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Importance of acyl-coenzyme A:cholesterol acyltransferase 1/2 dual inhibition for anti-atherosclerotic potency of pactimibe

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Abstract

Pactimibe sulfate, [7-(2,2-dimethylpropanamido)-4,6-dimethyl-1-octylindolin-5-yl]acetic acid hemisulfate, a novel Acyl-coenzyme A: cholesterol acyltransferase (ACAT) inhibitor, was investigated in vitro and in vivo to characterize its potential. Pactimibe exhibited dual inhibition for ACAT1 and ACAT2 (concentrations inhibiting 50% [IC_{50}s] at micromolar levels) more potently than avasimibe. Kinetic analysis revealed pactimibe is a noncompetitive inhibitor of oleoyl-CoA (K_i value: 5.6 μM). Furthermore, pactimibe markedly inhibited cholesteryl ester formation (IC_{50}: 6.7 μM) in human monocyte-derived macrophages, and inhibited copper-induced oxidation of low density lipoprotein more potently than probucol. Pactimibe exerted potent lipid-lowering and anti-atherosclerotic effects in atherogenic diet-fed hamsters. At doses of 3 and 10 mg/kg for 90 days, pactimibe decreased serum total cholesterol by 70% and 72%, and aortic fatty streak area by 79% and 95%, respectively. Despite similar cholesterol lowering, fatty streak area reduction was greater by 10 mg/kg. These results suggest that ACAT1/2 dual inhibitor pactimibe has anti-atherosclerotic potential beyond its plasma cholesterol-lowering activity.
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Keywords: ACAT (Acyl-coenzyme A:cholesterol acyltransferase); Hypercholesterolemia; Atherosclerosis; Pactimibe

1. Introduction

Acute coronary syndromes, the leading cause of hospitalizations among adults, are the sequelae of atherothrombotic events associated with thrombotic complications of fissure, erosion, or rupture of vulnerable atherosclerotic plaque (Davies, 1996; Falk et al., 1995; Libby, 1995). Acyl-coenzyme A:cholesterol acyltransferase (ACAT, EC 2.3.1.26) is the primary cellular enzyme that catalyzes the formation of cholesteryl esters from cholesterol and fatty acyl-coenzyme A. The development of vulnerable atherosclerotic plaque is associated with the accumulation of cholesteryl ester via ACAT1 in foam cells of the arterial wall. In contrast, ACAT2 plays a key role in very low density lipoprotein (VLDL) secretion from the liver and in dietary cholesterol absorption in the form of chylomicron from the intestine (Rudel et al., 2001). Dual inhibition of ACAT1 and ACAT2, therefore, represents an attractive therapeutic approach for preventing coronary artery disease by both anti-atherosclerotic and hypolipidemic activities (Sliskovic and White, 1991).

A number of ACAT inhibitors have been synthesized and their pharmacological profiles evaluated in animals and humans. However, several adverse effects, such as adrenal toxicity (Matsuo et al., 1996; Reindel et al., 1994; Vernetti et al., 1993), diarrhea (Kashiwa et al., 1997), hepatotoxicity (Ishi et al., 1994; Nakaya et al., 1994), and various elusive efficacies in humans (Hainer et al., 1994; Harris et al., 1990; Tardif et al., 2004) have been revealed and none of these compounds has so far succeeded in clinical development, most exhibiting low bioavailability as a consequence of their hydrophobicity.

Pactimibe sulfate, [7-(2,2-dimethylpropanamido)-4,6-dimethyl-1-octylindolin-5-yl] acetic acid hemisulfate, is a novel

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ACAT inhibitor we recently synthesized. The purposes of this study are: (1) to evaluate the in vitro inhibitory activity of pactimibe against ACAT using several enzyme sources; (2) to compare the anti-oxidant activity between pactimibe and probucol, which is known to have significant antioxidant action; and (3) to elucidate whether or not pactimibe has anti-atherosclerotic effects in vivo independent of its lipid-lowering effects by measuring both plasma lipid levels and atherosclerotic lesions in Western-type diet-fed hypercholesterolemic hamsters. The pharmacological profile of pactimibe indicates its potential as a therapeutic agent for both hypercholesterolemia and atherosclerotic diseases.

2. Materials and methods

2.1. Test compounds

Pactimibe sulphate, [7-(2,2-dimethylpropanamido)-4,6-dimethyl-1-octylindolin-5-yl] acetic acid hemisulfate (formerly named CS-505, $(C_{25}H_{40}N_2O_3)_2{\cdot}H_2SO_4$, M.W. 931.29) was synthesized at the Process Development Laboratories, Sankyo Co., Ltd. (Tokyo, Japan). Avasimibe (CI-1011, Pfizer Pharmaceuticals Inc., 2,6-bis(1-methylethyl)[[2,4,6,-tris(1-methylethyl)phenyl]acetyl]sulfamate, $C_{29}H_{43}NO_4S$, M.W. 501.73) was synthesized at Chemtech Labo., Inc. (Tokyo, Japan). The concentration of pactimibe is represented as that of the sulfate salt free form. For preparation of the dosing solution to animals, the sulfate salt form of pactimibe was suspended in 5% (w/w) gum arabic solution. All animal treatment protocols were reviewed by and are in compliance with the guidelines established by Sankyo's Institutional Animal Care and Use Committee. The structures of these compounds are shown in Fig. 1.

