ACAT inhibitors CL 283,546 and CL 283,796 reduce LDL cholesterol without affecting cholesterol absorption in African green monkeys

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Abstract Previous studies with a number of selective acyl-coenzyme A:cholesterol acyltransferase (ACAT) inhibitors in several animal models have demonstrated significant reductions in plasma cholesterol and, in some studies, triglyceride levels. This study was conducted to examine the effects of two ACAT inhibitors, CL 283,546 and CL 283,796, in cholesterol-high fat diet fed African green monkeys, a relevant primate model of hyperlipidemia and coronary artery atherosclerosis. Treatment with CL 283,546 or CL 283,796 resulted in significant reductions (ca. 25–30%) in total plasma cholesterol at both 10 and 30 mg/kg per day doses. This reduction in plasma cholesterol was due almost entirely to reduction in low density lipoprotein (LDL) cholesterol (ca. 45%) without significantly affecting high density lipoprotein (HDL) cholesterol, very low density lipoprotein + intermediate density lipoprotein (VLDL + IDL) cholesterol, or triglyceride concentrations. There were no significant effects on plasma concentrations of apolipoproteins A-I, E, or B and, thus, the reduction seen in LDL cholesterol appears to be due to a diminished cholesterol content of LDL particles. Our studies revealed that treatment with these compounds did not reduce cholesterol absorption, which was somewhat surprising as ACAT inhibitors are generally thought to exert their hypolipidemic effects, at least in part, by inhibition of intestinal cholesterol absorption. Our data are consistent with a principal activity of these drugs on the liver to reduce cholesteryl ester secretion in VLDL, leading to a diminished LDL-cholesterol content, and, presumably, enhanced biliary cholesterol-bile acid excretion.—**Wrenn, S. M., Jr., J. S. Parks, F. W. Immermann, and L. L. Rudel.** ACAT inhibitors CL 283,546 and CL 283,796 reduce LDL cholesterol without affecting cholesterol absorption in African green monkeys. *J. Lipid Res.* 1995. **36:** 1199–1210.

Supplementary key words mechanisms • apoB • apoA-I • apoE • LDL • HDL • triglycerides

The enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT, E.C. 2.3.1.26) appears to play an important role in cholesterol homeostasis (1), with activity on pathways of cholesterol absorption, secretion, and metabolism. In the intestine, ACAT is involved in chylomicron assembly and secretion and, thus, directly affects cholesterol absorption (2–14). By a related mechanism in the liver, ACAT has been shown to be involved in the assembly and secretion of apoB-100-containing lipoproteins (15–17) and is undoubtedly responsible for the majority of cholesteryl esters contained in VLDL. It has been reported that mRNA for apoB in HepG2 cells is constitutively expressed (18–20) and apoB that is not associated with lipid is degraded (19, 21, 22). Inhibition of ACAT in HepG2 cells has been shown to reduce the secretion of apoB (16–18) and addition of oleate, the preferred substrate for ACAT as oleoyl-CoA (23), has been shown to increase the secretion of apoB and apoB-containing lipoproteins (24), implicating ACAT as a potential regulatory enzyme for the production of hepatic lipoproteins. Some investigators have concluded, however, that apoB secretion from cultured cells may be more closely linked to triglyceride levels than cholesteryl esters (25). Similar findings on the relationship of hepatic cholesteryl esters to apoB secretion have also been obtained from perfused liver studies (15, 26–29) and analogous relationships have been seen for plasma levels of apoB, apoB-containing lipoproteins, and hepatic mRNA for apoB from studies in several species (15, 30–33).

In addition to the role in hepatic and intestinal lipoprotein formation and secretion, ACAT has been clearly associated with the formation of cholesteryl esters

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein.

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and appearance of foam cells at atherosclerotic sites (34–38). Two recent reports (39, 40) have indicated that treatment of rabbits and Yucatan pigs with the ACAT inhibitor CI-976 reduced cholesteryl ester content and accumulation of foam cells without increasing free cholesterol in atherosclerotic lesions, while minimally affecting total plasma cholesterol concentrations, consistent with direct antiatherosclerotic activity.

In view of these complementary activities of ACAT inhibitors, we investigated the activities of both CL 283,546 and CL 283,796 in cholesterol–fat-fed African green monkeys whose cholesterol metabolism and lipoprotein profiles resemble those of humans (41–44) and in which the extent and severity of coronary atherosclerosis have been shown to correlate with plasma LDL concentration and LDL cholesteryl ester content (15). Previous studies with the related compound, CL 277,082, a well-studied inhibitor of ACAT (3, 11, 13, 14, 45, 46), indicated that it was a good hypolipidemic agent that inhibited cholesterol absorption in several species including cynomolgus monkeys (12, 13, and E. E. Largis, A. S. Katocs, Jr., Y. Urano, A. Kato, and S. M. Wrenn, Jr. unpublished results), but not in normolipemic humans (47). Studies with the two compounds used in this study, CL 283,546 (12, 48, and A. S. Katocs, Jr., E. E. Largis, and S. M. Wrenn, Jr. unpublished data) and CL 283,796 (A. S. Katocs, Jr., E. E. Largis, Y. Urano, T. Ishikawa, A. Kato, and S. M. Wrenn, Jr. unpublished data), have demonstrated both to be potent and selective inhibitors of ACAT and have indicated potent hypocholesterolemic activities in rat, rabbit, hamster, and cynomolgus monkey (only CL 283,546 was evaluated in cynomolgus monkeys). Herein, we report the effects of both compounds on plasma lipids, cholesterol absorption, and apolipoprotein concentrations in the African green monkey.

