CL 277,082: a novel inhibitor of ACAT-catalyzed cholesterol esterification and cholesterol absorption

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Abstract CL 277,082 (I) was found to be a potent inhibitor of acyl CoA:cholesterol acyltransferase (ACAT, EC 2.3.1.26) in microsomes from a variety of tissues with IC_{50} values of 0.14 μM for intestinal mucosal microsomes, 0.74 μM for liver, and 1.18 μM for rat adrenal. I was also shown to inhibit ACAT in cultured smooth muscle cells (IC_{50} = 0.8 μM) and was found to be specific in inhibiting cholesterol esterification since it did not inhibit fatty acid incorporation into triglycerides or phospholipids. Also, other cholesterol esterifying enzymes such as lecithin:cholesterol acyltransferase (LCAT) and pancreatic cholesterol esterase were not inhibited by I, nor was esterification of retinol by acyl CoA:retinol acyltransferase (ARAT) from intestinal mucosal microsomes inhibited. I was a potent inhibitor of cholesterol absorption in cholesterol-fed rats by markedly inhibiting increases in liver and serum cholesterol concentration (ED_{50} = 5.2 mg/kg per day) while increasing the excretion of neutral ^{14}C-labeled sterol in the feces.—**Largis, E. E., C. H. Wang, V. G. DeVries, and S. A. Schaffer.** CL 277,082: a novel inhibitor of ACAT-catalyzed cholesterol esterification and cholesterol absorption. *J. Lipid Res.* 1989. **30:** 681–690.

Supplementary key words intestinal microsomes • cholesteryl ester • acyl-CoA:cholesterol acyltransferase • acyl-CoA:retinol acyltransferase

Acyl coenzyme A:cholesterol acyltransferase (ACAT, 2.3.1.26) is responsible for the intracellular esterification of cholesterol (1, 2). Enzyme activity increases when cultured cells (3–7) and microsomes (8–11) are exposed to cholesterol-rich lipoproteins. This effect may be due to an increase in the microsomal membrane cholesterol (12, 13) and/or regulation in ACAT activity via an ATP-dependent process in intact cells (14, 15). Since the intracellular accumulation of cholesteryl esters is a hallmark of the atherosclerotic plaque, controlling ACAT activity may be of importance in regulating the development of atherosclerosis.

Whereas ACAT is used by most cells of the body to store cholesterol as cholesteryl ester, it may also play a key role in the intestinal absorption of cholesterol (16–18). Cholesterol is internalized in the mucosal cells as free cholesterol (19), but more than 90% of the absorbed sterol is excreted into mesenteric lymph as cholesteryl ester (20). Significant ACAT activity has been observed in intestinal mucosal cells from several animal species (16, 21–23) including man (17). The site of greatest ACAT activity is the jejunum which coincides with the major site of cholesterol absorption (24). Also, mucosal ACAT activity has been shown to increase with increased dietary cholesterol (21, 22). Thus, a potent ACAT inhibitor could have utility as both a hypocholesterolemic agent and potentially as an anti-atherosclerotic compound.

In this report, we present data on a new class of ACAT inhibitor that is structurally unrelated to previously reported inhibitors (18, 25–28). CL 277,082, a trisubstituted urea, is an absorbable compound which is a potent inhibitor of ACAT and produces hypocholesterolemic effects in cholesterol-fed rats by inhibiting absorption of dietary cholesterol. The synthesis and preliminary description of biological properties of this compound have been published (29).

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Palmitoyl CoA (55 mCi/mmol), [4-^{14}C]cholesterol (60 mCi/mmol), [1,2-^{3}H]cholesterol (53 mCi/mmol), and [1-^{14}C]oleic acid (48 mCi/mmol) were purchased from New England Nuclear, Boston, MA. Taurocholic acid, oleic acid, cholesteryl oleate, retinyl palmitate, retinol, cholesterol, and porcine pancreas cholesterol esterase were obtained from Sigma Chemical Co., St. Louis, MO. Pentax® bovine albumin was purchased from Miles Laboratories.

Abbreviations: DTT, dithiothreitol; ACAT, acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26); ARAT, acyl-CoA:retinol acyltransferase (EC 2.3.1....); CL 277,082, N-(2,4-difluorophenyl)-N-(4-neopentylbenzyl)-N-(*n*-heptyl)urea; SMC, smooth muscle cells.

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CL 277,082 was synthesized by Mr. M. Dutia, Lederle Laboratories, Pearl River, NY. All other chemicals used were standard high purity commercial materials.

Preparation of microsomes

Male New Zealand rabbits were fed a 1% cholesterol-supplemented rabbit chow diet for 4 weeks. The rabbits were killed with an overdose of sodium pentobarbital. Microsomes from intestinal mucosal cells were prepared by removing a 50-cm segment of jejunum starting 20 cm from the stomach. The intestine was opened longitudinally, rinsed in cold Dulbecco's phosphate-buffered saline (Grand Island Biological Co.) and the mucosa was scraped off using a rubber spatula. The mucosa was homogenized in 10 ml of cold 0.25 M sucrose buffer, containing 1 mM ethylenediaminetetraacetic acid, 5 mM dipotassium hydrogen phosphate, pH 7.4, and 1 mM dithioerythritol, using a motor-driven Teflon pestle homogenizer (Kontes). The homogenate was filtered through surgical gauze and centrifuged at 15,000 *g* for 15 min. The supernatant was again filtered through surgical gauze and centrifuged at 100,000 *g* for 60 min at 4°C. The microsomal pellet was resuspended in 5 ml of sucrose buffer and centrifuged at 100,000 *g* for 30 min. The washed microsomal pellet was then resuspended in 3 ml of sucrose buffer to give a protein concentration of 4–8 mg protein/ml. Rabbit liver microsomes were prepared in the same manner using 2 g of the large lobe of the liver which was minced and homogenized in 10 ml of sucrose buffer. Microsomes were stored at −70°C until assayed.