2.2. Chemicals

[1-^{14}C]-Oleoyl-CoA (2083.1 MBq/mmol), [4-^{14}C]-cholesterol (2072.0 MBq/mmol), [11,12-^{3}H]-retinol (1853.7 GBq/mmol), [9,10-^{3}H]-palmitic acid (1591.0 GBq/mmol), cholesteryl [1-^{14}C]-oleate (2.2015 GBq/mmol), [9,10-^{3}H]-triolein (814 GBq/mmol), and DL-3-hydroxy-3-methyl-[3-^{14}C]-glutaryl-CoA (2131.2 GBq/mmol) were purchased from NEN™ Life Science Products, Inc. (Boston, MA, USA). All other chemicals used were of reagent grade.

2.3. Lipoprotein preparation

Rabbit beta-VLDL (β-VLDL, $d<1.006$ g/ml) was isolated from the plasma of Japanese white rabbits fed a diet containing 1% cholesterol for 2 weeks. Lipoproteins were subjected to second ultracentrifugation (Hatch, 1968), isolated at the same density, and exhaustively dialyzed against 150 mmol/l NaCl and 0.24 mmol/l EDTA (pH 7.4) (Havel et al., 1955). The cholesterol concentration in the β-VLDL was determined enzymatically using a commercial kit (Cholesterol E Test Wako, Wako Pure Chemical Industries, Ltd., Osaka Japan). The β-VLDL was sterilized by filtration and stored at 4 °C prior to use.

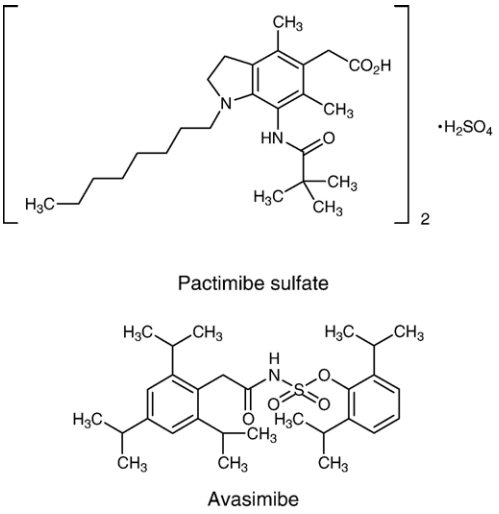

Fig. 1. Chemical structures.

2.4. Human and murine macrophage isolation

Human promonocytic THP-1 cell line originally from the American Type Culture Collection (ATCC) was maintained in suspension culture in RPMI 1640 medium (Life Technologies, Inc., NY, USA) containing 10% fetal bovine serum (FBS), 1% antibiotic mixture (50 units/ml penicillin and 50 μg/ml streptomycin), and 55 μM 2-mercaptoethanol. Monocytic differentiation of THP-1 cells was induced by exposure to 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co., St. Louis, MO, USA) for 3 days. A mouse macrophage-like J774A.1 cell line originally from the ATCC was cultured as cell monolayers in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and the 1% antibiotic mixture. Rat peritoneal macrophages were harvested from unstimulated male Sprague–Dawley (SD) rats (7 weeks old, Japan SLC, Hamamatsu, Japan) as previously described (Edelson and Cohn, 1974). Human primary monocyte-derived macrophages were obtained as previously described (Urbich et al., 2003). Peripheral blood mononuclear cells were individually isolated by Lymphoprep density-gradient centrifugation using peripheral blood from eight healthy volunteers. Immediately after isolation of peripheral blood mononuclear cells, CD14-positive monocytes were purified by positive selection with anti-CD14 microbeads (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) using a magnetic cell sorter device (LS column and Midi Magnets, Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). CD14-positive monocytes were incubated for 7 days in RPMI 1640 medium (Life Technologies, Inc., NY, USA) containing 10% FBS, the 1% antibiotic mixture, 55 μM 2-mercaptoethanol, and 50 ng/ml monocyte colony-stimulating factor to induce macrophage differentiation.

2.5. Preparation of enzyme source

Preparation of microsomal fraction was carried out according to the method of Fujioka et al. (Fujioka et al., 1997). Male

Sprague–Dawley (SD) rats (7 weeks old, Japan SLC, Hamamatsu, Japan) were fasted overnight, and the liver and small intestine were removed at sacrifice and homogenized by a POLYTRON® homogenizer (KINEMATICA AG, Switzerland) in a tenfold volume of homogenizing buffer (0.25 M sucrose buffered with 10 mM potassium phosphate, pH 7.4). The homogenate was centrifuged at 10,000×*g* at 4 °C for 15 min and the postmitochondrial fraction was centrifuged at 100,000×*g* at 4 °C for 60 min. The resulting pellet (microsomal fraction) was stored at −80 °C until use. The cells were washed twice with PBS, scraped into the homogenizing buffer, and sonicated twice for 15 s. The resulting cell suspension was used either for the preparation of the microsomal fraction for ACAT assay or the enzyme sources for esterase assay described later.

