Pharmacological Properties of R-755, a Novel Acyl-CoA:Cholesterol Acyltransferase Inhibitor, in Cholesterol-Fed Rats, Hamsters and Rabbits

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ABSTRACT—R-755 (*N*-(2,6-diethylphenyl)-*N'*-[3-(2-methylphenyl)-6,7-dihydro-5*H*-cyclopenta[*f*][*l*]benzothiophen-2-yl]urea), a novel acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, has been characterized in vitro, ex vivo and in vivo. R-755 potently inhibited ACAT activities, with IC₅₀ values from 2.5 to 64 nM, in rabbit intestinal microsomes and several cell lines (CaCo-2, THP-1 and J774A.1 cells). R-755 reduced serum cholesterol and triglyceride levels and liver cholesterol contents in cholesterol-fed rats, hamsters and rabbits. Rabbits were fed a high cholesterol diet for 2 weeks and further fed the same diet containing R-755 for 2 weeks. R-755 dose-dependently reduced cholesterol content and ACAT activity in the aorta. When phorbol 12-myristate 13-acetate-treated THP-1 and J774A.1 cells were incubated in the medium containing 20% of serum from rats administered R-755, the ACAT activities of the cells were inhibited. Rabbits were fed a high cholesterol diet for 8 weeks to establish aortic atherosclerosis and then fed a normal diet with or without R-755 for 8 weeks. R-755 dose-dependently reduced the surface area with atherosclerotic involvement and cholesterol contents in the aorta, although plasma cholesterol level did not differ from that in the control group. These results suggest that R-755 is a potent hypolipidemic agent and has a direct antiatherosclerotic activity at the arterial wall.

Keywords: Acyl-CoA:cholesterol acyltransferase (ACAT), Atherosclerosis, Hypercholesterolemia, ACAT inhibition, R-755

Acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26, ACAT) is a microsomal enzyme that catalyzes the esterification of cholesterol (1). ACAT, identified as a key enzyme in cholesterol homeostasis, was found in various tissues including the intestine, liver and arterial wall (1). In the intestine, ACAT is involved in the absorption of cholesterol. Cholesterol is absorbed in a free form and esterified by ACAT in the mucosal cell. Esterified cholesterol is incorporated into a chylomicron together with triglycerides, phospholipids and apolipoproteins; and then the chylomicron is secreted into the lymph (2). Therefore, inhibition of the ACAT activity in the intestine decreases cholesterol absorption (2–4). In the liver, ACAT plays important roles in lipoprotein cholesterol production and secretion (5, 6). Free cholesterol is esterified by ACAT in the hepatocyte and incorporated into very low density lipoprotein (VLDL), which is secreted into the blood. Therefore, inhibition of the ACAT activity in the liver decreases cholesterol secretion by inhibition of VLDL assembly. Indeed, stimulation of the ACAT activ-

ity increases VLDL cholesteryl ester secretion in cultured rat hepatocytes (5), and inhibition of the activity decreases VLDL cholesteryl ester and apolipoprotein B secretion in perfused monkey livers (6). In these manners, inhibition of intestine and liver ACAT lowered serum cholesterol level (4, 7, 8). The reduction of serum cholesterol level prevented progression of atherosclerosis in the arterial wall (9, 10). In addition, arterial ACAT relates to the progression of atherosclerosis. Excess free cholesterol is converted into the esterified form by ACAT and accumulated in monocyte-derived macrophages and smooth muscle cells on the arterial wall. This is an essential step in the formation of atherosclerotic lesions (11). It was suggested that ACAT inhibitors, CI-976, E5324 and FR145237, directly prevented cholesterol accumulation in the arterial wall not through the reduction of serum cholesterol level in cholesterol fed-rabbits (12–14). Therefore, ACAT inhibitors prevented atherosclerosis not only by hypocholesterolemic activity but also direct antiatherosclerotic activity that inhibits ACAT activity in monocyte-derived macrophages and smooth muscle cells on the arterial wall, and thus they are expected to be cholesterol lowering and antiatherosclerotic

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Fig. 1. Chemical structures of R-755 and CI-976.

agents. We discovered a novel ACAT inhibitor that is a benzothiophen derivative, R-755, and investigated in vitro ACAT inhibition and cholesterol lowering and antiatherosclerotic activities in cholesterol-fed animal models.

MATERIALS AND METHODS

Chemicals

R-755 (*N*-(2,6-diethylphenyl)-*N'*-[3-(2-methylphenyl)-6,7-dihydro-5*H*-cyclopenta[*f*][*l*]benzothiophen-2-yl]urea) and CI-976 (2,2-dimethyl-*N*-(2,4,6-trimethoxyphenyl)-dodecanamide) (Warner-Lambert compound) (Fig. 1) were synthesized at Nihon Nohyaku Research Center. [14 C]Oleoyl CoA (2.1 – 2.2 GBq/mmol), [9,10- 3 H(*N*)]oleic acid (340 – 370 GBq/mmol), [14 C]oleic acid (2.1 – 2.2 GBq/mmol) and [1,2,6,7(*N*)- 3 H]cholesteryl oleate (2.5 – 2.6 TBq/mmol) were purchased from New England Nuclear (Boston, MA, USA). All other chemicals were reagent grade.

Animals

In animal experiments, we followed the standard operation procedure of our institute that satisfies guiding principles for the care and use of laboratory animals approved by the Japanese Pharmacological Society. Male Sprague-Dawley (SD) rats (Nihon SLC, Shizuoka), male Golden Syrian hamsters (Nihon SLC) and male Japanese White (JW) rabbits (Kitayama Labes Co., Ltd., Nagano) were individually housed in metal cages in a room with controlled temperature ($23 \pm 2^\circ\text{C}$), humidity ($55 \pm 15\%$) and light (7:00 – 19:00 h). They were maintained on a commercial laboratory chow, MF (Funabashi farms, Chiba) for rats and hamsters or RC-4 (Oriental Yeast Co., Ltd., Tokyo) for rabbits.

Cells and cell culture

CaCo-2, a human colon carcinoma cell line; THP-1, human monocytic leukemia cell line; and J774A.1, murine macrophage cell line, were obtained from Dainippon pharmaceutical Co., Ltd. (Osaka), American Type Culture Collection (Rockville, MD, USA) and the JCRB Cellbank in NIHS (Tokyo), respectively.