Enzyme assays

ACAT assay. The ACAT enzyme activity was assayed using the method described by Hashimoto, Dayton, and Alfin-Slater (8). The reaction mixture included 0.5 ml of potassium hydrogen phosphate buffer (0.2 M; pH 7.4) which contained 15 mg/ml of bovine serum albumin (Pentax, Miles Laboratories, Inc.), 0.02 ml [^{14}C]palmitoyl coenzyme A (55.4 mCi/mmol, New England Nuclear) and 0.03 ml of microsome suspension (150–250 μg protein/assay).

CL 277,082 was dissolved in methanol at concentrations such that addition of 0.003 ml of the solution would yield the final desired concentration. These solutions were added to the buffer and microsomes and then preincubated for 10 min at 37°C. Control tubes received methanol only. The reaction was started by the addition of [^{14}C]palmitoyl CoA and the reaction was run for 4 min at 37°C in a shaker bath. The reaction was stopped by the addition of 1 ml of ethanol. The lipids from the reaction mixture were extracted by the addition of 3 ml of petroleum ether and vortexing. The lipids were separated using silica gel G thin-layer chromatography with a developing solvent of hexane–diethyl ether–acetic acid 80:16:4 and the cholesteryl ester band (R_f = 0.94) was identified with iodine vapor. The cholesteryl esters were assayed using scintillation counting. Extraction efficiency of ^{14}C-labeled cholesteryl ester added to microsomes was 89–95%.

Calculations of IC_{50} values were made using data from three individual experiments at various drug concentrations in which duplicate assay tubes were used at each concentration. The percent inhibition at each drug concentration was the average of the three experiments. IC_{50} values were then derived by linear regression analysis of the data.

ARAT assay. Acyl CoA:retinol acyltransferase was assayed by a method described previously (30). Microsomes isolated from rabbit jejunum mucosa were used in the assay. The reaction mixture contained 0.45 ml K_2HPO_4 buffer (pH 7.4) containing 2 mg/ml BSA, 15 μM retinol, and 20 μM [1-^{14}C]palmitoyl CoA. CL 277,082 was added by first dissolving the drug in methanol and adding 0.003 ml/tube to give the final desired concentration. Control tubes contained methanol without drug. The reaction was linear for at least 20 min at 37°C in a shaker bath. The reaction was stopped with 1 ml ethanol and retinyl [^{14}C]palmitate and cholesteryl [^{14}C]palmitate were extracted as described in the ACAT assay. The two radioactive products could be isolated on silica gel G plates (Analtech, Inc., Newark, DE) using hexane–diethyl ether–acetic acid 90:10:1 as the developing solvent. Cholesteryl oleate and retinyl palmitate were added as carrier for detection by iodine vapors. R_f values using this system were cholesteryl ester, 0.66; retinyl palmitate, 0.55; triglycerides, 0.19; and cholesterol, 0.05.

LCAT assay. Serum lecithin:cholesterol acyltransferase (EC 2.3.1.43) was assayed using the method described by Stokke and Norum (31). [^{3}H]Cholesterol was purified by TLC and eluted in acetone. This solution (0.5 ml) was added slowly with constant stirring to 5 ml of a bovine serum albumin solution (250 mg in 0.2 M K_2HPO_4 buffer, pH 7.1). This solution was placed under a stream of nitrogen until the odor of acetone was no longer detectable. The assay contained: 0.1 ml serum (cynomolgus monkey); 0.03 ml [^{3}H]cholesterol-albumin emulsions; 0.02 ml HDL (monkey), containing 60 μg cholesterol; and 0.002 ml methanol containing CL 277,082. Control tubes received methanol without drug. Assay was run for 1 h at 37°C in a shaker bath. The reaction was stopped by the addition of 0.5 ml ethanol and the [^{3}H]cholesteryl ester extracted and isolated by TLC as described in the ACAT assay. The [^{3}H]cholesterol esterification rate was linear for up to 3 h. At 1 h, 7.7% of the added [^{3}H]cholesterol was esterified in control tubes.

Cholesterol esterase assay. Porcine pancreas cholesterol esterase (Sigma, EC 3.1.1.13) was assayed for cholesterol esterification using the method described by Lange (32). Vesicles containing 5.0 μmol/ml phosphatidylcholine,

2 μmol/ml cholesterol, 6 μmol/ml oleic acid, and 1 μCi/ml [1-^{14}C]oleic acid were formed by homogenization with a Polytron® homogenizer in 50 mM potassium phosphate buffer (pH 6.0). The assay tubes contained 1.0 ml of vesicles, 0.2 ml sodium taurocholate (24 mM), 0.4 ml cholesterol esterase (150 μg protein), and 0.03 ml methanol containing CL 277,082. The tubes were incubated for 2 h at 37°C in a shaker bath and the reaction was stopped with the addition of 1 ml of ethanol. The cholesteryl oleate was isolated and counted by the procedure described for the ACAT assay.