2.6. Assay of microsomal ACAT activity

The assay of ACAT activity was performed essentially according to the method previously described with several modifications (Doolittle and Chang, 1982; Ross and Rowe, 1984). The assay of the ACAT activity from PMA-treated THP-1 cells at apparent maximum velocity of the enzyme was accomplished by addition of exogenous cholesterol in the form of cholesterol: phosphatidylcholine:cholic acid:*n*-tetradecane, molar ratio 0.4:1:1, with *n*-tetradecane in vesicles prepared by sonication in a bath sonicator. For the assay of ACAT from rat tissues or cells, vesicles without *n*-tetradecane were used. Microsomal protein from tissues or cells (0.04–1 mg) were assayed in 50 μM of [1-^{14}C]-oleoyl-CoA containing 335 μg of bovine serum albumin, 2 mM dithiothreitol, approximately 100 μg of cholesterol in vesicles, and 0.15 M potassium phosphate buffer (pH 7.4) (final volume, 0.2 ml). Test compounds were dissolved in dimethyl sulfoxide (DMSO); equivalent amounts of DMSO were added to control samples. The reaction was carried out at 37 °C for 4 min and stopped by the addition of 5 ml of chloroform/methanol (2:1, v/v) and 1 ml of saline. Cholesteryl [1-^{14}C]-oleate was separated by silica gel thin layer chromatography with a solvent system of *n*-hexane/diethyl ether/acetic acid (85:15:1, v/v/v) and quantified by liquid scintillation counting. For the kinetic study of ACAT from PMA-treated THP-1 microsomes, the assay was performed under the same conditions as described above, except that the final concentration of [1-^{14}C]-oleoyl-CoA (2083.1 MBq/mmol, not diluted with cold oleoyl-CoA) was changed from the one concentration of 50 μM to five concentrations of 3.552, 4.44, 5.92, 8.88, and 17.76 μM. Each assay was performed in duplicate.

2.7. Assay of ACAT activities of recombinant ACAT1 and ACAT2

Homogenates of High Five insect cells containing baculovirally expressed human ACAT1 and ACAT2 (GenBank accession nos. L21934 and AF099031, respectively) were used as a source of the enzymes as previously described (Cho et al., 2003). The activity of the recombinant human ACAT1 and ACAT2 was measured according to the method of Cases et al. (1998).

2.8. Assay of other related lipid-metabolizing enzymes

Both pactimibe and avasimibe were evaluated in vitro at relatively high concentrations up to 300 μM to determine whether they inhibited the activities of other related lipid-metabolizing enzymes such as lecithin:cholesterol acyltransferase (LCAT) in plasma from SD rats (Stokke and Norum, 1971); acyl-coenzyme A:retinol acyltransferase (ARAT) (Ross, 1982) and acyl-coenzyme A synthase (ACS) in liver microsomes from SD rats (Bar-Tana and Shapiro, 1975); acid and neutral cholesterol esterase in homogenate of J774A.1 macrophages (Ishii et al., 1992); lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) in post-heparin plasma from SD rats (Nilsson-Ehle and Schotz, 1976); and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase in liver microsomes from Wister–Imamichi rats (Kuroda and Endo, 1976). The test compounds dissolved in DMSO were added to the reaction mixtures. Each assay was performed in duplicate.

2.9. Copper-induced low density lipoprotein (LDL) oxidation

It was expected that pactimibe would have an antioxidant effect since this compound has an indoline backbone in its molecule (Fig. 1). Probucol was introduced as a reference antioxidant compound in control assays. LDL oxidation was performed as previously described (Kleinveld et al., 1992) with probucol (Sigma Chemical Co., St. Louis, MO), as a positive control. Briefly, fresh blood samples obtained from six healthy male volunteers were mixed with disodium ethylenediaminetetraacetic (EDTA) acid at a final concentration of 5 mM. LDL (d=1.019–1.063) was isolated from the supernatant of a 2000*g* spin (4 °C, 15 min) by sequential density ultracentrifugation (Hatch, 1968). The easily visible yellow LDL fraction was isolated and dialyzed against EDTA free phosphate buffered saline. Total protein was determined (Bradford, 1976) and the aqueous oxidation mix (200 μl) containing 10 μM $CuSO_4$ and 0.1 mg/ml of LDL protein was continuously monitored at 234 nm every 10 min for 10 h. In order to limit spontaneous oxidation of LDL, the desalting procedure was done at 4 °C within 45 min after LDL isolation. The change in absorbance at 234 nm versus time was divided into three consecutive phases: lag, propagation, and decomposition. Then lag time was calculated as previously described (Kleinveld et al., 1992). Three sequential time points with minimum absorbance change in the lag phase and three with maximum absorbance change in the propagation phase were picked up for the calculation of lag time. Lag time is defined as the intersection (min) of the linear least square slopes in both the lag phase and the propagation phase. The ED_{200} value, the concentration which prolongs the lag time to 200% of the control, was calculated by the linear least-square method. The measured lag-times correspond to the onset of oxidation of LDL and a short lag-time indicates a greater susceptibility of LDL to oxidation.

2.10. Analysis of cholesteryl ester formation in human monocyte-derived macrophages

Cholesterol esterification in macrophages is a critical step for foam cell formation, which is proposed to be a proatherogenic factor. Thus, in order to relate in vitro potency to inhibit ACAT activity with effect on production of cholesterol ester, cellular esterification of cholesterol was determined according to the method described by Brown et al. (1979) with some modifications. Briefly, each well treated with or without pactimibe for 24 h received [1-^{14}C]-oleic acid (1 μCi/ml, 0.2 mM) complexed with fatty acid-free bovine serum albumin (0.6 mg/ml) in assay medium (RPMI 1640 medium containing 10% (v/v) lipoprotein-deficient serum (Biomedical Technologies Inc., MA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 55 μM 2-mercaptoethanol, and 50 μg cholesterol/ml β-VLDL). The cells were then washed three times with phosphate-buffered saline (PBS), the lipid was extracted in situ, and cholesteryl ester was separated by TLC as described above. The remaining cellular protein was dissolved in 0.1 N NaOH and aliquots were assayed for protein determination according to the instructions of a Bio-Rad DC protein assay kit with BSA standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.11. Effects of pactimibe on serum lipid levels and atherosclerosis in hypercholesterolemic hamsters