CaCo-2 cells were grown on 6-well culture plates (Corning, NY, USA) in Eagle's medium (Nissui Pharma-

ceutical Co., Ltd., Tokyo) containing 10% fetal bovine serum (FBS; ICN Biomedicals Inc., Aurora, Ohio, USA), 2.1 mM glutamine, 1% nonessential amino acids and 60 mg/l kanamycin. J774A.1 cells (1.5×10^5 cells/ml) were plated in 6-well culture plates (Corning) in 2 ml of Dulbecco's modified Eagle's medium (Nissui Pharmaceutical) containing 10% FBS, 4.1 mM glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin and grown for 1 day.

THP-1 cells (1.5×10^3 cells/ml) were plated in 6-well culture plates (Corning) in 2 ml of RPMI1640 (Nissui Pharmaceutical) containing 10% FBS, 2.1 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO, USA) and cultured for 4 days. In order for the cells to differentiate to macrophages, PMA was added to medium.

All cell lines were maintained under a humidified atmosphere of 95% air, 5% CO_2 at 37°C .

Preparation of acetyl low density lipoprotein (AcLDL)

LDL ($d = 1.019 - 1.063$ g/ml) was isolated from normal human serum by sequential ultracentrifugation. The isolated LDL was dialyzed against 0.15 M NaCl, 1 mM EDTA (pH 7.4) at 4°C and sterilized by filtration ($0.45 \mu\text{m}$). AcLDL was prepared by reaction with acetic anhydride as described by Basu et al. (15).

Assay of microsomal ACAT activity

Male JW rabbits (2 – 3 kg) were sacrificed under anesthesia with pentobarbital sodium, and small intestines were excised immediately. Intestinal mucosal microsomes were prepared according to the method of Heider et al. (3). ACAT activity was determined essentially according to the method of Helgerud et al. (16).

Assay of ACAT activity in cultured cells

[^3H]Oleic acid-BSA complex (50 μM , specific activity: 100 dpm/pmol) and [^{14}C]oleic acid-BSA complex (5 mM, specific activity: 20 dpm/pmol) were prepared by the method of Kam et al. (17). For the assay of ACAT activity in CaCo-2 cells, cultures were used only after dome formation, 5 days after confluency. The medium was replaced by medium-199/Earle's (Gibco, Grand Island, NY, USA) containing 10 mM HEPES, pH 7.3 and each compound dissolved in DMSO was added. After incubation for 2 h, the medium was replaced with [^3H]oleic acid-BSA solution (1980 μl of medium plus 20 μl of [^3H]oleic acid-BSA complex) and the cells were incubated for 1 h.

The media of cultured J774A.1 and PMA-treated THP-1 cells were replaced by 1 ml of each medium containing 50 μg protein/ml AcLDL and the cells were incubated for 16 h. Then each compound dissolved in 1 μl of DMSO was added. After incubation for 2 h, 20 μl of [^{14}C]oleic acid-BSA complex was added and the mixture was incubated for

2 h. The radioactivity in esterified cholesterol was determined essentially according to the method of Field et al. (18).

Ex vivo ACAT assay in macrophages

Male SD rats (7-week-old) were orally administrated R-755 or vehicle (corn oil) after fasting for 16 h. At 6 h after the administration, blood samples were collected from the inferior vena cava under anesthesia with diethyl ether and the serums were obtained. J774A.1 and PMA-treated THP-1 cells were incubated in each medium containing 50 μ g protein/ml AcLDL for 16 h. The medium was replaced by 1 ml of the medium containing 20% rat serum and 50 μ g protein/ml AcLDL. After incubation for 4 h, ACAT activity was assayed by the method described above.

Experimental design for hypolipidemic activity in cholesterol-fed rats and hamsters

Male SD rats (6-week-old) were fed MF containing 1% cholesterol, 1% cholic acid and 8.5% coconut oil and male Golden Syrian hamsters were fed MF containing 0.5% cholesterol and 8% coconut oil ad libitum for 4 days. Animals in normal control group were fed MF. Each compound or vehicle (0.5% carboxymethylcellulose) was orally administered daily for 4 days from the day that high-cholesterol diet was started. At 24 h after the final administration, blood samples were collected from the inferior vena cava of nonfasted animals and livers were excised under anesthesia with diethyl ether. Serum cholesterol and triglyceride and liver cholesterol were measured.

Experimental design for hypolipidemic activity in cholesterol-fed rabbits

In experiment A (expt A), male JW rabbits (approximately 2.3 kg) were fed 100 g/day of RC-4 containing 1% cholesterol (HCD) for 14 days. Then the rabbits were divided into control and compound-treated groups with comparable mean and S.E.M. values of plasma cholesterol. The animals were fed 100 g/day of the HCD with or without compound for a further 14 days. Blood samples were collected from the ear vein at 0, 7, 14, 15, 18, 21 and 28 days.

In experiment B (expt B), male JW (approximately 2.2 kg) were fed 100 g/day of the HCD for 8 weeks to develop aortic atherosclerosis. Then the animals were divided into control and compound-treated groups in the same manner as in expt A. Thereafter, they were given 100 g/day of RC-4 with or without compound for 8 weeks. Blood samples were collected at 0, 2, 4, 6, 8, 9, 10, 11, 12, 14 and 16 weeks.

In both experiments, at the final point, rabbits were anesthetized with pentobarbital sodium and exsanguinated from the carotid. Livers and aortas were excised. Aortic adherent tissues were removed and aortas were opened longitudinally

along the arterial wall.

In expt A, plasma cholesterol and triglyceride, liver cholesterol, aortic cholesterol and aortic ACAT activity were measured. In expt B, the cholesterol in the plasma, liver and aorta were measured and the surface area of atherosclerotic involvement in the aorta was determined.

Measurement of plasma or serum lipids

Plasma or serum total cholesterol and triglyceride were measured by an auto analyzer (model 705; Hitachi, Tokyo) with associated reagents (Wako Pure Chemical Industries, Osaka).