ACAT in smooth muscle cells in culture

Smooth muscle cells (SMC) were cultured from thoracic aortas of African Green monkeys as described by Ross (33). Low density lipoproteins (LDL) were isolated from the serum of hypercholesterolemic cynomolgus monkeys, by addition of sodium bromide and centrifugation (34). The 1.017–1.063 g/ml density range was isolated as LDL. Cationized LDL (C-LDL) was prepared using 3-dimethylaminopropylamine (Aldrich) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Pierce Chemical Co.) according to the method of Basu et al. (35).

Smooth muscle cells were cultured in 35-cm^2 culture wells (Costar) for 48 h using Dulbecco's modified Eagle's medium and 10% fetal calf serum (GIBCO, Grand Island, NY) with LDL plus graded concentrations of CL 277,082 dissolved in methanol. Albumin-bound [^{14}C]oleate, prepared by the method of St. Clair and Leight (36) from albumin (Pentax®) and [^{14}C]oleate (New England Nuclear), were added and the cells were incubated for 2.5 h. Cells were removed from the culture dishes with trypsin–ethylenediaminetetraacetic acid and lipids were extracted using the Folch procedure (37). Cholesteryl ester was isolated by thin-layer chromatography and the cholesteryl ester was counted by liquid scintillation spectrometry.

Cholesterol-fed rats

Male rats (Sprague-Dawley, Charles River Farms) were fed a 1% (w/w) cholesterol–0.5% (w/w) cholic acid diet with CL 277,082. The cholesterol (Baker) and CL 277,082 were dissolved in chloroform and dispersed in a ground Purina rat chow diet. The chloroform was allowed to evaporate in a hood for 2 days and the cholic acid (Aldrich) was dry mixed into this feed.

Serum cholesterol and triglycerides were assayed by enzymatic techniques using a Centrifichem System 400 Autoanalyzer (Union Carbide Co.) and liver cholesterol was assayed by saponification of liver, extraction of cholesterol into organic solvent, and colorometric analysis of cholesterol (37–39).

RESULTS

Kinetic properties

The results of inhibition of ACAT from different tissues are shown in **Fig. 1.** In broken cell preparations, mucosal microsomes from rabbit jejunum were the most sensitive to I with an IC_{50} of 0.14 μM, followed by rabbit liver, 0.74 μM, and rat adrenal, 1.18 μM. When monkey aortic smooth muscle cells were exposed to I for 48 h, the inhibition of [^{14}C]oleate incorporation into cholesteryl ester resulted in an IC_{50} of 0.89 μM.

Kinetic studies indicated that inhibition of intestinal ACAT is noncompetitive with respect to palmitoyl CoA (**Fig. 2**). The apparent K_m for palmitoyl CoA was 2.2 μM and increasing concentrations of substrate could not reverse the inhibition of I. The K_i was 0.145 μM when estimated using the Dixon plot (40). We could observe no difference in the inhibitory activity of I or in the K_m with either palmitoyl CoA or oleoyl CoA as substrates. Since palmitoyl CoA is a more stable molecule in storage, it was routinely used for in vitro studies.

The inhibition of I appears to be reversible in that ACAT activity in liver microsomes inhibited by I can be restored by resuspending in BSA buffer and isolating the microsomal pellet by centrifugation. Three such resuspensions reversed an initial 82% inhibition to 35% inhibition (**Fig. 3**). Control microsomes, without drug, were run in parallel with no change in activity.

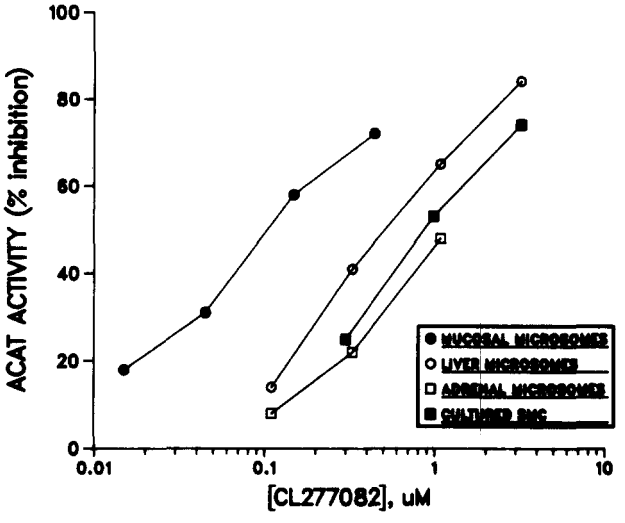

Fig. 1. Effect of CL 277,082 (I) on ACAT. I was tested for inhibition of ACAT from rabbit mucosal microsomes, rat liver, rat adrenal, and monkey thoracic aorta smooth muscle cells grown in tissue culture. Details of experimental procedure are given in Materials and Methods. IC_{50} values were mucosa, 0.14; liver, 0.74; adrenal, 1.18; and smooth muscle cells, 0.89 μM. The percentage inhibition at each drug concentration was the average of two experiments with duplicate assay tubes for each drug concentration. Values are mean ± SD and IC_{50} values were derived by linear regression analysis of the data.