MATERIALS AND METHODS

The ACAT inhibitors CL 283,546, N'-heptyl-N-{[4-(3-methylbutyl)phenyl]methyl}-N'-(2,4,6-trifluorophenyl) urea, and CL 283,796, N' -(4-chloro-2,6-dimethylphenyl)-N-heptyl-N-{[4-(3-methylbutyl)phenyl]methyl} urea, were synthesized at Lederle Laboratories by previously described methods (14). Structures of these compounds are shown in **Fig. 1** in comparison with the related analog CL 277,082.

Study design

Eighteen adult African green monkeys (12 males, 6 females) were used in this study. Prior to initiation of drug treatment, the animals were fed an atherogenic diet containing 35% of the calories as fat and 0.8 mg

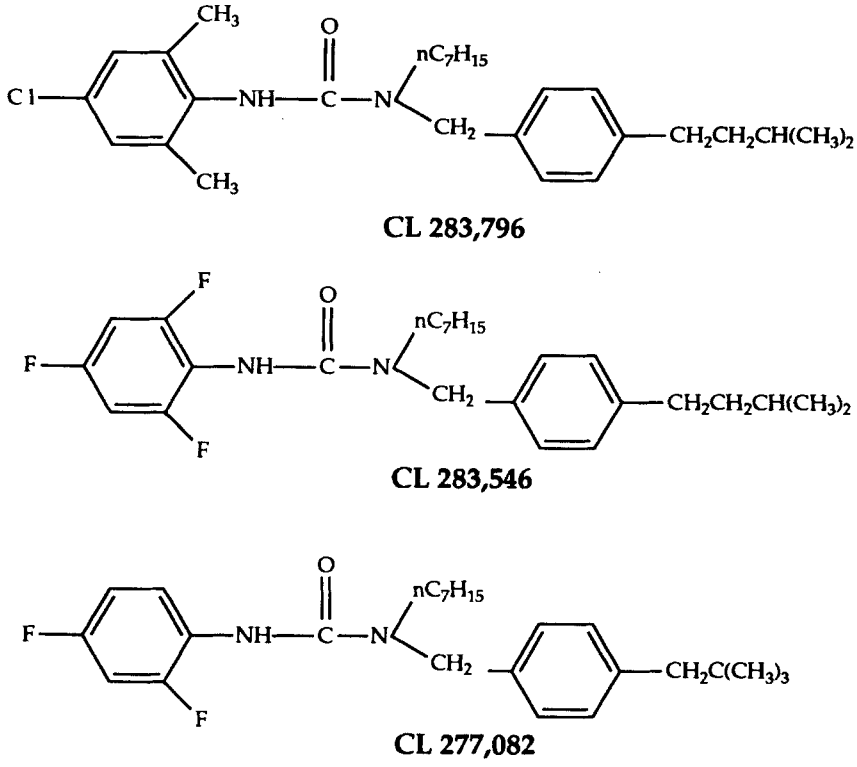

Fig. 1. Structures of the ACAT inhibitors CL 283,546, CL 283,796, and the related analog CL 277,082.

cholesterol/Kcal for approximately 6 weeks. Detailed diet compositions have been previously published (49); the diet used in this study contains lard (15.6 g/100 g of diet) and a small amount of safflower oil (0.8 g/100 g). Food was weighed for feeding and each animal received the equivalent of 90 kcal/kg per day in two meals per day, one in the morning and one in the afternoon. Drugs were admixed during the preparation of the diets and were, thus, administered with each feeding. On specified days, 18-h fasting blood samples were taken for determination of plasma lipids. All food was removed from the cages by 3:30 PM on the day preceding blood collection. At approximately 9:00 AM on the sampling day, animals were restrained with ketamine HCl (10 mg/kg), the skin of the thigh near the groin was swabbed with 70% isopropanol and 10 ml of blood was collected by venipuncture from the femoral vein into EDTA-Vacutainer tubes. Tubes containing blood were placed on ice at the time blood was drawn, after which they were centrifuged for 30 min at 1500 *g*. The plasma was drawn off with a Pasteur pipette and transferred into disposable screw-capped plastic tubes for subsequent analysis. Two weeks and 1 week prior to drug treatment, fasting blood samples were taken for determination of total plasma cholesterol, HDL cholesterol, and triglycerides. Body weight was also determined at this time and on a weekly basis. A fasting blood sample was also taken prior to drug treatment for determination of plasma apolipoprotein concentrations and lipoprotein cholesterol distribution. During the last week of the baseline period a cholesterol absorption study was performed. Animals were then placed into one of three groups: control (group 1), CL 283,546 treatment (group 2), or CL 283,796 treatment (group 3) so that each group contained six monkeys. Animal assignments were made such that the groups had similar mean total plasma cholesterol, HDL cholesterol, and body weight baseline values (**Table 1**).

During the first 5-week test period, animals were fed the high cholesterol–high fat diet alone (group 1), diet plus CL 283,546 at a dose of 10 mg/kg per day (group 2), or diet plus CL 283,796 at a dose of 10 mg/kg per day (group 3). During the next 5-week treatment period (weeks 6–10), group 1 was maintained on diet alone to serve as a reference control, but groups 2 and 3 were given escalated drug doses of 30 mg/kg per day in the same diet. These doses were selected based upon previous studies in cynomolgus monkeys with CL 283,546 (12, 48).