Determination of cholesterol contents in liver and aorta

Except for the aorta of the cholesterol-fed rabbits (expt A), the tissues were homogenized in 0.9% NaCl. The aorta (expt B) was homogenized after determination of the surface area of atherosclerotic involvement. The aorta (expt A) was homogenized in buffer containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris (pH 7.4) for the assay of ACAT activity. The lipids of the homogenate were extracted with *n*-hexane/isopropyl alcohol (3:2, v/v), and the concentrations of total and free cholesterol were measured by enzymatic methods using commercial kits (Wako). Esterified cholesterol was calculated as the difference between total and free cholesterol.

Assay of aortic ACAT activity

The homogenate of the aorta (expt A) was centrifuged for 5 min at $300 \times g$ to remove cellular debris and the supernatant was used. ACAT activity was determined essentially by the method of Burrier et al. (19). The reaction mixture consisted of 200 μ l of 200 mM potassium phosphate buffer, pH 7.4, containing 4 mM DTT, 180 μ M BSA and 100 μ l of the supernatant of the aorta homogenate. The reaction mixture was preincubated at 37°C for 10 min. Twenty microliters of 252 μ M [$1\text{-}^{14}\text{C}$]oleoyl CoA (specific radioactivity: 50 dpm/pmol) was added to mixture, following by incubation at 37°C for 10 min. The radioactivity in esterified cholesterol was determined essentially according to the method of Field et al. (18).

Determination of surface area of atherosclerotic involvement

The aortas (expt B) were fixed in buffered formalin and stained with oil red O. The intimal surface and positively stained areas were measured by an image analyzer (SP500; Olympus Co., Ltd., Tokyo). Surface area of atherosclerotic involvement was defined as the percentage of stained area to total intimal surface.

Protein determination

Protein concentration was determined by the method of

Lowry et al. (20).

Statistical analyses

Each value was expressed as the mean $\pm$ S.E.M. The significance of differences between the groups was evaluated by Dunnett's multiple comparison test. Student's *t*-test was used in the comparison between two groups. Significance was accepted at $P < 0.05$.

RESULTS

In vitro potency

The effects of R-755 and CI-976 on ACAT activities in

Table 1. Inhibitory effects of R-755 and CI-976 on ACAT activities in rabbit intestinal mucosal microsomes and cultured cell lines

	IC ₅₀ values (nM)	
	R-755	CI-976
Microsomes	2.5	70
CaCo-2	6.1	280
J774A.1	64	730
THP-1	25	about 300

Several concentrations of each test compound were examined in triplicate assays. Using the consequent inhibition curves, IC₅₀ values were determined.

rabbit intestinal microsomes and several cell lines are shown in Table 1. R-755 and CI-976 inhibited all these ACAT activities with IC₅₀ values of 2.5 to 64 nM and 70 to 730 nM, respectively.

Hypolipidemic activity in cholesterol-fed rats and hamsters

R-755 and CI-976 were administrated to cholesterol-fed rats and hamsters for 4 days. Animals treated with compounds showed no significant change in body weight except for rats treated with 100 mg/kg of CI-976. The final body weight at this dose of CI-976 in rats (205 ± 6 g) was significantly different ($P < 0.05$) from that of the control group (229 ± 3 g). Hypolipidemic effects are shown in Table 2. Feeding of a high cholesterol diet resulted in a significant increase in serum total cholesterol and triglyceride levels to about 2.7 and 1.8-fold of the normal control levels in rats and about 2.8 and 5.0-fold in hamsters, respectively. R-755 and CI-976 dose-dependently lowered serum total cholesterol and triglyceride levels in rats and hamsters. R-755 at a dose of 30 mg/kg lowered serum total cholesterol and triglyceride levels to the normal control levels in rats. As for the serum total cholesterol level, ED₅₀ values of R-755 were estimated to be at 0.84 and 1.74 mg/kg in rats and hamsters, in comparison with 65.6 and 62.0 mg/kg of CI-976.

Total cholesterol contents in the liver were 7.6 and

Table 2. Hypolipidemic effects of R-755 and CI-976 in cholesterol-fed rats and hamsters

Species	Treatment	Diet	Dose (mg/kg)	Serum (mg/dl)		Liver (mg/g liver)			
				TC	TG	TC	FC	EC	
Rat	Vehicle	Normal	0	64 ± 3 [#]	143 ± 6 [#]	1.9 ± 0.0 [#]	1.4 ± 0.1 [#]	0.5 ± 0.1 [#]	
		HCD	0	171 ± 14	252 ± 21	14.4 ± 0.9	2.3 ± 0.2	12.1 ± 0.8	
	R-755	HCD	0.03	167 ± 17	222 ± 22	11.4 ± 0.9*	2.1 ± 0.1	9.4 ± 0.8*	
			0.3	141 ± 12	225 ± 43	6.6 ± 0.4*	2.1 ± 0.1	4.5 ± 0.3*	
			3	91 ± 15*	183 ± 43	5.2 ± 0.4*	1.7 ± 0.2	3.5 ± 0.5*	
			30	61 ± 1*	95 ± 11*	2.9 ± 0.1*	1.6 ± 0.1	1.3 ± 0.1*	
	CI-976	HCD	30	146 ± 7	308 ± 91	7.3 ± 0.5*	1.9 ± 0.2	5.3 ± 0.6*	
			100	90 ± 6*	120 ± 8	3.5 ± 0.2*	1.7 ± 0.0*	1.8 ± 0.8*	
	Hamster	Vehicle	Normal	0	172 ± 5 [#]	272 ± 30 [#]	10.4 ± 1.3	2.8 ± 0.1 [#]	7.6 ± 1.2
			HCD	0	484 ± 37	1361 ± 188	14.7 ± 1.7	3.4 ± 0.1	11.3 ± 1.6
R-755		HCD	0.03	454 ± 25	1149 ± 297	10.7 ± 1.2	3.0 ± 0.1	7.7 ± 1.1	
			0.3	354 ± 29*	702 ± 201*	7.9 ± 1.2*	2.6 ± 0.1*	5.2 ± 1.1*	
			3	327 ± 25*	690 ± 97*	7.4 ± 1.3*	2.6 ± 0.1*	4.8 ± 1.5*	
			30	276 ± 32*	513 ± 46*	6.5 ± 1.1*	2.4 ± 0.1*	4.0 ± 1.2*	
CI-976		HCD	30	372 ± 44	692 ± 89*	8.9 ± 1.2*	2.8 ± 0.2*	6.1 ± 1.0*	
			100	276 ± 20*	663 ± 95*	8.5 ± 0.4*	2.7 ± 0.1*	5.8 ± 0.3*	

Each value is a mean $\pm$ S.E.M. ($n = 5 - 10$). [#] $P < 0.05$ between normal and HCD control. * $P < 0.05$ vs HCD control. TC: total cholesterol, TG: triglyceride, FC: free cholesterol, EC: esterified cholesterol.