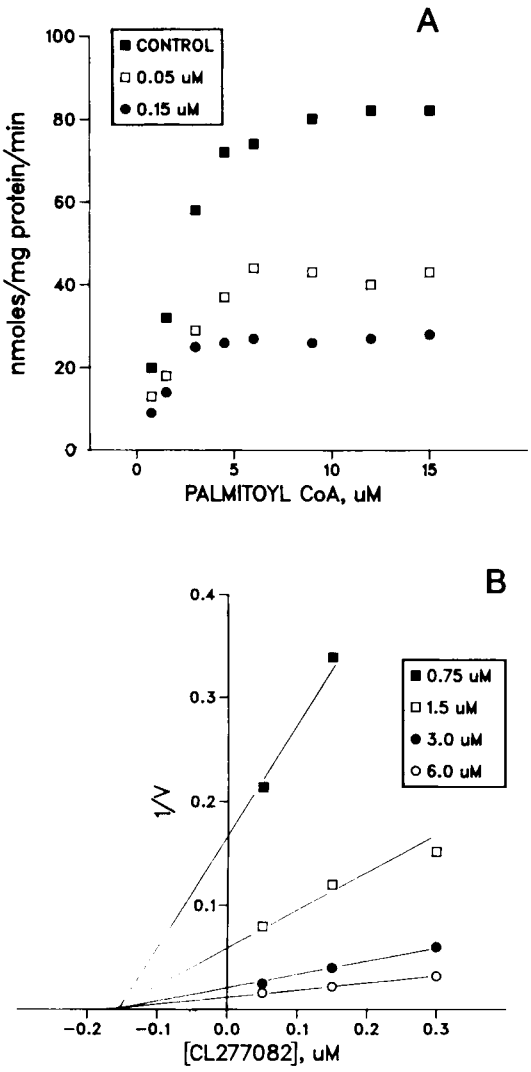

Fig. 2. Effect of I on ACAT from rabbit intestinal mucosal microsomes. A. Effect of I at 0.05 μM, 0.15 μM, and control plotted against increasing concentrations of palmitoyl CoA. Microsomal protein per assay was 100 μg and the apparent K_m for palmitoyl CoA was 2.2 μM. B. Kinetic data using palmitoyl CoA-albumin ratios of 1:1 and the final palmitoyl CoA concentrations were 0.75, 1.5, 3.0 and 6.0 μM. Microsomal protein concentration was 150 μg/assay tube.

SMC in tissue culture

I was also effective in inhibiting ACAT activity in cultured monkey aortic smooth muscle cells (SMC). Cells exposed to cationized-LCL (C-LCL) and I for 48 h resulted in inhibition of ACAT activity with an IC_{50} of 0.89 μM (Fig. 1). In other studies, cells were exposed to C-LDL for 48 h to elevate ACAT activity, then exposed to 5 μM of I. This resulted in a rapid inhibition of [^{14}C]oleate incorporation into cholesteryl ester within 2 h with no inhibition of [^{14}C]oleate incorporation into the triglyceride or phospholipid fraction (**Fig. 4**). There was a significant increase in [^{14}C]oleate incorporation into the triglyceride and phospholipid fraction after a 2-h exposure to I, but this elevation returned to control values after 4 and 24 h. This appears to be a transient event and may be due to an increase in [^{14}C]oleate available for esterification into the triglyceride and phospholipid fractions with the inhibition of esterification into the cholesteryl ester fraction. Also, the decrease in [^{14}C]oleate into cholesteryl ester is not due to an inhibition of the albumin–oleate complex into the cells since incorporation into the triglyceride and phospholipid fractions, which represents 86% of oleate esterification into lipid, was not inhibited.

The effects of I on ACAT in SMC could be reversed by removal of I from the media. Cells were exposed to C-LDL and C-LDL plus I (5 μM) for 48 h (Fig. 4). There was a 79% decrease in ACAT activity after 48 h exposure to I. Removal of I and washing of cells with media resulted in a significant increase in ACAT activity within 2 h which continued to increase 24 h after removal of I.

In these experiments, I did not inhibit the uptake of C-LDL by the SMC since I did not decrease the cholesterol content (**Table 1**). The addition of C-LDL for 48 h elevated total cholesterol 3.6-fold, free cholesterol 1.5-fold, and cholesteryl ester by 18.5-fold over control cells. Addition of I to C-LDL-treated cells resulted in a further 12% increase in total cholesterol, 32% increase in free cholesterol, and no change in cholesterol ester (Table 1).