Determinations of total plasma cholesterol, triglycerides, and HDL cholesterol values were made using Lipid Research Clinic methodology (50) on a Technicon RA-500 analyzer (26). Plasma apolipoproteins A-I, E, and B were quantified by the enzyme-linked immunosorbent assays previously described (26, 51). Lipoprotein cholesterol distribution was quantified after separation of plasma lipoproteins by ultracentrifugation and FPLC size exclusion chromatography on Superose 6B (52) as previously described (53). Cholesterol absorption was measured by the dual isotope method of Grundy, Ahrens, and Salen (54). Briefly, a single oral dose of [1,2(n)-^{3}H]cholesterol (0.45 μCi) and [4-^{14}C]β-sitosterol (0.1 μCi) was fed to each animal on a piece of apple. All feces were subsequently collected from each animal over the next 4 days. After blending each fecal sample with a minimum volume of water to assure uniformity, an aliquot was taken for analysis. These aliquots were saponified in ethanolic KOH and neutral sterol was extracted into hexane. After bleaching under UV light, a portion of the hexane solution was taken for liquid scintillation counting to determine the ^{3}H: ^{14}C ratio. Cholesterol absorption was calculated from these values by the method of Borgstrom (55).

Cholesterol absorption was determined on week −1 (predrug dosing), and again at weeks 4–5 (10 mg/kg per day dose) and 9–10 (30 mg/kg per day dose). At weeks 0 (predrug dosing), 5, and 10, fasting blood samples were also taken for assay of plasma apolipoproteins A-I, B, and E and for lipoprotein cholesterol distribution.

Statistical methods

Statistical analyses were performed using analysis of variance (ANOVA) methods for the following response parameters: total plasma cholesterol, plasma triglycer-

TABLE 1. Mean baseline plasma lipid values and body weights

Group	Total Plasma Cholesterol	HDL-C	Triglycerides	Body Weight	Male/Female Distribution
	mg/dl	*mg/dl*	*mg/dl*	*kg*	
1 (n = 6)	384 ± 83	74 ± 7	32 ± 7	4.97 ± 0.38	4/2
2 (n = 6)	396 ± 55	76 ± 5	45 ± 13	4.86 ± 0.42	4/2
3 (n = 6)	386 ± 115	76 ± 4	38 ± 7	4.68 ± 0.72	4/2

All plasma lipid values are for means ± SEM, based on two separate samples for each animal determined within 2 weeks prior to beginning drug treatment; HDL-C, HDL-cholesterol.

TABLE 2. Percent change from baseline body weight in CL 283,546- and CL 283,796-treated African green monkeys

Day	Mean Percent Change Control	CL 283,546	CL 283,796
	%	%	%
1		Start 10 mg/kg dosing	
3	0.4	- 0.2	0.5
11	2.3	0.6	0.7
17	- 1.6	2.7[a]	- 2.4
24	- 0.4	8.8[a]	- 1.0
30	- 0.1	4.0[a]	0.1
36		Start 30 mg/kg dosing	
38	- 2.9	4.1[a]	1.4
45	0.7	5.4	1.6
52	1.1	5.8	2.0
58	1.2	4.2	1.7
66	1.6	5.7	2.3

Mean values are for n = 6 in each group. Percent changes are from baseline values indicated in Table 1.

[a] $P \leq 0.05$; *P*-values from two-sided *t*-tests comparing treatment groups to the control group.

ides, HDL cholesterol, LDL cholesterol, VLDL + IDL cholesterol, apolipoproteins A-I, B, and E, and body weight. Separate ANOVAs were performed for each parameter and sampling time. The dependent variable in the ANOVAs was percent change from baseline; treatment group was the independent variable. Two-sided *t*-tests were used to compare means for groups treated with CL 283,546 or CL 283,796 to control group means. All *t*-statistics were based on error terms from the appropriate ANOVAs.

RESULTS

Effects on body weight

Treatment with CL 283,546 initially resulted in small but statistically significant increases in body weights in comparison with control group values, but treatment with CL 283,796 did not effect body weights (**Table 2**). Overall the effects on body weights were small, and the statistically significant increase associated with CL 283,546 treatment seen in the first few weeks of the study had disappeared towards the end of the study period.

Effects on lipoprotein lipids

Drug treatment with CL 283,796 or CL 283,546 at 10 mg/kg per day resulted, respectively, in 24.1% and 26.2% reductions in total plasma cholesterol after 30 days of dosing (**Fig. 2** and **Table 3**). Onset of drug effects was rapid: significant hypolipidemic effects were seen after treatment for between 1 and 2 weeks at this dose level. Upon increasing the dose to 30 mg/kg per day for an additional 5 weeks, no further decrease in total plasma cholesterol was seen for CL 283,796, but CL 283,546 dose escalation resulted in an approximate 5 to 10% further reduction in total plasma cholesterol levels (Table 3). In the 10 mg/kg CL 283,796 treatment group there was a highly significant correlation of the reduction in total plasma cholesterol of individual animals with baseline cholesterol values (**Fig. 3**). Similar, but insignificant ($P \leq 0.13$), effects were also seen in the 10 mg/kg CL 283,546 treat-