1.4 times higher in the control group than the normal control group in rats and hamsters, respectively. Although free cholesterol content was significantly increased in the control group, the amount of free cholesterol was very low compared with that of total cholesterol. Therefore, the increased amount of total cholesterol is essentially attributable to the increase in esterified cholesterol content. R-755 and CI-976 dose-dependently reduced liver total, esterified and free cholesterol contents in rats and hamsters. As for liver total cholesterol content, ED_{50} values of R-755 and CI-976 were estimated at to be 0.24 mg/kg and <30 mg/kg in rats. R-755 at a dose of 0.3 mg/kg and CI-976 at a dose of 30 mg/kg reduced total and esterified cholesterol contents to under the normal control level in hamsters. Liver cholesterol contents were more sensitive to R-755 and CI-976 than serum cholesterol level.

Hypolipidemic activity in cholesterol-fed rabbits (expt A)

Rabbits were fed a high cholesterol diet for 14 days and further fed the same diet containing compounds for 14 days. Animals treated with compounds showed no significant change in body weight (data not shown).

Figure 2 shows changes of plasma cholesterol and triglyceride levels. Plasma total cholesterol and triglyceride levels were about 23 and 35 mg/dl before feeding the high cholesterol diet (0 day) and reached about 800 and 70 mg/dl at the time of initial compound treatment (14 days). In the control group, plasma total cholesterol and triglyceride

levels further increased to 1260 ± 131 and 115 ± 19 mg/dl at the 28th day. R-755 and CI-976 markedly reduced plasma cholesterol and triglyceride levels. R-755 at doses of 0.1 and 1 mg/kg dose-dependently lowered plasma total cholesterol level by 50.6% and 85.2% from the control level at the 28th day, in comparison with the 60.4% by CI-976 at a dose of 10 mg/kg. Also R-755 dose-dependently lowered plasma triglyceride level by 73.0% and 76.5%, in comparison with the 62.6% by CI-976.

In the primary study, cholesterol contents in the liver and aorta and ACAT activity in the aorta were determined in normal rabbits ($n = 3$). The esterified, free and total cholesterol contents were 0.38, 2.07 and 2.45 mg/g tissue in the liver; 0, 0.44 and 0.44 mg/g tissue in the aortic arch; and 0.01, 0.58 and 0.59 mg/tissue in the thoracic aorta, respectively. The ACAT activity in the aortic arch and thoracic aorta were 73.9 and 54.5 pmol $\cdot$ mg protein $^{-1} \cdot$ min $^{-1}$, respectively. Animals fed a high cholesterol diet (control group) showed an increase in liver and aortic cholesterol contents and aortic ACAT activity from those in normal rabbits (Figs. 3 – 5).

Figure 3 shows the cholesterol contents in the liver. R-755 significantly reduced liver cholesterol contents in a dose-dependent manner. R-755 at a dose of 1 mg/kg reduced esterified, free and total cholesterol contents by 88.8%, 58.2% and 77.8%, respectively, in comparison with the 88.0%, 47.9% and 56.3% reduction by CI-976 at a dose of 10 mg/kg.

Fig. 2. Changes of plasma total cholesterol and triglyceride levels in cholesterol-fed rabbits (expt A). Rabbits were fed a diet containing 1% cholesterol (HCD) for 14 days and fed the HCD with or without compound for a further 14 days. Plasma total cholesterol and triglyceride were measured as described in Materials and Methods. Open circles: control; closed circles: R-755, 0.1 mg/kg; closed triangles: R-755, 1 mg/kg; closed diamonds: CI-976, 10 mg/kg. Each value is a mean $\pm$ S.E.M. ($n = 5$). * $P < 0.05$ vs control.

Fig. 3. Cholesterol contents in the liver in cholesterol-fed rabbits (expt A). After 14-day treatment with compounds, liver cholesterol contents were determined as described in Materials and Methods. Each value is a mean $\pm$ S.E.M. ($n = 5$). Closed columns: esterified cholesterol, open + closed columns: total cholesterol. * $P < 0.05$ vs control.

Figure 4 shows the aortic cholesterol contents. In the aortic arch of the control group, esterified and free cholesterol contents were greater than those in the thoracic aorta. R-755 and CI-976 tended to reduce esterified and free cholesterol contents in the aortic arch. In consequence, total cholesterol content was significantly reduced. In the thoracic aorta, R-755 at a dose of 1 mg/kg tended to reduce esterified

and total cholesterol contents. However, CI-976 at a dose of 10 mg/kg did not affect cholesterol contents.

ACAT activities in the aortic arch and thoracic aorta were also assayed, and the result is shown in Fig. 5. R-755 at doses of 0.1 and 1 mg/kg significantly reduced the ACAT activities in the aortic arch (56.3% and 50.8%, respectively) and thoracic aorta (55.2% and 49.4%, respectively). CI-976 also significantly reduced ACAT activities in the aortic arch and thoracic aorta by 57.3% and 48.2%, respectively.

Inhibition of ex vivo ACAT activity in macrophages

Effects of the serum from rat treated with R-755 on ACAT activities in J774A.1, a murine macrophage cell line, and PMA-treated THP-1, a human monocytic cell line differentiated to macrophages, were investigated (Fig. 6). AcLDL significantly stimulated ACAT activities about 11 and 52-fold of those of AcLDL (–) control in J774A.1 and PMA-treated THP-1 cells, respectively. The rat serum treated with R-755 at a dose of 10 mg/kg significantly reduced ACAT activities in J774A.1 and PMA-treated THP-1 cells by 75.3% and 78.2%, respectively.