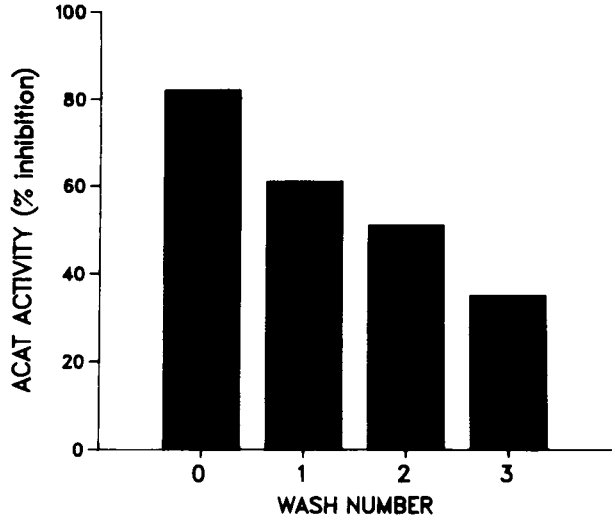

Fig. 3. Reversibility of I on ACAT activity in rabbit liver microsomes. Microsomes (2.8 mg/ml) were incubated with I (1.5 μg/ml) in 0.2 M K_2HPO_4 buffer (pH 7.4) containing 2 mg/ml BSA for 10 min at 37°C. A portion of the microsomes was removed for assay (Materials and Methods). The remaining portion of microsomes was diluted with 5 ml of buffer containing BSA, incubated for 5 min at 37°C, and centrifuged at 100,000 *g* for 30 min at 4°C. The microsomal pellet was resuspended in buffer and assayed for ACAT activity. This procedure was repeated three times. Control microsomes, without I, were run in parallel with no change in ACAT activity.

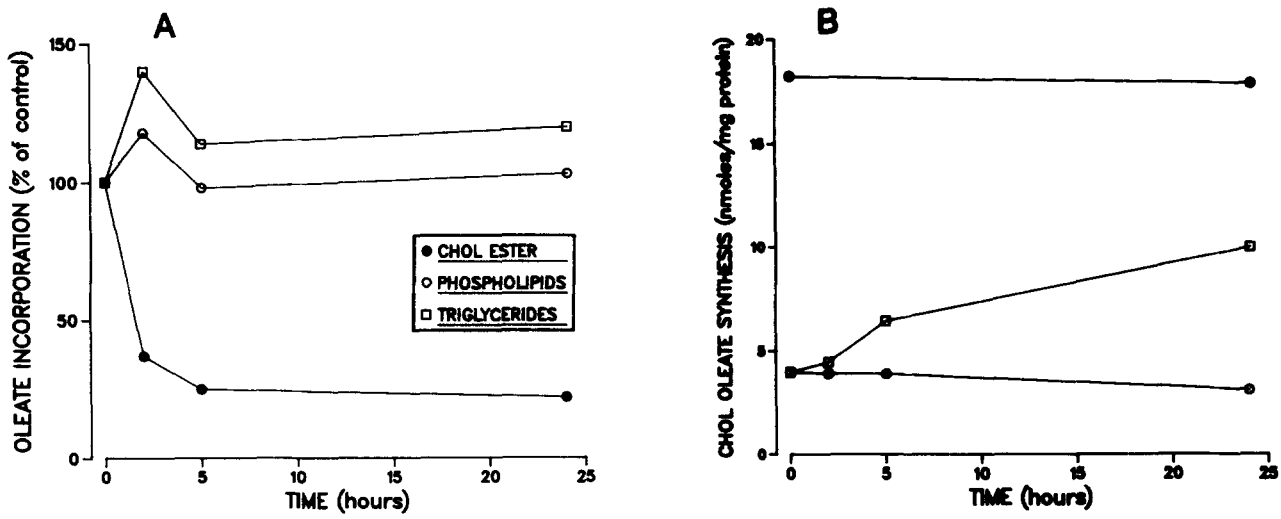

Fig. 4. Effect of I on [^{14}C]oleate incorporation into lipids of SMC in tissue culture. Confluent cultures of SMC from African Green monkey thoracic aorta were exposed to cationized-LDL (C-LDL, 90 μg cholesterol/ml) for 48 h. I (5 μm) was added, along with fresh media containing 10% FCS and C-LDL. Cells were harvested at 2, 5, and 24 h. Incorporation of [^{14}C]oleate into cholesterol, triglycerides, and phospholipids was followed by extracting lipids with Folch reagent followed by isolation on TLC. B. Effect of I on [^{14}C]oleate incorporation into cholesteryl esters. Confluent SMC cultures were exposed to C-LDL (90 μg cholesterol/ml) and I for 48 h. I was then removed (□) or continued (○) for 2, 5, and 24 h in the presence of C-LDL. Control cells (●) were exposed to C-LDL only.

Effect of I on other esterifying enzymes

Lecithin:cholesterol acyltransferase (LCAT). I was tested for effect on LCAT activity in cynomolgus monkey serum. When I was added to serum, there was no decrease in incorporation of [^{3}H]cholesterol into cholesteryl ester fraction with I concentrations up to 300 μM (**Table 2**). This is 2–3 orders of magnitude higher than the concentration needed to inhibit ACAT (Fig. 1). Also, no change in the serum ratio of cholesterol:cholesteryl ester was found in rabbits or monkeys given I for up to 6 months (Largis, E. E., C. H. Wang, V. G. DeVries, and S. A. Schaffer, unpublished observations).

Porcine pancreatic esterase (PCE). Cholesterol esterase from the pancreas has been shown to reversibly form cholesteryl esters from cholesterol, and in the presence of bile acids and at an acid pH. It has been proposed that this may be an important mechanism mediating cholesterol absorption from the gut (41). When I was tested for effects on PCE, there was no inhibition of cholesteryl ester formation with concentrations of I up to 100 μM (**Table 3**).