2.11.1. Experimental design

F1B hamsters at 11 weeks old weighing 110–120 g were obtained from Charles River Japan Inc. (Kanagawa, Japan). Animals were housed in a room controlled for temperature (21–25 °C), humidity (45–65%), and light (7 AM–7 PM). Food (commercial laboratory chow diet, F-2, Funabashi Farm Co., Ltd., Chiba, Japan) and water were given ad libitum until the start of the experiment (at 12 weeks old). Nine hamsters received the basic chow diet all through the experimental period. We defined this group as the normal group. A total of 27 hamsters received the basic diet plus 10% (w/w) hydrogenated coconut oil and 0.05% cholesterol (atherogenic diet, Funabashi Farm Co., Ltd.) till the end of the experimental period. Body weights of hamsters fed the atherogenic diet were measured at 18 weeks old. Animals were divided into 3 groups ($n=9$ in each group) where the mean values of body weights per group were almost equivalent. Subsequently, groups were assigned at random to control and pactimibe (3 and 10 mg/kg) groups.

Pactimibe was orally administered to the hamsters once daily for 90 days at doses of 0 (control), 3, or 10 mg/kg body weight. The first atherogenic diet-loading day was defined as Day 0, and each compound was administered from Day 52 to Day 141.

2.11.2. Lipid analysis

At the end of the experimental period, animals were fasted for 17 h, then blood samples were taken from the abdominal aorta under general anesthesia (Nembutal® sodium solution, 40 mg/kg, i.p.). The liver was also collected at necropsy. Serum was obtained by centrifugation (2000×*g*, 4 °C, 15 min). The serum and liver samples were stored at −20 °C until measurements of serum lipid concentrations and hepatic lipid content, respectively. The levels of serum total cholesterol, triglycerides, and phospholipids were determined enzymatically using a HITACHI type 7250 automatic analyzer (Hitachi, Ltd., Tokyo, Japan). High density lipoprotein (HDL) cholesterol was measured after precipitation of VLDL and LDL using a commercial kit (HDL-Cholesterol Precipitant Set, Wako Pure Chemical Industries, Ltd.). The level of non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol. Lipids in the liver were extracted as previously described (Folch et al., 1957). The lipid extract was evaporated under a stream of nitrogen gas, and redissolved with an aliquot of isopropyl alcohol. Total cholesterol, unesterified cholesterol, and triglyceride concentrations were determined using appropriate chemical assay kits (Cholesterol C-II Test Wako, Free Cholesterol C Test Wako, and Triglyceride E Test Wako, respectively, Wako Pure Chemical Industries, Ltd.). Esterified cholesterol was calculated as the difference between total and free cholesterol.

2.11.3. Quantification of aortic fatty streak area

Quantification of the aortic fatty streak area was carried out as previously described (Kowala et al., 1993). Briefly, the aortic arches were excised, cleaned, fixed with 10% buffered formalin, and immersed in Oil Red O. Each aortic arch was cut open along the outer curvature, laid flat on a glass slide with the transparent endothelium facing up, slipped into aqueous mounting medium, and examined en face by light microscopy. The total Oil Red O stained area of each aortic arch and size of the arch were analyzed to calculate the fatty streak area (μm^2) per aortic arch area (mm^2) using a computerized image analysis system (Image Technology, NJ, USA) attached to a compound light microscope (Olympus Optical Company Ltd., Tokyo, Japan).

2.12. Statistical methods

Each value is expressed as the mean±standard error of the mean. In the analyses of serum and hepatic concentrations, data sets that failed Bartlett's test ($P<0.05$) were analyzed using nonparametrical Steel's test. In the analysis of aortic Oil Red O stained areas, to normalize the data and stabilize the variance, the data were converted to $\log_{10}$ values before analysis was performed. Further post hoc analyses were performed using Student's or Aspin–Welch *t*-test to evaluate the relationship between the groups. A difference was considered to be statistically significant when the *P* value was less than 0.05. All analyses were performed using the Statistical Analysis System (SAS®, version 6.12, SAS Institute Inc. Cary, NC, USA).

3. Results

3.1. Effects on cell-free ACAT activity

Effects of pactimibe and avasimibe on microsomal ACAT activities in various rat tissues and cells (liver, small intestine,

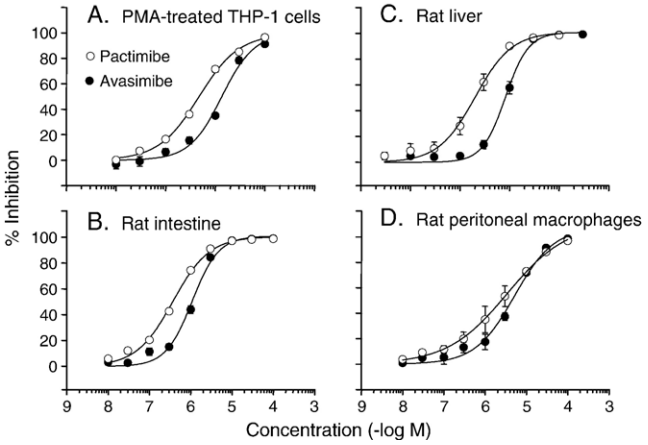

Fig. 2. Dose-dependent inhibition of ACAT activities by pactimibe and avasimibe. Microsomal ACAT activities were determined as described in the text. Data represent the mean±S.E.M. of three distinct experiments (each in duplicate). The control activities of PMA-treated THP-1 cells, rat intestine, rat liver, and rat peritoneal macrophages were 220±10, 685±146, 488±15, and 208±58 pmol/mg protein/min, respectively.