Effect on pre-established atherosclerosis in cholesterol-fed rabbits (expt B)

Effects of R-755 and CI-976 on pre-established atherosclerosis were investigated to clarify the direct effect of the compounds in the aortic wall not through the reduction of serum cholesterol level. Rabbits were fed a high cholesterol diet for 8 weeks to establish aortic atherosclerosis and then fed a normal diet with or without compounds for 8 weeks. Rabbits treated with compounds showed no significant

Fig. 4. Cholesterol contents in the aortic arch (a) and thoracic aorta (b) in cholesterol-fed rabbits (expt A). After 14-day treatment with compounds, aortic cholesterol contents were determined as described in Materials and Methods. Each value is a mean $\pm$ S.E.M. ($n = 5$). Closed columns: esterified cholesterol, open + closed columns: total cholesterol. * $P < 0.05$ vs control for total cholesterol.

Fig. 5. ACAT activities in the aortic arch (a) and thoracic aorta (b) in cholesterol-fed rabbits (expt A). After 14-day treatment with compounds, aortic ACAT activity was measured as described in Materials and Methods. Each value is a mean $\pm$ S.E.M. ($n = 5$). * $P < 0.05$ vs control.

Fig. 6. Effects of rat serum treated with R-755 on ACAT activities in J774A.1 (a) and THP-1 (b) cells. J774A.1 and PMA-treated THP-1 cells were incubated in the medium containing AcLDL for 16 h. The medium was replaced by a medium containing 20% rat serum treated with R-755 and AcLDL. After incubation for 4 h, ACAT activity was assayed as described in Materials and Methods. Each value is a mean $\pm$ S.E.M. ($n = 4$). # $P < 0.05$ between AcLDL (-) control and AcLDL (+) control. * $P < 0.05$ vs AcLDL (+) control.

changes in body weight and food consumption (data not shown). As shown in Fig. 7, the plasma total cholesterol level decreased rapidly after feeding was switched from a high cholesterol diet to a normal diet, and there were no significant differences between the control and treated groups in this parameter.

Table 3 shows the percentage of the surface area of atherosclerotic involvement (area positively stained by oil red O) in the aorta, aortic cholesterol contents and liver

cholesterol contents. In the aortic arch, the percentage of the surface area of atherosclerotic involvement was greater than that in the thoracic and abdominal aorta. R-755 and CI-976 did not affect the surface area of atherosclerotic involvement in the aortic arch. R-755 tended to reduce the surface area of atherosclerotic involvement in the thoracic aorta and abdominal aorta in a dose-dependent manner. The highest dose of R-755 (10 mg/kg) reduced those by about 50%; however, these differences were not significant. CI-

Fig. 7. Changes of plasma total cholesterol level in cholesterol-fed rabbits (expt B). Rabbits were fed a HCD for 8 weeks to establish aortic atherosclerosis and fed a normal diet with or without compound for a further 8 weeks. Open circles: control; closed circles: R-755, 0.1 mg/kg; closed triangles: R-755, 1 mg/kg; closed squares: R-755, 10 mg/kg; closed diamonds: CI-976, 30 mg/kg. Each value is a mean $\pm$ S.E.M. ($n = 5 - 6$). No statistically significant differences between control and compound-treated groups were observed.

976 at a dose of 30 mg/kg tended to reduce the surface area of atherosclerotic involvement in the thoracic aorta, but did not affect that in the abdominal aorta.

Animals fed a high cholesterol diet (control group) showed an increase in aortic and liver cholesterol contents from those in normal rabbits. In the aortic arch of the control group, esterified and free cholesterol contents were greater than those in the thoracic and abdominal aorta, as well as in the case of expt A. R-755 and CI-976 did not affect the cholesterol contents in the aortic arch. R-755 tended to reduce the cholesterol contents in the thoracic and abdominal aorta. The highest dose of R-755 (10 mg/kg) reduced total cholesterol content in thoracic and abdominal aorta by about 50%. CI-976 at a dose of 30 mg/kg reduced this content in the thoracic and abdominal aorta by about 30%. These results were almost consistent with that of the surface area of atherosclerotic involvement.

R-755 at a dose of 1 mg/kg significantly reduced esterified, free and total cholesterol contents in the liver. CI-976 does not significantly reduce these contents in the liver.

Table 3. Surface area of atherosclerotic involvement in aorta, aortic cholesterol contents and liver cholesterol contents in cholesterol-fed rabbits (expt B)