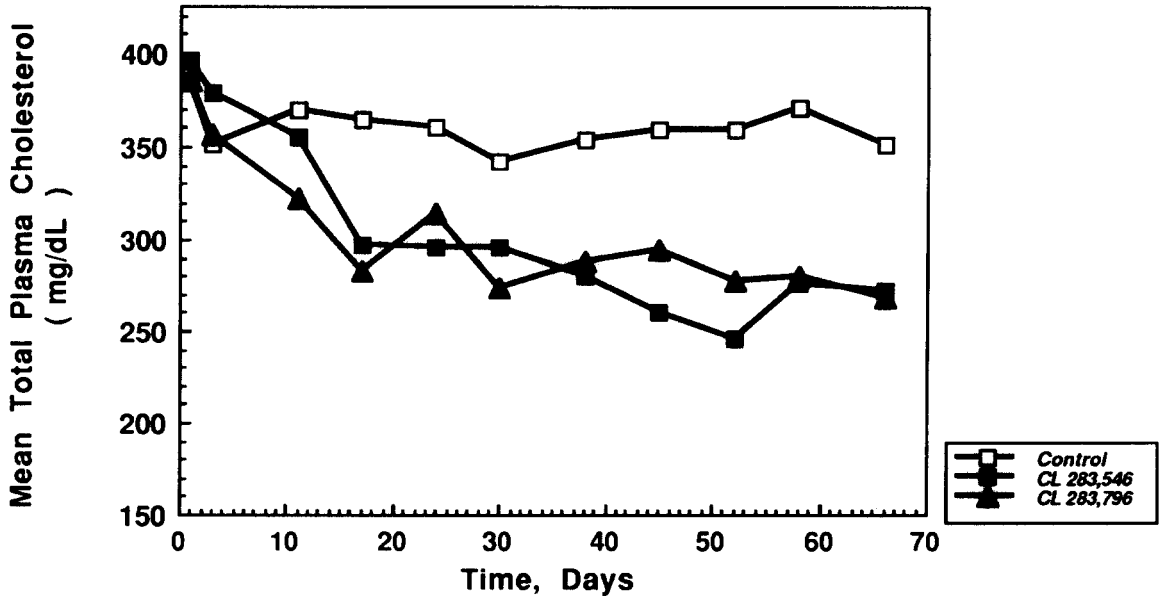

Fig. 2. Values for weekly determinations of total plasma cholesterol in African green monkeys in the three treatment groups. Therapy was begun in groups 2 and 3 on day 1 and was escalated to a higher dose on day 36. More detailed analysis of the data, including SEMs, can be seen in Table 3.

TABLE 3. Effects of CL 283,546 and CL 283,796 on total plasma cholesterol in African green monkeys

Day	Controls Group 1, (n = 6)	CL 283,546-Treated Group 2, (n = 6)	CL 283,796-Treated Group 3, (n = 6)
1 (Start 10 mg/kg/day)[a]	384 ± 83	396 ± 55	386 ± 115
3	351 ± 78	379 ± 46	357 ± 98
11	370 ± 80	355 ± 39	322 ± 91
17	365 ± 77	298 ± 46[b]	283 ± 67[b]
24	361 ± 69	296 ± 43[c]	315 ± 107[b]
30	342 ± 75 (−10.2%)	296 ± 41 (−24.1%)	274 ± 77[b] (−26.2%)
36 - (Start 30 mg/kg/day)			
38	354 ± 83	280 ± 33[c]	288 ± 89[b]
45	359 ± 83	260 ± 42[d]	295 ± 86[b]
52	359 ± 80	246 ± 46[d]	277 ± 80[c]
58	371 ± 89	276 ± 50[d]	280 ± 80[c]
66	351 ± 85 (−8.6%)	272 ± 47[d] (−31.9%)	269 ± 76[c] (−26.9%)

All total plasma cholesterol values are for means ± the standard error of the mean and are in mg/dl. Other values are for percent change from baseline for each group.

[a] Day 1 values are baseline means ± SEM for the previous two values in each group obtained within the 2 weeks prior to initiation of drug dosing.

[b,c,d] *P*-value from two-sided *t*-test comparing treatment group value to control group value: [b], $P \leq 0.05$; [c], $P \leq 0.01$; [d] $P \leq 0.002$.

ment group (Fig. 3). As there was no appreciable difference between the effects and mechanism of each drug, we calculated the correlation for the combined effects of the two individual drugs, which also was significant ($r = 0.759$, $P \leq 0.004$) at 5 weeks. Similar correlation of reduction in plasma cholesterol with baseline cholesterol value was also seen for each compound at the 30 mg/kg dose level (data not shown). Thus, the higher the baseline cholesterol value of an individual animal, the greater the reduction in total plasma cholesterol seen upon drug treatment.

Whether drug effects had reached equilibrium by the end of each dosing period is difficult to determine due to week to week variability in the values, but it appears that a plateau in mean cholesterol concentrations was being