Acyl CoA:retinol acyltransferase (ARAT). I was tested for inhibition of the enzyme responsible for absorption of retinol from the gut. The activity of ARAT (250 nmol/min per mg protein) was approximately twice the ACAT activity (130 nmol/min per mg protein) when assayed in

TABLE 1. Cholesterol content of aortic smooth muscle cells exposed to I (5 μM) and C-LDL for 48 h

	Cholesterol (Mean ± SEM)		
	Total	Free	Ester
		μg/mg protein	
Control	29.2 ± 1.4	25.7 ± 0.5	3.5 ± 1.5
C-LDL	105 ± 8[a]	40 ± 5[a]	65 ± 5[b]
C-LDL + I	118 ± 10[c]	53 ± 5[d]	65 ± 6[b]

Confluent smooth muscle cells in tissue culture were exposed to C-LDL (90 μg/ml cholesterol) and I (5 μM) for 48 h. Cells were harvested and cholesterol was assayed as described in Materials and Methods.
[a] $P < 0.001$ compared to control cells.
[b] $P < 0.0001$ compared to control cells.
[c] $P = 0.041$ compared to C-LDL cells.
[d] $P = 0.0018$ compared to C-LDL cells.

TABLE 2. Effect of I on lecithin:cholesterol acyl transferase (LCAT) in monkey serum

Treatment	Drug Concentration	[^{3}H]Cholesteryl Ester	% of Total [^{3}H]Cholesterol
	μM	*cpm/assay*	
Control		36,875 ± 2,300 (SEM)	6.4
CL 277,082	30	34,050[a]	6.1
CL 277,082	100	34,450[a]	6.2
CL 277,082	300	30,025[a]	5.5

Assay conditions and isolation of [^{3}H]cholesterol are described in Materials and Methods.
[a]Mean of duplicate values.

TABLE 3. Effect of I on cholesterol esterase from porcine pancreas

CL 277,082 Concentration	Cholesteryl Ester[a]
μM	*μmoles/mg protein*
0	257
10	218
30	215
100	227

[a]Mean of duplicate values that did not vary by more than 10%. Assay conditions and isolation of cholesteryl ester are described in Materials and Methods.

mucosal microsomes from rabbit jejunum. Concentrations of I that produced 90–100% inhibition of ACAT have no effect on ARAT (**Fig. 5**).

Cholesterol absorption in rats

When administered to rats fed a 1% cholesterol–0.5% cholic acid-supplemented diet for 2 weeks, I inhibited elevations in both liver and serum cholesterol with an ED_{50} of 5.4 mg/kg per day (**Table 4**). I did not affect food consumption. Triglycerides were decreased by 36% at the high dose.

When [^{14}C]cholesterol was administered to rats fed a 1% cholesterol–0.5% cholic acid-containing diet for 7 days, there was a large reduction in [^{14}C]cholesterol in the serum of I-treated rats (**Fig. 6**) when measured at 2, 4, 6, 8, and 24 h post-dosing. Fecal analysis revealed an 80% increase in neutral sterol excretion in the first 24 h after an oral dose of [^{14}C]cholesterol (Fig. 6). The percentage of the administered dose appearing in the liver 2 days after dosing was decreased by 70% in I-treated rats, and by 6 days the liver [^{14}C]cholesterol was decreased 90% from controls (**Table 5**). When jejunal and ileal portions of the intestine were analyzed for total cholesterol and [^{14}C]cholesterol, there was no increase in either cholesterol content or in [^{14}C]cholesterol levels in I-treated compared to control rats (Table 5). Therefore, I did not cause an accumulation of cholesterol in these intestinal segments in rats.

DISCUSSION

The kinetics of I appear to be noncompetitive with respect to acyl CoA, and the inhibition is reversible. There are three lines of evidence supporting this. First, washing microsomes, previously incubated with I, with BSA-containing buffer can reverse the inhibition. Second, inhibition of ACAT in SMC in tissue culture can be reversed by removing drug from the incubation medium. Third, inhibition of cholesterol absorption in rats can be reversed when I is removed from the diet (Largis, E. E., C. H. Wang, V. G. DeVries, and S. A. Schaffer, unpublished observations).

While the kinetic data indicate that I is a noncompetitive inhibitor with respect to acyl-CoA, such data should be viewed with caution since the assay involves an impure enzyme preparation. ACAT is not the only enzyme binding the acyl CoA substrate, and binding of acyl CoA to a second protein could obscure the kinetic data. Acyl CoA hydrolase has been reported to be present in microsomes in liver and intestine (42, 43) as well as triglyceride esterification enzymes (28) and ARAT (17). Also, while microsomes used in these studies were from cholesterol-fed rabbits, the cholesterol available to the ACAT enzyme may not be saturating, and an accurate evaluation of ACAT inhibitors may have to await testing on purified preparations of the enzymes.