Dose (mg/kg)	Control		R-755		CI-976	
	0	0.1	1	10	30	
Surface area of atherosclerotic involvement (%)						
Aortic arch	73.7 $\pm$ 10.0	66.6 $\pm$ 9.3 (9.6)	68.5 $\pm$ 8.6 (7.1)	68.1 $\pm$ 7.6 (7.6)	74.5 $\pm$ 4.4 (-1.1)	
Thoracic aorta	23.6 $\pm$ 9.4	16.4 $\pm$ 8.4 (30.5)	11.9 $\pm$ 7.7 (49.6)	11.4 $\pm$ 7.8 (51.7)	17.7 $\pm$ 4.4 (25.0)	
Abdominal aorta	26.9 $\pm$ 6.2	22.9 $\pm$ 8.2 (14.9)	14.1 $\pm$ 4.9 (47.6)	14.6 $\pm$ 4.7 (45.7)	26.3 $\pm$ 6.7 (2.2)	
Cholesterol contents (mg/g tissue)						
Aortic arch	TC	12.81 $\pm$ 2.59	10.25 $\pm$ 2.64 (20.0)	10.63 $\pm$ 3.66 (17.0)	10.07 $\pm$ 1.62 (21.4)	11.73 $\pm$ 1.36 (8.4)
	FC	3.99 $\pm$ 1.07	3.37 $\pm$ 0.80 (15.5)	3.08 $\pm$ 0.97 (22.8)	2.51 $\pm$ 0.45 (37.1)	3.20 $\pm$ 0.44 (19.8)
	EC	8.83 $\pm$ 1.57	6.88 $\pm$ 1.99 (22.1)	7.56 $\pm$ 2.69 (14.4)	7.56 $\pm$ 1.33 (14.4)	8.53 $\pm$ 1.14 (3.4)
Thoracic aorta	TC	2.23 $\pm$ 0.82	2.46 $\pm$ 1.42 (-10.3)	1.71 $\pm$ 1.06 (23.3)	1.15 $\pm$ 0.61 (48.4)	1.50 $\pm$ 0.41 (32.7)
	FC	0.63 $\pm$ 0.27	0.93 $\pm$ 0.59 (-47.6)	0.52 $\pm$ 0.29 (17.5)	0.32 $\pm$ 0.13 (49.2)	0.34 $\pm$ 0.07 (46.0)
	EC	1.60 $\pm$ 0.56	1.54 $\pm$ 0.71 (3.8)	1.19 $\pm$ 0.77 (25.6)	0.83 $\pm$ 0.43 (48.1)	1.17 $\pm$ 0.35 (26.9)
Abdominal aorta	TC	2.03 $\pm$ 0.47	1.55 $\pm$ 0.34 (23.6)	1.24 $\pm$ 0.48 (38.9)	1.07 $\pm$ 0.29 (47.3)	1.48 $\pm$ 0.44 (27.1)
	FC	0.59 $\pm$ 0.12	0.46 $\pm$ 0.12 (22.0)	0.45 $\pm$ 0.14 (23.7)	0.36 $\pm$ 0.08 (39.0)	0.36 $\pm$ 0.07 (39.0)
	EC	1.45 $\pm$ 0.37	1.09 $\pm$ 0.23 (24.8)	0.79 $\pm$ 0.34 (45.5)	0.71 $\pm$ 0.21 (51.0)	1.12 $\pm$ 0.38 (22.8)
Liver	TC	5.86 $\pm$ 1.60	4.69 $\pm$ 0.93 (20.0)	2.53 $\pm$ 0.14* (56.8)	2.47 $\pm$ 0.10* (57.8)	3.20 $\pm$ 0.41 (45.4)
	FC	3.15 $\pm$ 0.58	2.79 $\pm$ 0.38 (11.4)	1.93 $\pm$ 0.06* (38.7)	1.80 $\pm$ 0.12* (42.9)	2.27 $\pm$ 0.16 (27.9)
	EC	2.71 $\pm$ 1.03	1.90 $\pm$ 0.54 (29.9)	0.60 $\pm$ 0.10* (77.9)	0.67 $\pm$ 0.12 (75.3)	0.93 $\pm$ 0.28 (65.7)

Surface area of atherosclerotic involvement was defined as the percentage of the surface area stained by oil red O to total intimal surface. After 8 weeks treatment with compound, surface area of atherosclerotic involvement, aortic cholesterol contents and liver cholesterol contents were determined as described in Materials and Methods. Each value is a mean $\pm$ S.E.M. ($n = 5 - 6$). Each value in the parentheses is the reduction rate from the value of the control. * $P < 0.05$ vs control. TC: total cholesterol, FC: free cholesterol, EC: esterified cholesterol.

DISCUSSION

ACAT plays important roles in intestinal absorption of dietary cholesterol, hepatic secretion of VLDL and vascular cholesterol deposition (2, 5, 6, 12 – 14). Therefore, ACAT inhibitors are expected to be cholesterol lowering and anti-atherosclerotic agents. In the present study, we investigated the pharmacological properties of R-755, a novel ACAT inhibitor that is a benzothiophen derivative.

R-755 inhibited ACAT in rabbit intestinal microsomes and several cell lines more potently than CI-976. The IC_{50} value of CI-976 in intestinal microsomes is consistent with that (73 nM) reported by Krause et al. (7). The inhibitory activity of R-755 was about 10 – 45 times more potent than that of CI-976. This was especially marked for the ACAT activities in rabbit intestinal microsomes and CaCo-2 cells. It is reported that the inhibition of intestinal ACAT activities should result in a reduction of cholesterol absorption (2 – 4). The potent inhibitory activities of R-755 in intestinal microsomes and CaCo-2 cells suggest that R-755 potentially reduces intestinal absorption of dietary cholesterol. Therefore, we investigated the efficacy of R-755 in cholesterol-fed animal models and compared it with that of CI-976.

In cholesterol fed animal models, R-755 reduced serum (or plasma) total cholesterol level and liver cholesterol contents more potently than CI-976. The serum (or plasma) cholesterol lowering activity of R-755 was about 35 – 80 times more potent than that of CI-976. This is in reasonable agreement with the *in vitro* activity. R-755 at doses of 0.3, 3 and 30 mg/kg reduced liver cholesterol contents to under the normal level in cholesterol-fed hamsters. In this study, R-755 did not affect serum glutamic-pyruvic transaminase and alkaline phosphatase, which are biochemical parameters of liver function, and urea nitrogen, which is a parameter showing renal function (data not shown). TMP-153, an ACAT inhibitor, reduced liver cholesterol contents in normal hamsters (21). ACAT inhibitors reduced liver cholesterol contents in normal hamsters because the liver cholesterol contents in hamsters were more than those in normal rats and rabbits. These facts suggest that the effect of R-755 on liver cholesterol contents was efficacy but not toxicity.

The dietary hypercholesterolemia primarily results from increased absorption of cholesterol in the intestine. Furthermore, Sakuma et al. reported that FR129169, an ACAT inhibitor that is not orally absorbed, lowered liver cholesterol contents by the reduction of plasma cholesterol level (22). These facts support that lowering of serum and liver cholesterol by R-755 practically resulted from inhibition of intestinal cholesterol absorption. Krause et al. reported that CI-976 reduced plasma cholesterol level by inhibiting intestinal absorption of cholesterol and hepatic secretion of

VLDL in cholesterol-fed rats (7). Liver cholesterol contents were more sensitive to R-755 and CI-976 than serum cholesterol level. Furthermore, R-755 significantly reduced liver cholesterol contents in cholesterol-fed rabbits in which the effect on plasma cholesterol level could be cancelled (expt B). These results suggest that R-755 directly acted in the liver. Therefore, R-755 may have cholesterol-lowering activities not only by inhibiting cholesterol absorption in the intestine but also by inhibiting cholesterol secretion in the liver as well as CI-976.