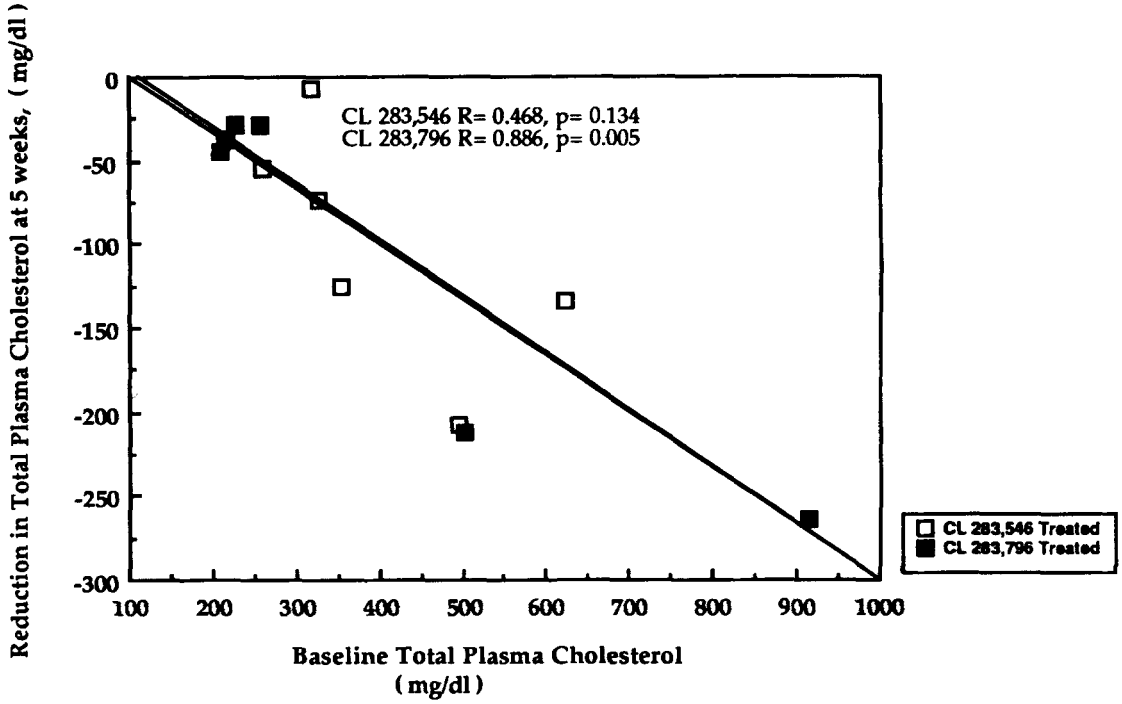

Fig. 3. Correlation of the reduction in total plasma cholesterol at 5 weeks (10 mg/kg treatment) with baseline cholesterol values for individual monkeys. Correlation coefficients and *P*-values are indicated for both the CL 283,796- and CL 283,546-treated groups.

TABLE 4. Effects of CL 283,546 and CL 283,796 on plasma triglyceride levels in African green monkeys

Day	Controls Group 1, (n = 6)	CL 283,546-Treated Group 2, (n = 6)	CL 283,796-Treated Group 3, (n = 6)
1 (Start 10 mg/kg/day)[a]	32 ± 7	45 ± 13	38 ± 7
3	27 ± 7	49 ± 23	26 ± 10
11	33 ± 15	51 ± 20	25 ± 10
17	42 ± 5	73 ± 19	96 ± 37
24	28 ± 5	60 ± 22	36 ± 11
30	37 ± 9 (+15.9%)	73 ± 35 (+32.9%)	30 ± 9 (−20.3%)
36 - (Start 30 mg/kg/day)			
38	31 ± 8	55 ± 20	29 ± 8
45	26 ± 8	69 ± 21[b]	23 ± 7
52	26 ± 8	73 ± 23[c]	21 ± 6
58	25 ± 9	55 ± 20	41 ± 17
66	30 ± 11 (−8.8%)	53 ± 21 (0.0%)	21 ± 5 (−46.3%)

All triglyceride values are for means ± the standard error of the mean and are in mg/dl. Other values are for percent change from baseline for each group.

[a] Day 1 values are baseline means obtained from the determination of two plasma triglyceride values within the 2 weeks preceding initiation of drug dosing.

[b,c] *P*-value from two-sided *t*-test comparing treatment group value to control group value: [b], $P \leq 0.05$; [c], $P \leq 0.01$.

approached near the end of each 5-week dosing period. The protocol design cannot eliminate time and carryover effects from earlier dosing, but as there were generally small differences between the effects of the two doses, these effects are estimated to be small.

Triglyceride values in the treated animals were generally more variable than plasma cholesterol levels (**Table 4**), but there were no consistent and significant effects of drug treatment on plasma triglyceride values in comparison with controls at either dose level. Additionally, there were no statistically significant differences in HDL cholesterol for CL 283,546- or CL 283,796-treated animals at any time points in the treatment period (**Table 5**).

To determine effects of drug treatment on cholesterol content in different lipoprotein classes, plasma from the monkeys was fractionated into LDL, HDL, and VLDL + IDL and cholesterol concentrations were determined for each fraction. For both drugs, the reductions in total plasma cholesterol could be attributed to reduction in LDL cholesterol, with small and statistically insignificant

TABLE 5. Effects of CL 283,546 and CL 283,796 on plasma HDL cholesterol levels in African green monkeys

Day	Controls Group 1, (n = 6)	CL 283,546-Treated Group 2, (n = 6)	CL 283,796-Treated Group 3, (n = 6)
1 (Start 10 mg/kg/day)[a]	74 ± 7	76 ± 5	76 ± 4
3	80 ± 7	74 ± 6	77 ± 5
11	83 ± 10	77 ± 5	77 ± 5
17	70 ± 9	67 ± 4	71 ± 6
24	88 ± 9	89 ± 6	83 ± 7
30	79 ± 9 (+6.2%)	77 ± 6 (+1.8%)	80 ± 6 (+5.4%)
36 - (Start 30 mg/kg/day)			
38	83 ± 10	82 ± 5	87 ± 5
45	94 ± 5	87 ± 5	91 ± 5
52	87 ± 10	84 ± 7	86 ± 6
58	85 ± 10	82 ± 8	84 ± 5
66	87 ± 10 (+17.5%)	88 ± 5 (+18.2%)	88 ± 5 (+15.9%)

All HDL cholesterol values are for means ± the standard error of the mean and are in mg/dl. Other values are for percent change from baseline for each group. There were no significant differences between treated groups and the control group values at any time.

[a] Day 1 values are baseline means obtained from the determination of two plasma HDL cholesterol values within the 2 weeks preceding initiation of drug dosing.