and peritoneal macrophages) and cultured human cells (PMA-treated THP-1) were investigated. Both compounds inhibited microsomal ACAT activities in a dose-dependent fashion (Fig. 2). The estimated IC_{50} values (the concentrations inhibiting the ACAT activities by 50% of the control) of each compound are summarized in Table 1. The inhibitory effects of pactimibe were consistently more potent than those of avasimibe. Furthermore, we evaluated pactimibe in a cell-free assay system using recombinant human ACAT1 and ACAT2 produced in insect cells. Pactimibe inhibited both ACAT1 and ACAT2 with IC_{50} values of 8.3 and 5.9 μM, respectively (Fig. 3). Lineweaver–Burk plot analysis for PMA-treated THP-1 microsomal ACAT activity revealed that pactimibe is a noncompetitive inhibitor of ACAT with respect to oleoyl-CoA with a K_i value of 5.6 μM in comparison to a K_m value of 14 μM for oleoyl-CoA (Fig. 4).

Table 1
Inhibitory effects of pactimibe and avasimibe on microsomal ACAT activities originating from various tissues and cells

Species	Tissue or cells	Specific activity (pmol/mg protein/min)	IC_{50} (μM)	
			Pactimibe	Avasimibe
Experiment 1				
Human	PMA-treated THP-1 cells	237	5.0	13
Rat	Intestine	740	0.37	1.2
Rat	Liver	506	1.2	7.4
Rat	Peritoneal macrophages	313	3.7	4.5
Experiment 2				
Human	PMA-treated THP-1 cells	222	4.9	14
Rat	Intestine	409	0.36	0.89
Rat	Liver	457	1.8	7.1
Rat	Peritoneal macrophages	114	0.92	3.3
Experiment 3				
Human	PMA-treated THP-1 cells	201	4.3	14
Rat	Intestine	907	0.38	1.1
Rat	Liver	500	3.0	10
Rat	Peritoneal macrophages	198	3.6	5.2
Avegage				
Human	PMA-treated THP-1 cells		4.7	14
Rat	Intestine		0.37	1.1
Rat	Liver		2.0	8.2
Rat	Peritoneal macrophages		2.7	4.3

Microsomal ACAT activities were determined as described in the text by incorporation of [1-^{14}C]-oleoyl-CoA into cholesteryl oleate with exogenous cholesterol as described in Materials and methods. The IC_{50} (the concentration inhibiting the ACAT activities by 50% of those of the control) was interpolated from the consequent sigmoidal inhibition curves shown in Fig. 2.

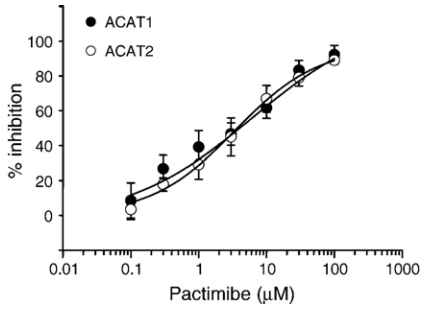

Fig. 3. Dose-dependent inhibition of ACAT activities by pactimibe. ACAT activities of recombinant ACAT1 and ACAT2 were determined as described in Materials and methods. Data represent the mean±S.E.M. of three distinct experiments (each in duplicate). The control activities of ACAT1 and ACAT2 were 236±47 and 1108±13 pmol/mg protein/min, respectively. IC_{50} values against ACAT1 and ACAT calculated from the sigmoidal dose response regression curve were 8.3 and 5.9 μM, respectively.

3.2. Effects on other lipid metabolizing enzyme activities

Essentially no inhibiting activities against ARAT, ACS, acid or neutral cholesterol esterase, LPL, HTGL, or HMG-CoA reductase were observed even at concentrations 8000-fold higher than those required to inhibit ACAT by 50% (0.37 vs. 300 μM). Both pactimibe and avasimibe exhibited dose-dependent inhibition on the activities of LCAT originating from rat plasma with IC_{50} values of 71 and 67 μM, respectively.

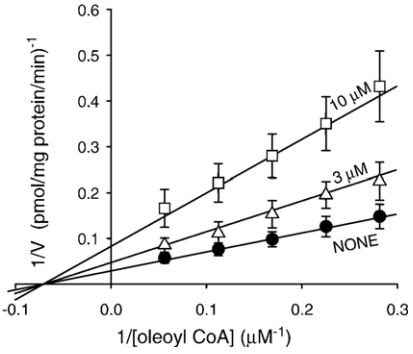

Fig. 4. Lineweaver–Burk plot for inhibition of ACAT activity by pactimibe. The assay was performed in the absence of pactimibe (●), and in the presence of 3 μM (△) and 10 μM (□) of pactimibe. The final concentrations of oleoyl-CoA were 3.552, 4.44, 5.92, 8.88, and 17.76 μM. The results are expressed as the mean±S.E.M. of three distinct experiments (each in duplicate) as described in Materials and methods.