I appears to be specific in inhibiting ACAT-catalyzed cholesterol esterification since it did not inhibit LCAT or

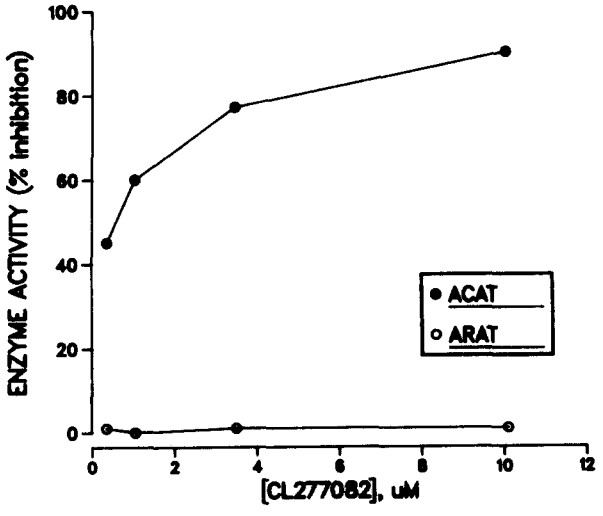

Fig. 5. Effect of I on ACAT and ARAT activities in mucosal microsomes from rabbit jejunum. Each point is the mean of triplicate tubes which were within 10% of the mean value.

TABLE 4. Effect of I on liver and serum lipids in rats fed a 1% cholesterol-0.5% cholic acid diet for 7 days

	Dose	AFC	Liver Cholesterol	Serum Cholesterol	Triglyceride
	% diet		*mg/g*	*mg/dl*	
Control		21	24.1 ± 1.5	352 ± 85	120 ± 37
CL 277,082	0.01	20	5.9[a] ± 0.9	84[a] ± 2	77 ± 29
CL 277,082	0.003	21	15.6[a] ± 1.1	211[b] ± 44	121 ± 16
CL 277,082	0.001	20	23.0 ± 2.8	357 ± 67	132 ± 20

Rats (six/group) were housed in pairs and food consumption was measured daily. Data are expressed as mean ± SD; AFC, average daily food consumption (g/rat per day).
[a] $P < 0.002$.
[b] $P < 0.02$.

pancreatic cholesterol esterase (PCE). It has been suggested that PCE promotes uptake of cholesterol by mucosal cells via cholesteryl ester synthesis on the luminal surface and hydrolysis on the cytoplasmic side (44, 45). ACAT would then esterify the free intracellular cholesterol for transport into chylomicrons. It is clear from our data that I does not inhibit the synthetic activity of PCE and probably does not inhibit the esterase activity since I inhibited the absorption of dietary cholesterol and cholesteryl oleate to the same degree in rats (Largis, E. E., C. H. Wang, V. G. DeVries, and S. A. Schaffer, unpublished observations). Considering that I is a potent inhibitor of ACAT and the lack of inhibition seen on PCE, the role of PCE in cholesterol transport under conditions of high cholesterol-containing diets is questionable.

The specificity of I is also shown by a lack of its effect on other cellular acyltransferases. Acyl CoA:retinol acyltransferase (ARAT) is located in microsomes of intestinal mucosa (30) and liver (28) and has many properties in common with ACAT (30). However, dietary manipulations