TABLE 6. Effects of the ACAT inhibitors CL 283,546 and CL 283,796 on lipoprotein cholesterol levels in African green monkeys

	LDL Cholesterol			HDL Cholesterol			VLDL ¢ IDL Cholesterol		
Time, Weeks	Control	283,546	283,796	Control	283,546	283,796	Control	283,546	283,796
0 (Baseline)	318 ± 93	314 ± 56	288 ± 93	80 ± 10	86 ± 7	82 ± 4	19 ± 7	13 ± 2	26 ± 7
5 (10 mg/kg)	249 ± 79	204 ± 42	178 ± 77[a]	81 ± 9	80 ± 7	82 ± 6	11 ± 4	12 ± 3	14 ±6
10 (30 mg/kg)	265 ± 96	177 ± 44[b]	171 ± 73[b]	91 ± 11	88 ± 8	86 ± 7	15 ± 4	12 ± 2	23 ± 14

All values are for the mean ± SEM for n = 6 in each group (reported as mg/dl).
P-values are from two-sided *t*-tests comparing treatment groups to control group values: [a]$P = 0.13$, [b]$P = 0.03$.

effects seen on HDL cholesterol or VLDL + IDL cholesterol values (**Table 6**). LDL cholesterol concentrations, in comparison with controls, were significantly reduced only at the 30 mg/kg dose for each drug (Table 6), whereas significant reductions in total plasma cholesterol were seen at both the 10 mg/kg and 30 mg/kg doses (Table 3). This difference between effects on LDL and total plasma cholesterol may be due (in whole or in part) to the larger decrease from baseline observed in control animals for LDL cholesterol. The reduction in LDL and slight increase in HDL cholesterol resulted in favorable increases of 81% and 76%, respectively, in the HDL/LDL cholesterol ratios in the CL 283,546- and CL 283,796-treated animals, whereas the ratio in the control animals increased by 37% (from Table 6). Similarly, the TPC/HDL cholesterol ratios decreased by 41% and 40%, respectively, after 10 weeks in the CL 283,546- and CL 283,796-treated monkeys (from Tables 3 and 5).

Effects on apolipoproteins

As there were differential effects of drug treatment on the cholesterol concentrations of various lipoproteins, we measured the apolipoprotein concentrations to determine whether this was due to a diminished lipoprotein plasma concentration or reduced cholesterol content of each lipoprotein fraction. Apolipoproteins A-I, B, and E were measured in the plasma of control and treated animals prior to initiation of drug dosing and near the completion of each 5-week dosing period. ApoA-I values increased from baseline in control and both drug treatment groups. These results indicate no significant effect of drug treatment on apoA-I levels, which is consistent with the lack of effect of both drugs on HDL cholesterol values at equivalent treatment times (Tables 6 and 7).

As seen in **Table 7,** when comparisons were made for apoB and apoE values between these groups, there were no statistically significant effects of drug treatment. Thus, we found no correlation of apolipoprotein B or E values with the reduction observed in LDL cholesterol in the drug-treated monkeys.

Effects on cholesterol absorption

As most of the published data on ACAT inhibitors, including our findings with these two compounds in rat and rabbit and CL 283,546 in the cynomolgus monkey (A. S. Katocs, Jr., E. E. Largis, and S. M. Wrenn, Jr., unpublished data), indicated reductions in systemic cholesterol absorption, we examined the effect of both CL 283,546 and CL 283,796 on cholesterol absorption in the African green monkey. As seen in **Table 8,** at times near the end of each dosing period, there was no significant effect of treatment on cholesterol absorption, which was somewhat surprising as we had expected inhibition of cholesterol absorption to be one of the major effects of these drugs. Several possible interpretations of these results are discussed below.

TABLE 7. Effects of the ACAT inhibitors CL 283,546 and CL 283,796 on apolipoprotein A-I, B, and E values in African green monkeys

	ApoA-I			ApoB			ApoE		
Time Weeks	Control	283,546	283,796	Control	283,546	283,796	Control	283,546	283,796
0 (Baseline)	230 ± 35	264 ± 14	247 ± 17	146 ± 31	144 ± 39	92 ± 15	9.5 ± 3.2	8.7 ± 1.0	9.3 ± 3.9
5 (10 mg/kg)	318 ± 17	337 ± 42	364 ± 44	136 ± 28	117 ± 18	82 ± 25	6.6 ± 1.9	4.2 ± 0.3	5.6 ± 2.1
10 (30 mg/kg)	300 ± 39	285 ± 30	361 ± 23	132 ± 30	108 ± 26	75 ± 24	5.8 ± 1.6	4.7 ± 0.8	5.3 ± 2.1

All values (mg/dl) are for mean ± SEM for n = 6 in control and CL 283,796 groups and n = 5 for the CL 283,546 group. *P*-values from two-sided *t*-tests comparing treatment group to control group values. All *P*-values are insignificant: $0.16 \leq P \leq 0.96$.

TABLE 8. Effect of ACAT inhibitors on cholesterol absorption in African green monkeys

Time	Percent Absorption of Cholesterol		
	Controls Group 1	CL 283,546 Group 2	CL 283,796 Group 3
wk		%	
−1 (Pretreatment Baseline)	46.2 ± 2.7	41.3 ± 3.9	37.8 ± 4.0
4–5 (10 mg/kg)	45.7 ± 2.4	37.3 ± 5.1	32.9 ± 3.5
9–10 (30 mg/kg)	39.4 ± 2.7	38.8 ± 2.9	34.8 ± 3.5

All values are for mean percent absorption ± SEM (n = 6 for each group). There are no statistically significant differences in the comparisons of treatment groups with control group values and change from baseline values for each group.

DISCUSSION

Both CL 283,546 and CL 283,796 have been shown to be specific and potent inhibitors of ACAT in a number of tissues from different species (12 and E. E. Largis, T. Ishikawa, A. Kato, and S. M. Wrenn, Jr., unpublished data) and in vivo studies in rat, rabbit, and hamster demonstrated significant reductions in plasma cholesterol levels (12, 48 and A. S. Katocs, Jr., E. E. Largis, Y. Urano, A. Kato, and S. M. Wrenn, Jr., unpublished data).

