended in 0.25 M sucrose solution. The suspension was centrifuged at 900 x 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 x g for 15 min, and the supernatant was used for the ACAT assay.

The ACAT activity was determined by the incorporation of 14C-oleoyl-CoA into cholesteryl oleate according to the method of Heider et al. (14). The reaction mixture (total volume of 0.5 ml) consisting of 0.154 M potassium phosphate buffer (pH 7.4), 2 nM bovine serum albumin and 1.85 kBq 14C-oleoyl-CoA was incubated for 5 min at 37°C. After that, 10 μg of the homogenate protein was added to the mixture, and the incubation was carried out in duplicate assays for 10 min at 37°C. The total lipid fraction in the reaction mixture was extracted according to the method of Folch et al. (15), and the radioactive product (cholesteryl oleate) in the fraction was isolated by the thin layer chromatography. The radioactivity was detected with the liquid scintillation counter (LSC) (LSC-1000, Alokτ).

Dual isotope ratio method

The dual isotope ratio method employed was that described by Zilversmit (16). 3H-Cholesterol was dissolved in saline at a concentration of 370 kBq/ml as the tracer for intravenous injection (intravenous tracer). Six milligrams of cholesterol, 185 kBq of 14C-cholesterol and 156 mg of triolein were added to 2 ml of cholic acid solution (3.75 mg/ml), and the mixture was sonicated. The resulting emulsion was used as the tracer for oral administration (oral tracer).

The rats were fed with the cholesterol diet for 3 days, and F-1394 at a dose of 30 mg/kg, p.o. was also administered orally once a day for the 3 days. On the 3rd day, the rats received an oral tracer (2 ml/body, p.o.), and F-1394 at a dose of 30 mg/kg, p.o. was administered to the rats immediately after the application of the tracer. The whole blood was collected 4, 16, 21 and 85 hr after the application, and the radioactivity was measured as described in the dual isotope ratio method.

Hypocholesterolemic action

Total cholesterol (TC) levels in the serum from the rats fed with the cholesterol diet for one night were 136±6 mg/dl (immediately before the administration of F-1394). In the normolipidemic rats, the serum TC levels were 50-60 mg/dl (data not shown). As shown in Fig. 2, F-1394 (3-30 mg/kg) decreased the serum TC levels in a dose-dependent manner and the duration of the hypocholesterolemic action was dependent on the dosage of F-1394. In recovery group, the TC levels were decreased gradually and returned to the normal levels 12 hr after the application of its vehicle.

In the comparison of F-1394 with DL-MA, the
Fig. 2. Hypcholesterolemic action by single oral administration of F-1394 in the cholesterol diet-fed rats. The rats were fed the high cholesterol diet for 24 hr before the administration of F-1394. The rats in the recovery group were fed with normal chow after the administration of its vehicle. Serum cholesterol levels were measured by the enzymatic/colorimetric method. Each symbol represents the mean±S.E. of 6 rats. ●: control; △: F-1394, 3 mg/kg, p.o.; □: F-1394, 10 mg/kg, p.o.; ■: F-1394, 20 mg/kg, p.o.; ○: F-1394, 30 mg/kg, p.o.; ○: recovery. *P<0.05, **P<0.01, ***P<0.001, as compared with the control group by Dunnett’s test or Student’s t-test.

Fig. 3. Comparison of the hypcholesterolemic action between F-1394 and DL-melinamide in the cholesterol diet-fed rats. The rats were fed the high cholesterol diet for 24 hr before the administration of the drugs. Blood was collected 1.5, 3 and 18 hr after the administration of the drugs. Serum cholesterol levels were measured by the enzymatic/colorimetric method. ●: F-1394, 30 mg/kg, p.o.; ○: DL-melinamide, 30 mg/kg, p.o. Each symbol represents the mean±S.E. of 8 rats.

Fig. 4. Comparison of hypocholesterolemic action between F-1394 and CL-277,082 in the cholesterol diet-fed rats. The rats were fed the high cholesterol diet for 24 hr before the administration of the drugs. Blood was collected 1.5 and 3 hr after the administration of the drugs. Serum cholesterol levels were measured by the enzymatic/colorimetric method. ●: F-1394, 30 mg/kg, p.o.; ○: CL-277,082, 30 mg/kg, p.o. Each symbol is given as the mean±S.E. of 6 rats.

hypocholesterolemic action of F-1394 at a dose of 30 mg/kg, p.o. rapidly reached the maximum within 3 hr. In contrast, DL-MA at a dose of 30 mg/kg, p.o. did not affect the serum TC levels in the cholesterol diet-fed rats up to at least 3 hr after the administration. After 18 hr, the degree of the TC decrease by DL-MA, being 30%,
Table 1. Effect of F-1394 on the activity of ACAT in the rat small intestine (ex vivo)

| Treatment | N  | ACAT activity (pmol/mg protein) |
|-----------|----|---------------------------------|
| Vehicle   | 10 | 49.6±8.1                        |
| F-1394    | 10 | 20.4±1.3**                      |

The rats were fed the high cholesterol diet for 24 hr before the administration and throughout the experiment. Rats were orally administered F-1394 at a dose of 30 mg/kg, and their small intestines were quickly removed 3 hr after the administration. ACAT activity was determined by the incorporation of $^{14}$C-oleoyl-CoA into cholesteryl oleate. The incubation was carried out for 10 min at 37°C. Lipid in the mixture was extracted by using chloroform : methanol (2:1), and the radioactive product was isolated by TLC. Each value represents the mean±S.E. of 4-5 rats. **P<0.01, as compared with the vehicle group by Student’s t-test.