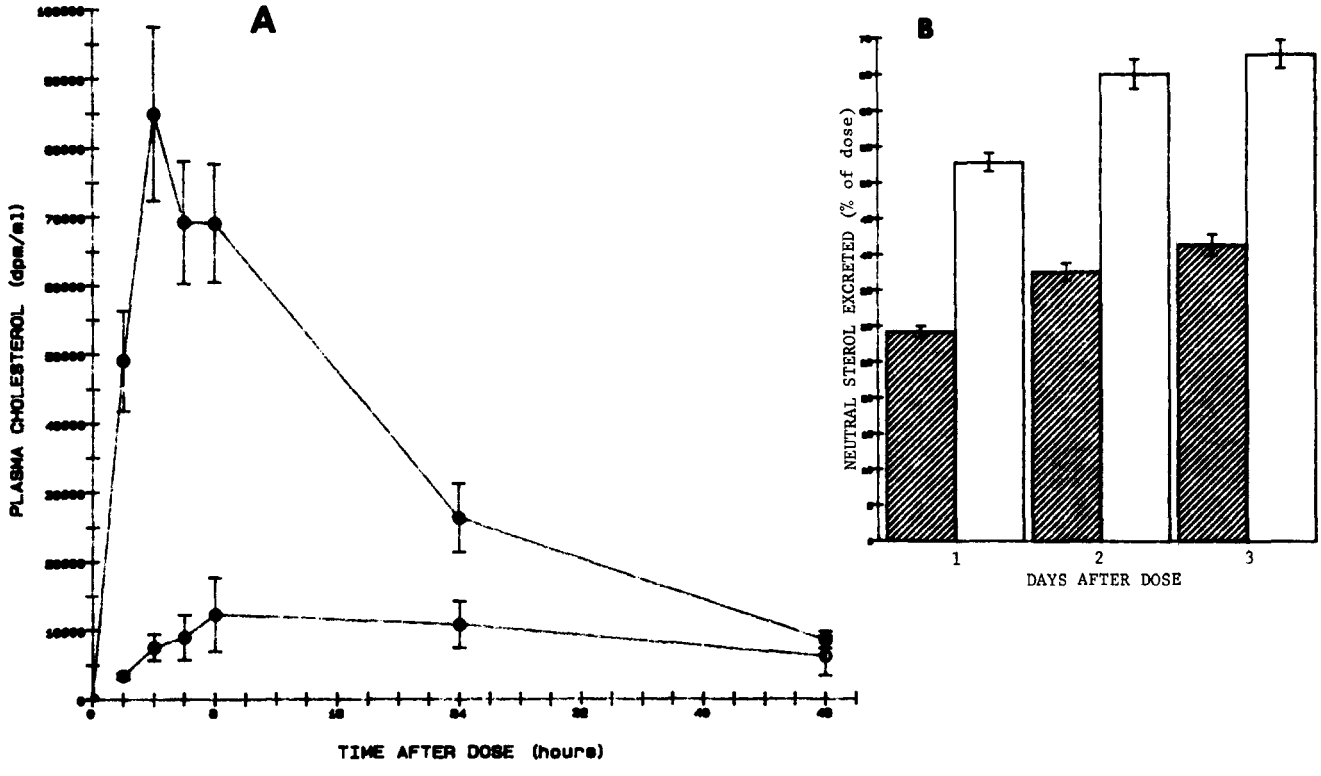

Fig. 6. Effect of I on [^{14}C]cholesterol levels in serum and ^{14}C-labeled neutral sterol excretion in feces from rats. Rats were fed a 1% cholesterol 0.5% cholic acid diet for 7 days. Drug-treated rats received I in the diet at 0.01%. Rats were given 5 mg/kg [4-^{14}C]cholesterol (3 μCi/mg) via gavage. Rats were maintained on the cholesterol diet. A. Serum [^{14}C]cholesterol. Control (●) and I treated (○) rats. B. Cumulative fecal ^{14}C-labeled neutral sterol. Control (hatched bars) and I treated (open bars) rats.

TABLE 5. Effect of I on uptake of an oral dose of [^{14}C]cholesterol in rat liver and intestine

	Days After Dose	Liver		Intestine			
				Jejunal		Ileal	
		Cholesterol	[^{14}C]Cholesterol	Cholesterol	[^{14}C]Cholesterol	Cholesterol	[^{14}C]Cholesterol
		mg/g	*% dose*	*mg/g*	*cpm/g*	*mg/g*	*cpm/g*
Control[a]	2	28.7 ± 5.6	31.9 ± 4.8	3.2 ± 0.2	9,013 ± 480	3.3 ± 0.4	10,746 ± 1,964
Control	6	40.7 ± 5.7	28.5 ± 5.9				
I[b]	2	5.7 ± 1.9[c]	6.8 ± 3.2[c]	3.3 ± 0.6	10,411 ± 3,695	3.1 ± 0.8	11,393 ± 2,428
I	6	7.4 ± 1.6[c]	2.8 ± 1.3[c]				

[a]Rats fed diet of 1% cholesterol:0.5% cholic acid for 7 days and then given an oral dose of 10 mg/kg [4-^{14}C]cholesterol (1.6 Ci/mg). Rats continued on the cholesterol diet for another 6 days.
[b]Same protocol as above (*a*) with addition of I mixed in the diet at 0.01%.
[c]$P < 0.005$ compared to control.

of cholesterol and fat in the diet modified ACAT activity in intestinal and hepatic microsomes, but did not have parallel effects on ARAT (21). Our findings that I is a potent inhibitor of intestinal ACAT activity without inhibiting ARAT further demonstrate differences between these two acyltransferase enzymes.

A number of compounds have been reported to inhibit ACAT. Bell (46) has reported that a number of local anesthetics, such as lidocaine, inhibit ACAT activity over a concentration range of 0.25–5.0 mM. Major tranquilizers, such as chlorpromazine, were reported to be somewhat more potent with an IC_{50} of 0.1 mM in rabbit arterial microsomes (25). The hypolipidemic agents, bezafibrate and clofibrate, have also been shown to inhibit cholesterol esterification in cultured SMC, fibroblasts, and macrophages (26, 47). Like the anesthetics, these hypolipidemics have IC_{50} values in the range of 0.5–2 mM. More potent inhibitors of ACAT include progesterone (27) and the experimental drugs 57-118 (18), 58-035 (28), and melinamide (48). Of all these compounds, only 57-118, 58-035, and melinamide have IC_{50} values that are below 1 μM and have also been shown to inhibit cholesterol absorption in rabbits (18) and rats (44, 49). We have observed that a requirement for an ACAT inhibitor to be effective at inhibiting cholesterol absorption is that the compound must have an IC_{50} of less than 1 μM when tested on intestinal mucosa microsomes. Also, as can be seen in Fig. 1, an inhibitor can have a wide range of IC_{50} values on ACAT from different tissues ranging from 0.14 μM in intestine to 1.18 μM in adrenal. Such wide variation of inhibition on the same enzyme would suggest that ACAT may have individual properties depending on the source of the enzyme. Indeed, incubation of bovine adrenal cortical microsomes with ATP/$MgCl_2$, in the presence of 100,000 *g* supernatant, will decrease ACAT activity (50), while the same experimental procedures will increase ACAT activity in intestinal and liver microsomes (14, 15, 50). This would suggest that ACAT may be regulated differently in various tissues, and indeed, different ACAT isozymes may exist.

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