Previous studies with these two compounds (12, 48 and A. S. Katocs, Jr., E. E. Largis, Y. Urano, A. Kato, and S. M. Wrenn, Jr., unpublished data), as well as related analogs (3, 11, 13, 45, 46), indicated that their hypocholesterolemic activity was due, at least in part, to inhibition of intestinal ACAT and concomitant reduction in cholesterol absorption. Studies with other ACAT inhibitors (4–8, 10) have also shown that inhibition of cholesterol absorption was responsible for or contributed to their hypolipidemic effects. For example, studies with the ACAT inhibitor, DuP 128, in African green monkeys (56) indicated modest, but significant, inhibition of cholesterol absorption (−14%) and even greater reductions in total plasma cholesterol (−39%) and apoB levels (−28%), albeit at a relatively high dose of 150 mg/kg. Also, the recent studies of Marzetta et al. (30) have shown that CP-113,818 inhibited cholesterol absorption and reduced plasma cholesterol, predominantely VLDL + LDL cholesterol, in African green monkeys. Therefore, the results obtained in our study in the same species, indicating significant hypocholesterolemic activity without inhibition of cholesterol absorption, were not anticipated.

Because there are substantial differences between the relative potencies and effects of ACAT inhibitors among species, under different dietary conditions, and in different tissues (12, 30, 31, and unpublished), our results may relate to the relative importance by which ACAT pathways contribute to cholesterol absorption under these different situations. Previous studies with other ACAT inhibitors have shown that moderate reduction in cholesterol absorption may result in even larger effects on plasma cholesterol levels (30, 56), whereas other drugs that produce similar reductions in cholesterol absorption have been reported to have no effect on serum cholesterol levels (57). Thus, the mechanistic activities of various drugs can impact significantly on plasma lipid values independent of their abilities to inhibit cholesterol absorption. Certainly, ACAT activity appears to be related to the magnitude of hypercholesterolemia, since we see an effect of drug treatment on plasma cholesterol that is directly proportional to the starting baseline cholesterol values of individual animals (Fig. 3), a finding also seen in other laboratories (30).

One possible interpretation of our findings is that intestinal ACAT may, in fact, be inhibited in the African green monkey leading to decreased lymphatic secretion of cholesterol in chylomicrons, while cholesterol absorption, as measured by the method we used (54), appears unaffected. Under these conditions, labeled cholesterol could remain trapped in the enterocyte due to inhibition of intestinal ACAT and, unless retrograde egress and/or sloughing of enterocytes into the lumen of the intestine occurred during the period of stool collection, cholesterol absorption could appear unaltered. As cholesterol secretion from the enterocyte is polarized (58) towards the lymphatics, as ACAT inhibition does not appear to inhibit intestinal cholesterol uptake (59), and as unesterified cholesterol accumulates in the intestine of hamsters upon inhibition of ACAT (60), this possibility seems tenable. However, although Burrier et al. (61) found that ACAT inhibition diminished only esterified cholesterol and not free cholesterol transcellular movement across the intestine, such activity would be expected to decrease net cholesterol flux into the enterocyte, thereby reducing total cholesterol uptake and cholesterol absorption, as measured by the technique used in our studies. Furthermore, it should be pointed out that studies with two related ACAT inhibitors, using the same method in the same species (30, 56), resulted in decreased cholesterol absorption. Thus, it seems unlikely that CL 283,546 and CL 283,796 are exerting their hypolipidemic activity by affecting intestinal cholesterol absorption in the African green monkey.

The mechanism that we feel is likely the most germane suggested by our findings is the inhibition of hepatic ACAT leading to reduced secretion of cholesteryl esters in VLDL. There have been a number of recent reports which indicate that hepatic activity is responsible, at least in part, for the hypocholesterolemic effects of ACAT inhibitors. Recently Carr, Parks, and Rudel (15) have reported that hepatic ACAT activity in African green monkeys is highly correlated with plasma LDL-cholesteryl ester enrichment and coronary atherosclerosis. Similarly, Krause et al. (10, 62) and Burrier et al. (63) have shown in rats, rabbits, and hamsters that hepatic effects of ACAT inhibitors are responsible in whole or in part for the hypocholesterolemia and reduced liver cholesterol ester content. Recently, Huff et al. (31) reported that the effect of the ACAT inhibitor DuP 128, in minature pigs fed a cholesterol-free diet, was due primarily to inhibition of hepatic cholesterol esterification which resulted in the reduction of LDL and VLDL cholesterol and apoB levels. Additionally, Marzetta et al. (30) have reported that reduction in plasma cholesterol levels in non-human primates, seen upon treatment with the ACAT inhibitor CP-113,818, was due in significant part to hepatic effects to reduce VLDL cholesterol secretion, whereas in rats, hamsters, and rabbits the hypolipidemic effects were attributed principally to reduction in intestinal cholesterol absorption.

In our studies in African green monkeys, treatment with CL 283,546 or CL 283,796 resulted in significant reductions in LDL cholesterol but non-significant effects on VLDL cholesterol values. If hepatic activity of these drugs contributed to the hypocholesterolemic effects, one would anticipate comparable reductions in LDL cholesterol and its precursor, VLDL. However, as lipolytic activity in the monkey is high and VLDL is rapidly converted to LDL, effects that are initially produced on VLDL are more easily observed in the product of this reaction, the LDL fraction, which has much higher plasma concentrations (Table 6) and is, thus, more accurately measured. Therefore, it is not surprising that marginal and insignificant effects were seen on VLDL + IDL cholesterol levels, but significant reductions in LDL cholesterol and favorable increases in HDL/LDL cholesterol ratios (Table 6) were seen in our studies, consistent with effects on the liver.