3.3. Inhibitory effect on LDL oxidation

Treatment of LDL isolated from six healthy volunteers with 0.6, 2.1, or 6.4 μM pactimibe retarded the onset of oxidation of LDL from all volunteers, i.e., increased the lag time, in a dose-dependent manner (mean lag time: 98 min, 124 min, and 202 min, respectively, versus 75 min for control). The ED200 value was individually calculated and the mean ED200 value was 3.7 μM. In contrast, probucol did not exhibit potent antioxidative activity under our experimental conditions. Probucol at final concentrations of 1.9, 5.8, and 19 μM did not significantly affect the lag time in 2 of 3 volunteers, whereas it retarded the onset of LDL oxidation in 1 of 3 volunteers in a dose-dependent manner (lag time: 85 min at 19 μM and 295 min at 5.8 μM [no propagation observed at 1.9 μM], versus 77 min for control).

3.4. Effect on cholesteryl ester formation in human monocyte-derived macrophages

The effect of pactimibe on cholesteryl ester formation in human monocyte-derived macrophages was evaluated by measuring incorporation of [1-^{14}C]-oleate into cholesteryl esters (Fig. 5). Pactimibe dose-dependently inhibited the cholesteryl ester formation with an IC_{50} value of 6.7 μM. The inhibitory effect of pactimibe on cholesteryl ester formation was comparable with the result from the cell-free ACAT assay system (IC_{50} value for PMA-treated THP-1 cells, 4.7 μM).

3.5. Hypocholesterolemic and anti-atherosclerotic activities in hypercholesterolemic hamsters

The atherogenic diet caused hyperlipidemia in hamsters (serum total cholesterol >450 mg/dl and serum triglycerides >1000 mg/dl). There were no significant differences in body weight or in food intake throughout the study period among the atherogenic diet-fed groups. Once daily oral administration of pactimibe to hamsters at doses of 3 or 10 mg/kg for 90 days significantly decreased serum total cholesterol levels and non-HDL cholesterol levels, without significantly affecting HDL cholesterol levels in the hyperlipidemic hamsters. Pactimibe significantly decreased not only serum triglyceride levels but also serum phospholipid levels (Fig. 6). Pactimibe at doses of 3 and 10 mg/kg reduced the free cholesterol content in addition to drastic reduction in total cholesterol and esterified cholesterol contents in the liver. Pactimibe also significantly decreased hepatic triglyceride content (Table 2). Fig. 7 shows the effect of pactimibe on aortic fatty streak areas in the hamsters. The mean fatty streak area in the control group was greater than that in the normal group, although without statistical significance ($P=0.1147$) because the severest spontaneous atheromatous lesion was observed in the normal group. Pactimibe at doses of 3 and 10 mg/kg significantly

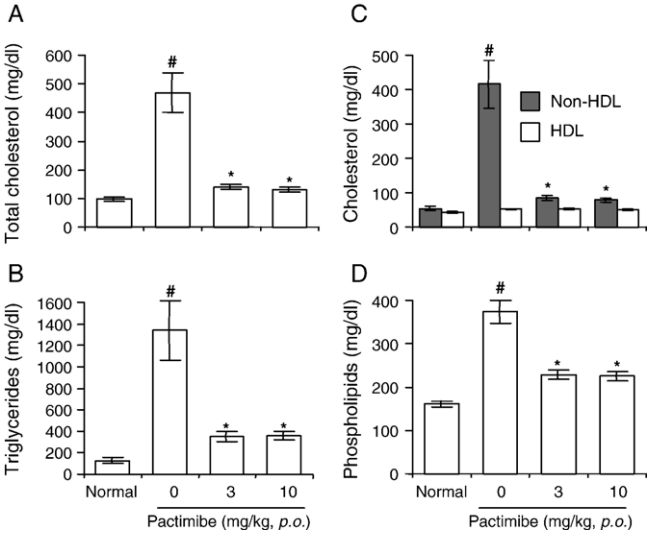

Fig. 6. Effect of pactimibe on serum lipid levels in hamsters. Hamsters were fed an atherogenic diet for 142 days. Pactimibe was orally administered once daily to hamsters for 90 days from Day 52. Serum lipid levels were determined as described in the text. Each value represents the mean±S.E.M. ($n=9$). #$P<0.05$, as 29/30 compared to the normal group using Aspin–Welch's t-test or Student's t-test. *$P<0.05$, as compared to the control (0 mg/kg) group using Dunnett's multiple comparison test or Steel's test.

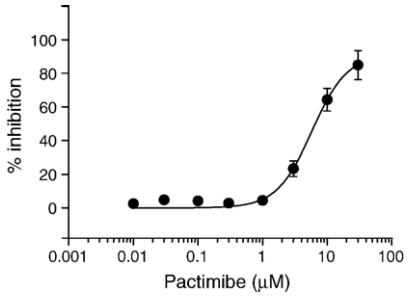

Fig. 5. Effect of pactimibe on whole-cell ACAT activity in human monocyte-derived macrophages. Human monocyte-derived macrophages from eight healthy volunteers were cultured individually with or without pactimibe at 0.01–10 μM. Whole cell-ACAT activity was determined by measuring the incorporation of [1-^{14}C] oleate into cholesteryl esters in duplicate per subject. Results are expressed as the mean±S.E.M. ($n=8$).