Table 2. Effect of F-1394 on the cholesterol absorption in rats fed a high cholesterol diet (dual isotope ratio method)

| Treatment | N  | 24 hr later | 96 hr later |
|-----------|----|-------------|-------------|
| Vehicle   | 4–5| 46.5±2.3    | 53.7±1.8    |
| F-1394    | 3–4| 34.2±1.8**  | 39.8±1.3**  |

The rats were fed the high cholesterol diet for 3 days, and F-1394 at a dose of 30 mg/kg was also administered orally once a day for the 3 days. After fasting overnight, on the 4th day, the rats received 185 kBq of $^{14}$C-cholesterol (2.1 GBq/mmole, p.o.) and $^{3}$H-cholesterol (1890.7 GBq/mmole, i.v. 30 min after the last administration of F-1394. Blood was collected 24 and 96 hr after the application of the tracers. Each value represents the mean±S.E. of 3–5 rats. **P<0.01, as compared with the vehicle group by Student’s t-test.

Ex vivo ACAT assay

In rats of the vehicle group, the ACAT activity in the small intestinal mucosa was 49.6±8.1 pmol/mg protein/min. The ACAT activity in the small intestinal mucosa was significantly inhibited (20.4±1.3 pmol/mg protein/min) 3 hr after the administration of F-1394 at a dose of 30 mg/kg, p.o. (Table 1).

Dual isotope ratio method

In rats of the vehicle group, the rates of dietary cholesterol absorption were 46.5±2.3% and 53.7±1.8%, respectively, 24 and 96 hr after the application of the tracers. The absorption was significantly suppressed by the treatment with F-1394 compared with that of the vehicle group (Table 2).

$^{14}$C-cholesterol absorption

Experiment 1: In rats of the vehicle group, the radioactivity of $^{14}$C-cholesterol in the blood was 248±23 Bq/ml 5 hr after the application of the tracer. F-1394 at a dose of 30 mg/kg, p.o. markedly prevented the appearance of the radioactivity in the whole blood at that time. The decay curve of vehicle group only slightly differed from that of the F-1394 group from 21 hr to 85 hr (Fig. 5).

Experiment 2: In rats of the vehicle group, the radioactivity of $^{14}$C-cholesterol in the blood was 664±153 Bq/ml 4 hr after the application of the tracer. When F-1394 (30 mg/kg, p.o.) was administered 2 hr before the application of the tracer, the appearance of the radioactivity in the blood was decreased by 89%. In the case of the administration of F-1394 1 hr before or immediately after the application of the tracer, F-1394 (30 mg/kg) completely inhibited the appearance of the radioactivity in the circulation by 95% or 93%. Furthermore, when F-1394 (30 mg/kg) was administered 1 hr after the application of the tracer, the inhibitory percentage was 82%. In rats orally given F-1394 2 hr after the appli-
cation of the tracer, the appearance of the radioactivity in the blood was reduced by 62% (Table 3).

DISCUSSION

The present study was designed to clarify the hypocholesterolemic action by F-1394, a potent, selective and competitive inhibitor of ACAT (1), and its mechanism in the cholesterol diet-fed rat.

TC levels in the serum from the rats fed with the cholesterol diet for a day were twofold and more greater than those from normolipidemic rats: 136 mg/dl versus 50-60 mg/dl. This result indicates that dietary hypercholesterolemia is indelible in rats by one day-feeding with the cholesterol diet. Single administration of F-1394 to these rats decreased the serum TC levels in a dose-dependent manner, and the duration of the hypocholesterolemic action was depended on the dosage (Fig. 2). Especially, when 20 or 30 mg/kg, p.o. of F-1394 was given, the TC levels decreased to normal levels (50 - 60 mg/dl) 3 hr after the administration. The ACAT activity in the small intestinal mucosa excised from the rats receiving oral F-1394 was inhibited significantly 3 hr after the administration (Table 1). The study by the dual isotope ratio method showed that F-1394 prevented dietary cholesterol absorption via the gut (Table 2). In the experiment of 14C-cholesterol absorption, although F-1394 did not affect the decay curve of 14C-cholesterol in the elimination phase, F-1394 markedly decreased the appearance of the radioactivity in the blood in the absorption phase of 14C-cholesterol (Fig. 5). Furthermore, the preliminary distribution study with radiolabelled F-1394 demonstrated that the small intestine contained a sufficient mass of F-1394 to inhibit the activity of intestinal ACAT (data not shown). These results reveal that the oral administration of F-1394, a selective inhibitor of ACAT (1), inhibits the activity of ACAT in the small intestinal mucosa and subsequently contributes much to the reduction of cholesterol absorption via the gut, resulting in the decrease in the serum cholesterol levels in the cholesterol diet-fed rats. Many investigators reported that the intestinal ACAT has been implicated as a key enzyme involved in dietary cholesterol absorption in rats (3, 6), guinea pig (4), rabbits (14, 17) hamsters (18) and humans (5). In the streptozotocin-induced diabetic rats, the marked increase in the small intestinal ACAT activity and severe hypercholesterolemia were induced by the feeding the rats a high cholesterol diet (19), and the inhibition of the ACAT activity brought about cholesterol-lowering action in this model (20, 21). In Wistar fatty rats, a new model for non-insulin-dependent diabetes mellitus (22), the enhanced ACAT activity in the small intestine and hypercholesterolemia were observed (23). Thus, F-1394 may have a therapeutic potential for dietary hypercholesterolemia including diabetes-associated hypercholesterolemia.