R-755 and CI-976 significantly reduced serum (or plasma) triglyceride level in a dose-dependent manner. R-755 at a dose of 30 mg/kg reduced serum triglyceride level to under the normal level in cholesterol-fed rats, but the difference was not significant. The adverse effect was not observed in normal rats treated with R-755 at a dose of 50 mg/kg for 14 days (data not shown). This result suggests that the effect of R-755 on serum triglyceride level was efficacy but not toxicity. The reduction of plasma triglyceride level by ACAT inhibitors, CI-976 and U-73482, in rats was also previously reported (7, 23). It is possible that ACAT inhibitors reduce plasma triglyceride level through the two mechanisms. One is inhibition of triglyceride absorption in the intestine through the interference with the assembly of chylomicrons by inhibition of esterified cholesterol formation and the other is inhibition of triglyceride secretion in the liver through the interference with the assembly of VLDL. In rats with hepatic overproduction of VLDL by sucrose feeding, CI-976 dose-dependently reduced plasma triglyceride level (7). This result suggests that CI-976 inhibits triglyceride secretion in the liver. The inhibitory effect of CI-976 on triglyceride absorption in the intestine has not been clarified. However, in animal models with intestinal overproduction of chylomicrons by cholesterol feeding, the inhibition of triglyceride absorption in the intestine may mainly be attributable to the reduction of serum (or plasma) triglyceride level by R-755 and CI-976.

Hypercholesterolemia promotes the progression of atherosclerosis. In the monocyte-derived macrophages and smooth muscle cells on the arterial wall, free cholesterol is converted into the esterified form by ACAT and then accumulated (11). Therefore, ACAT inhibitors prevented cholesterol accumulation in the arterial wall by hypocholesterolemic activity and inhibition of ACAT activity in these cells. R-755 and CI-976 reduced plasma cholesterol level in cholesterol-fed rabbits (expt A), and they reduced cholesterol contents and inhibited ACAT activity in the aortic arch in this experiment. It is possible that the cholesterol contents are reduced by reduction of the plasma cholesterol level and inhibition of ACAT activity in the aortic arch. However, ACAT activity correlates well with the concentration of substrate cholesterol in the atherosclerotic microsomes (24). Therefore, this inhibition of ACAT activity may be

due to reduction of cholesterol contents by their hypocholesterolemic activities, but not direct inhibition of ACAT activity by R-755 in the arterial wall. However, in the thoracic aorta, R-755 and CI-976 inhibited ACAT activity without significant reduction of cholesterol contents. This result suggests the possibility that R-755 directly inhibits ACAT activity in the arterial wall not through the reduction of plasma cholesterol level. However, there is a conflict that cholesterol contents in the thoracic aorta were not reduced despite inhibition of ACAT activity. It may be due to the small increase in total cholesterol content in the thoracic aorta by cholesterol feeding. Feeding of a high cholesterol diet increased the total cholesterol content in the thoracic aorta only 1.7-fold of that in normal rabbit, in comparison with 5.6-fold in aortic arch.

We performed the next experiment to support a direct inhibition of ACAT activity by R-755 in monocyte-derived macrophages on the arterial wall. The serum from rats treated with R-755 at a dose of 10 mg/kg significantly inhibited ACAT activities in macrophages (J774A.1 and PMA-treated THP-1). Moreover, when R-755 was orally administered to rats at a dose of 10 mg/kg, plasma concentration of R-755 was 750 ng/ml (1650 nM) at 6 h after treatment (data not shown). This concentration was about 25–65 times higher than the IC_{50} in macrophages (J774A.1: 64 nM, PMA-treated THP-1: 25 nM). These results strongly support the possibility that R-755 directly inhibits ACAT activity in monocyte-derived macrophages on the arterial wall.

To clarify the prevention of cholesterol accumulation by R-755 action in the arterial wall and not through hypocholesterolemic activity, we performed an experiment (expt B) in cholesterol-fed rabbits in which the effect of ACAT inhibitors on the plasma cholesterol level could be eliminated. In this experiment, aortic atherosclerosis had been established by feeding the animals a high cholesterol diet for 8 weeks, and thereafter, R-755 and CI-976 were given with a normal diet. The changes in the plasma cholesterol level in the treated groups were almost the same as that in the control group. However, the percentage of the surface area of atherosclerotic involvement (area positively stained by oil red O) and cholesterol contents in the thoracic and abdominal aorta were reduced by about 50% in the R-755 (10 mg/kg)-treated group. These results suggest that R-755 prevents cholesterol accumulation by direct inhibition of ACAT activity in the arterial wall not through the hypocholesterolemic activity. Furthermore, the effect of R-755 was more potent than that of CI-976. In the aortic arch, R-755 and CI-976 did not reduce the percentage of the surface area of atherosclerotic involvement and cholesterol contents. This result may be due to the establishment of severe atherosclerosis in the aortic arch.

In this study, we showed that R-755 was a potent ACAT

inhibitor and had a potent hypolipidemic activity in cholesterol-fed animal models. Furthermore, R-755 had an anti-atherosclerotic activity by direct inhibition of ACAT activity at the arterial wall in the experimental animal model. These pharmacological efficacies of R-755 were more potent than that of CI-976. These results suggest that R-755 can be expected to be a therapeutically useful drug that not only has lowering effects on plasma cholesterol and triglycerides but also has an anti-atherosclerotic effect by direct inhibition of ACAT activity at the arterial wall.