Table 2
Effect of pactimibe on hepatic lipid levels in hamsters

Group	Dose (mg/kg)	Total cholesterol	Free cholesterol	Esterified cholesterol	Triglycerides
Normal	None	3.40±0.3	2.47±0.08	0.92±0.23	6.72±0.34
Control	0	10.74±0.53[a]	3.71±0.14[a]	7.03±0.41[a]	7.42±0.48
Pactimibe	3	2.72±0.12[b]	2.33±0.06[b]	0.39±0.06[b]	5.16±0.30[b]
Pactimibe	10	2.48±0.04[b]	2.24±0.03[b]	0.24±0.02[b]	5.34±0.29[b]

Hamsters were fed an atherogenic diet for 142 days. Pactimibe was orally administered once daily to hamsters for 90 days from day 52. Hepatic total cholesterol, free cholesterol, esterified cholesterol, and triglyceride levels (mg/g liver) were determined as described in the text. Each value represents the mean±S.E.M. ($n=9$).

[a] $P<0.05$, as compared to the normal group using Aspin–Welch's t-test or Student's t-test.

[b] $P<0.05$, as compared to the control group using Dunnett's multiple comparison test or Steel's test.

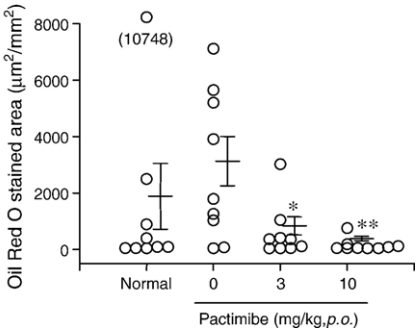

Fig. 7. Effect of pactimibe on aortic Oil Red O stained areas in hamsters. Hamsters were fed an atherogenic diet for 142 days. Pactimibe was orally administered to hamsters once daily for 90 days from Day 52. Aortic Oil Red O stained areas were determined as described in the text. Distribution of Oil Red O areas is shown. The horizontal bars represent the mean of each group. Spontaneous and severe lesion formation was found in the normal group (value shown in parentheses). The asterisks indicate a statistically significant difference compared with the control (0 mg/kg) group at $*P<0.05$ and $**P<0.01$ according to Dunnett's multiple comparison test.

reduced the aortic fatty streak area by 79% and 95%, respectively, compared to the control group.

4. Discussion

The studies described in this report have shown pactimibe not only to be a potent inhibitor of ACAT in microsomes prepared from the liver, intestinal tissues, and peritoneal macrophages of rats and human macrophage cell line, but also to have hypocholesterolemic and anti-atherosclerotic activities in vivo.

We have shown that pactimibe inhibits both macrophage ACAT and intestinal ACAT activities more potently than avasimibe in cell-free assays using native enzymes. The inhibitory activities of avasimibe were consistent with those reported in a previous paper (Lee et al., 1996). In mammals, two ACAT genes, ACAT1 and ACAT2, have been discovered (Anderson et al., 1998; Buhman et al., 2000, 2001; Cases et al., 1998; Chang et al., 1993, 2001; Oelkers et al., 1998; Rudel et al., 2001). As shown in Fig. 3, pactimibe inhibited both ACAT1 and ACAT2, and displayed approximately equivalent IC_{50} values. Therefore, pactimibe is a dual type ACAT1/2 inhibitor with a new type of structure. In the enzyme selectivity study, pactimibe did not affect ARAT, ACS, acid or neutral cholesterol esterase, LPL, HTGL, or HMG-CoA reductase. These results strongly suggest that pactimibe does not impact on biologically important key steps for vitamin A storage, utilization of very long chain fatty acids, stored cholesterol, the metabolism or inter-conversions of lipoproteins, or cholesterol synthesis. Although pactimibe inhibited the activity of LCAT with an IC_{50} value of 71 μM, the inhibitory potency against the enzyme was about 15 to 190 times less than that against ACAT, IC_{50} values on rat microsomal ACAT ranging from 0.37 to 2.7 μM. Avasimibe also inhibited LCAT with an IC_{50} value of 67 μM. In the clinical trial, avasimibe even at a high dose (750 mg) did not show LCAT inhibition-related unfavorable responses such as lowering of HDL cholesterol levels or elevation of triglyceride levels (Insull et al., 2001). Therefore, we believe that the effect of pactimibe on LCAT activity is not a major issue in humans. These results suggest that pactimibe is a highly specific ACAT1/2 dual inhibitor. The kinetic analysis data indicate that pactimibe is a noncompetitive inhibitor with respect to oleoyl-CoA under the condition of saturation of microsomal cholesterol levels. However, such data should be viewed with caution since the assay contains impure enzyme preparation under the condition in this study, ACAT is not the only enzyme binding to the oleoyl-CoA substrate, and possible binding of oleoyl-CoA to a second protein could compromise the kinetic analysis data in our assay. Triglyceride biosynthetic enzymes and ARAT also could utilize oleoyl-CoA in the microsomes from macrophages used in this study. Our preliminary kinetic analysis using semi-purified preparation of the enzyme from recombinant human ACAT1 also showed pactimibe inhibits ACAT1 in a noncompetitive manner for oleoyl-CoA. Presumably, pactimibe may interact with an allosteric site of ACAT because ACAT is considered to be an allosteric enzyme regulated by cholesterol.