The TC levels in the rats of the recovery group were returned to the normal levels 12 hr after the exchange from the cholesterol diet to the normal chow (Fig. 2). This result indicates that the serum TC levels change sensitively in response to an existing mass of dietary cholesterol at the gut in rats.

In the comparison of F-1394 with DL-MA or CL-277,082, the onset of TC reduction in the serum by F-1394 was faster than that by DL-MA or CL-277,082 after the administration (Figs. 3 and 4). Furthermore, the 14C-cholesterol absorption study showed that orally administered F-1394 immediately caused marked inhibition of cholesterol absorption (Table 3). From these results, the hypocholesterolemic action of F-1394 had a faster onset than those of DL-MA and CL-277,082, and the administration of F-1394 seems to be suitable for administration immediately after a meal. Therefore, F-1394 may have a hypocholesterolemic potential for postprandial hyperlipidemic patients by oral administration immediately after a meal. Although there have been numerous reports on the hypocholesterolemic action by other ACAT inhibitors in cholesterol diet-fed animals (17, 18, 20, 21, 24–27), the immediate effect after single oral administration of these compounds has not been demonstrated. In this study, we demonstrated the immediate effect of F-1394 on hypercholesterolemia induced by dietary cholesterol feeding. Although the reason for the immediate effect of F-1394 still remains unclear, there are two possible explanations for the effect of F-1394: First, the transit time of F-1394 through the stomach might be

| Treatment | Blood 14C-cholesterol levels (Bq/ml) | Inhibition (%) |
|-----------|--------------------------------------|---------------|
| Vehicle   | 663.9±153.1                          |               |
| F-1394    |                                      |               |
| 2 hr before | 72.9±13.7*                          | 89.0          |
| 1 hr before | 35.2±0.7*                           | 94.7          |
| immediately before | 49.4±10.8*                      | 92.6          |
| 1 hr after   | 116.9±3.2*                           | 82.4          |
| 2 hr after   | 253.2±22.2                           | 61.9          |

The rats were fed the high cholesterol diet for one night before the application of 14C-cholesterol (2.1 GBq/mmol). F-1394 at a dose of 30 mg/kg was orally administered 1–2 hr before/after or immediately after the application of the isotope, and their blood was collected 4 hr after the application of the isotope. Each value represents the mean±S.E. of 3 rats. *P<0.05, as compared with the vehicle group by Student’s t-test.
shorter than those of other ACAT inhibitors. Secondly, F-1394 might penetrate easily into the intestinal mucosal cell, whereas DL-MA and CL-277,082 may not. Previously, we reported that the in vitro inhibitory potency of F-1394 on the activity of the small intestinal mucosal ACAT from the rabbit was less than that of CL-277,082, but not that of DL-MA (1). Thus, it is suggested that the onset of the hypocholesterolemic action of these drugs in our study is little correlated with the inhibitory potency on the activity of ACAT in the enzyme preparations such as the microsomal fraction or tissue homogenates.

In the 14C-cholesterol absorption study, the radioactivity of 14C-cholesterol at 85 hr after the application of the oral tracer in the blood from the rats of the F-1394 group was significantly lower than that in the vehicle group, whereas the decay curve in F-1394 group in the elimination phase of 14C-cholesterol did not so differ from that in the vehicle group (Fig. 5). These results suggest that F-1394 slightly enhanced the disappearance of cholesterol from the circulation. However, further detailed studies are necessary to clarify its mechanism. Recently, several investigators reported that ACAT inhibition inhibited foam cell formation (28) and prevented release into the circulation. However, further detailed studies are necessary to clarify its mechanism. Recently, several investigators reported that ACAT inhibition inhibited foam cell formation (28) and prevented release into the circulation. However, further detailed studies are necessary to clarify its mechanism. 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In summery, F-1394 at the doses of 3-30 mg/kg inhibits the activity of the small intestinal ACAT in vivo, and subsequently contributes much to the prevention of dietary cholesterol absorption and causes hypocholesterolemic action in the cholesterol diet-fed rats. Furthermore, the effect of F-1394 appears immediately after its administration.

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