REFERENCES

- 1 Suckling KE and Stange EF: Role of acyl-CoA:cholesterol acyltransferase in cellular cholesterol metabolism. *J Lipid Res* **26**, 647–671 (1985)
- 2 Bennett CS and Tercyak AM: Reduced cholesterol transmucosal transport in rats with inhibited mucosal acyl CoA:cholesterol acyltransferase and normal pancreatic function. *J Lipid Res* **25**, 148–159 (1984)
- 3 Heider JG, Pickens CE and Kelly LA: Role of acyl CoA:cholesterol acyltransferase in cholesterol absorption and its inhibition by 57–118 in the rabbit. *J Lipid Res* **24**, 1127–1134 (1983)
- 4 Sugiyama Y, Ishikawa E, Odaka H, Miki N, Tawada H and Ikeda H: TPM-153, a novel ACAT inhibitor, inhibits cholesterol absorption and lowers plasma cholesterol in rats and hamsters. *Atherosclerosis* **113**, 71–78 (1995)
- 5 Devron CA, Engelhorn SC and Steinberg D: Secretion of very low density lipoproteins enriched in cholesteryl esters by cultured rat hepatocytes during stimulation of intracellular cholesterol esterification. *J Lipid Res* **21**, 1065–1071 (1980)
- 6 Carr TP, Hamilton RL Jr and Rudel LL: ACAT inhibitors decrease secretion of cholesteryl esters and apolipoprotein B by perfused livers of african green monkeys. *J Lipid Res* **36**, 25–36 (1995)
- 7 Krause BR, Anderson M, Bisgaier CL, Bocan T, Bousley R, DeHart P, Essenburg A, Hamelehle K, Homan R, Kieft K, McNally W, Stanfield R and Newton RS: In vivo evidence that the lipid-regulating activity of the ACAT inhibitor CI-976 in rats is due to inhibition of both intestinal and liver ACAT. *J Lipid Res* **34**, 279–294 (1993)
- 8 Azuma Y, Kawasaki T, Ikemoto K, Obata K, Ohno K, Sajiki N, Yamada T, Yamasaki M and Nobuhara Y: Cholesterol-lowering effects of NTE-122, a novel acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor, on cholesterol diet-fed rats and rabbits. *Jpn J Pharmacol* **78**, 355–364 (1998)
- 9 Kroon AA, Stalenhoef AFH, Buikema H, Demacker PMN, de Wilde PCM, Leijten PA and van Gilst WH: The effect of cholesterol reduction on the endothelial function and progression of atherosclerosis in WHHL rabbits. *Atherosclerosis* **103**, 221–230 (1993)
- 10 Shiomi M, Ito T, Watanabe Y, Tsujita Y, Kuroda M, Arai M, Fukami M, Fukushima J and Tamura A: Suppression of established atherosclerosis and xanthomas in mature WHHL rabbits by keeping their serum cholesterol levels extremely low. Effect of pravastatin sodium in combination with cholestyramine. *Atherosclerosis* **83**, 69–80 (1990)
- 11 Brown MS, Ho YK and Goldstein JL: The cholesteryl ester

- cycle in macrophage foam cells. Continual hydrolysis and reesterification of cytoplasmic cholesteryl esters. *J Biol Chem* **255**, 9344 – 9352 (1980)
- 12 Bocan TMA, Bak MS, Uhlendorf PD, Newton RS and Krause BR: Comparison of CI-976, an ACAT inhibitor, and selected lipid-lowering agents for antiatherosclerotic activity in iliac-femoral and thoracic aortic lesions. *Arteriosclerosis Thromb* **11**, 1830 – 1843 (1991)
- 13 Tanaka H, Ohtsuka I, Kogushi M, Kimura T, Fujimori T, Saeki T, Hayashi K, Kobayashi H, Yamada T, Hiyoshi H and Saito I: Effect of the acyl-CoA:cholesterol acyltransferase inhibitor, E5324, on experimental atherosclerosis in rabbits. *Atherosclerosis* **107**, 187 – 201 (1994)
- 14 Matsuo M, Ito F, Konto A, Aketa M, Tomoi M and Shimomura K: Effect of FR145237, a novel ACAT inhibitor, on atherogenesis in cholesterol-fed and WHHL rabbits. Evidence for a direct effect on the arterial wall. *Biochim Biophys Acta* **1259**, 254 – 260 (1995)
- 15 Basu SK, Goldstein JL, Anderson RGW and Brown MS: Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc Natl Acad Sci USA* **73**, 3178 – 3182 (1976)
- 16 Helgerud P, Saarem K and Norum KR: Acyl-CoA:cholesterol acyltransferase in human small intestine: its activity and some properties of the enzymic reaction. *J Lipid Res* **22**, 271 – 277 (1981)
- 17 Kam NTP, Albright E, Mathur SN and Field FJ: Inhibition of acylcoenzyme A:cholesterol acyltransferase activity in CaCo-2 cells results in intracellular triglyceride accumulation. *J Lipid Res* **30**, 371 – 377 (1989)
- 18 Field FJ, Albright E and Mathur SN: Regulation of cholesterol esterification by micellar cholesterol in CaCo-2 cells. *J Lipid Res* **28**, 1057 – 1066 (1987)
- 19 Burrier RE, Deren S, McGregor DG, Hoos LM, Smith AA and Davis HR Jr: Demonstration of a direct effect on hepatic acyl CoA:cholesterol acyltransferase (ACAT) activity by an orally administered enzyme inhibitor in the hamster. *Biochem Pharmacol* **47**, 1545 – 1551 (1994)
- 20 Lowry OH, Rosebrough NJ, Farr AL and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265 – 275 (1951)
- 21 Sugiyama Y, Odaka H, Itokawa S, Ishikawa E, Tomari Y and Ikeda H: TMP-153, a novel ACAT inhibitor, lowers plasma cholesterol through its hepatic action in Golden hamsters. *Atherosclerosis* **118**, 145 – 153 (1995)
- 22 Sakuma Y, Hagihara H, Ohne K, Nagayoshi A, Mutho S, Ito Y, Notsu Y and Okuhara M: Plasma cholesterol reducing effect of FR129169, a novel acyl-CoA:cholesterol acyltransferase inhibitor, in the rat. *Jpn J Pharmacol* **70**, 35 – 41 (1996)
- 23 Bell FP, Gammill RB and St Jhon LC: U-73482: A novel ACAT inhibitor that elevates HDL-cholesterol, lower plasma triglyceride and facilitates hepatic cholesterol mobilization in the rat. *Atherosclerosis* **92**, 115 – 122 (1992)
- 24 Hashimoto S and Dayton S: Influence of microsomal cholesterol concentrations on the cholesterol-esterifying activity of normal and atherosclerotic aortas. *Artery* **1**, 308 – 318 (1975)