Circulating monocytes adhere to endothelial cells, migrate into the subendothelial space, and differentiate into macrophages, which leads to foam cell formation (Faggiotto and Ross, 1984; Faggiotto et al., 1984). Thus, conversion of foam cells from tissue macrophages via cholesterol ester accumulation by ACAT1 activity is a critical step for atherogenesis. On the other hand, paradoxically, complete inhibition of ACAT1 in macrophages in atherosclerosis-susceptible mice was associated with accelerated atherosclerosis (Fazio et al., 2001; Su et al., 2005). Increased free cholesterol, which is cytotoxic, in macrophages in the vessel wall is a one of the proposed mechanisms responsible for the proatherogenic phenomenon in mice (Tabas, 2002, 2005). Therefore, it is important to consider the two aspects of ACAT1 inhibition: decreased cholesteryl ester in macrophages (anti-atherosclerotic) and increased free cholesterol in macrophages (proatherogenic). In our study with human monocyte-derived macrophages, pactimibe at all concentrations tested exhibited no apparent cytotoxic effect presumably due to its moderate inhibitory activities. These results suggested that, at least in vitro, moderate ACAT inhibition in monocyte-derived macrophages is anticipated to inhibit foam cell formation by not only inhibiting accumulation of cholesteryl ester but also enhancing cholesterol efflux from the cells. Indeed, two independent ACAT inhibitors have shown enhanced cholesterol efflux from macrophages (MCC-147, synthesized by Mitsubishi Pharma Corporation) (Sugimoto et al., 2004) and fibroblasts (F12511, Pierre Fabre Research Institute) (Yamauchi et al., 2004). Thus, pactimibe may have potential to exhibit anti-atherosclerotic activity through inhibiting cholesteryl ester formation and/or stimulating cholesterol efflux from macrophages in the arterial wall in humans.

Of note, in in vitro studies on copper-induced oxidation of LDL, pactimibe exhibited potent antioxidant activity at a clinically relevant concentration. This antioxidant activity is considered to be promising mechanism of action for therapeutic agents for atherosclerosis (Steinberg et al., 1989). Indeed,

probucol (Carew et al., 1987) and vitamin E (Prasad and Kalra, 1993) exhibited anti-atherosclerotic activity in laboratory animals by the suppression of oxidative stress. Also potential clinical effect of probucol as antioxidant agent was shown in a previous publication (Tardif et al., 2003). Therefore, it is expected that pactimibe might have additional anti-atherosclerotic effects comparing other ACAT inhibitors.

In hyperlipidemic hamsters, pactimibe was found to be orally active: once daily oral administration of pactimibe by gavage showed potent hypocholesterolemic and anti-atherosclerotic activities without any indication of toxicity in clinical blood chemistries. The hamster is a widely studied animal model whose cholesterol and bile acid metabolisms are similar to those in humans (Kris-Etherton and Dietschy, 1997; Suckling et al., 1991). Moreover, the diet used in the current study contained a similar amount of saturated fat and cholesterol to that consumed by Westerners, 0.05% cholesterol. Since most of the cholesterol and triglycerides in these hamsters is carried in the atherogenic lipoprotein fractions, macrophage-derived foam cells and extracellular lipid droplets consistently accumulate in a well-defined region along the inner curvature of the aortic arch, hence called "lesion-prone area". Therefore, the hamster represented a suitable animal model for our aim.

In our study, hypercholesterolemia in hamsters was primarily ascribed to the dietary cholesterol loading. Therefore, it is reasonable to speculate that the decrease in serum cholesterol by pactimibe resulted from the inhibition of intestinal cholesterol absorption. It is well known that systemically bioavailable ACAT inhibitors can affect hepatic ACAT and reduced plasma LDL-C by inhibiting VLDL cholesterol secretion without affecting intestinal cholesterol absorption (Burnett et al., 1999; Wrenn et al., 1995). Taking account of the additional facts that pactimibe efficiently inhibited intestinal cholesterol absorption, determined by a dual isotope method (Zilversmit and Hughes, 1974), and inhibited VLDL secretion, determined by the Triton WR-1339 method (Risser et al., 1978) (manuscripts in preparations), these results suggest that orally absorbable ACAT inhibitor pactimibe reduced serum total cholesterol not only by preventing cholesterol absorption via the gut but also by reducing VLDL cholesterol secretion from the liver into the circulation in hamsters. Pactimibe also decreased both serum triglyceride and phospholipid levels. Considering the facts that pactimibe reduced hepatic cholesteryl ester and triglyceride, the core lipids of VLDL, pactimibe may reduce VLDL secretion from the liver. The decrease in phospholipid levels could be attributed to the decrease in the number of VLDL. This hypothesis will be proved by the additional examination.

We found that dose-related reduction in aortic atherosclerotic lesion area was observed without obvious differences in their serum lipid levels. These data suggest that pactimibe reduced the atherosclerotic lesion in this model in a lipid-independent manner. In a satellite group, the peak plasma concentration of pactimibe was determined: around 1.0 μM was achieved after the 7th administration. The concentration obtained in the hamsters was pharmacologically relevant to inhibit ACAT in monocyte-derived human macrophages. Our data suggest that ACAT1 inhibition by decreasing cholesteryl ester production in macrophage accounts for the anti-atherosclerotic activity of pactimibe. This anti-atherosclerotic activity may be synergistically potentiated by its antioxidant activity.

In summary, pactimibe appears to be a highly potent systemic ACAT inhibitor with potent antioxidative activity that displays strong hypocholesterolemic and anti-atherosclerotic properties in hamsters. In in vitro assays, pactimibe was found to have multiple mechanisms of action for anti-atherosclerotic activity: 1) dual inhibition against both ACAT1 for macrophage foam cell formation and ACAT2 for cholesterol-lowering; and 2) structure-derived antioxidative activity. These findings suggest that pactimibe has the potential to be an effective therapeutic agent for hypercholesterolemia and atherosclerosis in humans.

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