In view of the likely activity of CL 283,546 and CL 283,796 on the liver and as cholesteryl ester biosynthesis has been reported to co-regulate with apoB and VLDL secretion in hepatocytes in culture (16, 17) and in perfused liver studies (27, 29), and with apoB secretion in minature pigs (31), we examined the effect of both drugs on plasma apolipoprotein concentrations. We hoped to determine whether the hypolipidemic effect seen upon treatment with ACAT inhibitors was due to reduced lipoprotein secretion or, alternatively, secretion of lipoproteins with reduced total cholesterol content. Our results indicate that the reduction in plasma cholesterol levels seen in the drug treatment groups is not correlated with apoB, apoE, or apo A-I levels, which remain unchanged from controls. Therefore, treatment with CL 283,546 or CL 283,796 appears to primarily reduce cholesterol content and alter composition of LDL and, if one assumes one apoB per particle, does not appear to reduce the blood concentration of LDL particles.

As previous studies (27, 31, 64) demonstrated a positive correlation of apolipoprotein B-100 concentration with cholesteryl ester and VLDL secretion, the lack of effect on apoB concentration seen in this study also would be consistent with a mechanism that did not involve hepatic activity. However, the results of Carr et al. (15) and Parks et al. (26) in African green monkeys have shown that decreased hepatic ACAT activity can result in decreased LDL cholesterol content without affecting apoB levels, a result analogous to our findings. These data, which contrast with results from perfused liver and cell culture experiments (17, 27, 64) and studies in minipigs (31, 40) showing that inhibition of ACAT reduced apoB and cholesteryl ester secretion in VLDL, may be related to the relative inhibition of ACAT (15). Only when ACAT is highly inhibited are both apoB and cholesteryl ester secretion reduced, but when ACAT is more moderately inhibited, cholesteryl ester secretion may be reduced without affecting apolipoprotein secretion (15). Alternatively, as suggested by Wu et al. from studies in HepG2 cells (25), triglycerides may regulate the secretion of apoB-containing lipoproteins, but in hepatocytes, perfused liver, and animal studies, others have shown that the regulation of secretion of these lipoproteins may be influenced by triglyceride levels but is generally dependent upon hepatic cholesteryl ester concentration (18, 62, 64). ACAT inhibition may influence apoB secretion, but, depending upon conditions, principally result in secretion of apoB-containing lipoproteins with decreased cholesterol content, as our findings indicate. Thus, our results are consistent with a mechanism whereby these drugs appear to be exerting their activity upon hepatic ACAT leading to secretion of cholesterol ester depleted lipoproteins.

Hepatic effects of these drugs, leading to reduced secretion of cholesterol in VLDL, would be expected to increase free cholesterol concentration in the hepatocyte leading to down-regulation of LDL receptor expression (65), whereas inhibition of intestinal absorption of cholesterol would be anticipated to up-regulate LDL receptor and HMG-CoA reductase expression as a consequence of decreased cholesterol delivery to the liver (11, 66). In view of the lack of effect of drug treatment on apoB levels, which would be anticipated to be altered if LDL receptor pathways were modulated, it appears that hepatic drug effects produce decreased LDL cholesterol concentrations without feedback regulation on LDL

clearance pathways. As the cholesterol pool which is a substrate for ACAT appears to be distinct from the pool that is responsible for regulation of the LDL receptor (67-69), this possibility seems likely. Additionally, both Krause et al. (62) and Huff et al. (31) found that ACAT inhibitors reduced plasma cholesterol without regulation of LDL receptor expression, consistent with this hypothesis.

As most studies with ACAT inhibitors have not shown an accumulation of cholesterol or cholesteryl esters in the liver or decreased LDL-receptor expression, inhibition of hepatic ACAT may result in increased biliary excretion of cholesterol and/or bile acids. Nervi et al. (70) have shown a high negative correlation of hepatic ACAT activity and biliary cholesterol excretion in the rat. Sampson et al. (71) observed similar effects on bile acid and cholesterol secretion in primary rat hepatocytes upon inhibition of ACAT. Additionally, the recent studies of Krause et al. (10) indicate that treatment of cholesterol-fed rats with the ACAT inhibitor CI-976 resulted in increased biliary cholesterol and bile acid excretion and similar interpretations were made by Huff et al. (31) from their studies in minipigs. Intestinal drug effects leading to reduced cholesterol delivery to the liver would likely result in low hepatic cholesterol contents, but would not be expected to increase biliary cholesterol excretion. Thus, inhibition of hepatic ACAT by CL 283,546 or CL 283,796, leading to decreased secretion of VLDL cholesterol and enhanced biliary excretion of cholesterol and bile acids, without down-regulation of hepatic LDL receptor clearance pathways, would appear to be an appropriate mechanism accounting for the effects observed in this study.

In conclusion, the favorable hypolipidemic activities exhibited by these ACAT inhibitors in animal models, along with the promising potential for direct antiatherosclerotic effects, an area under investigation by a number of laboratories (72), suggests promise for this drug class as therapy for coronary artery and other atherosclerotic diseases in humans. To date the clinical studies with ACAT inhibitors have been disappointing with only marginal activity seen on plasma lipids or cholesterol absorption in humans (47, 72, 73). There has been speculation as to the reasons for this (47, 74), but one certainly must consider mechanistic and specificity differences of individual drugs, including intestinal and hepatic effects, an issue addressed by the results of this paper, as well as fundamental differences between species regarding the regulation of gene expression by cholesterol (62) and the role of ACAT in these pathways.

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