Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits

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Abstract **Cholesteryl ester transfer protein (CETP) inhibitors increase high density lipoprotein-cholesterol (HDL-C) in animals and humans, but whether CETP inhibition will be antiatherogenic is still uncertain. We tested the CETP inhibitor torcetrapib in rabbits fed an atherogenic diet at a dose sufficient to increase HDL-C by at least 3-fold (207 ± 32 vs. 57 ± 6 mg/dl in controls at 16 weeks). CETP activity was inhibited by 70–80% throughout the study. Non-HDL-C increased in both groups, but there was no difference apparent by the study's end. At 16 weeks, aortic atherosclerosis was 60% lower in torcetrapib-treated animals (16.4 ± 3.4% vs. 39.8 ± 5.4% in controls) and aortic cholesterol content was reduced proportionally. Sera from a separate group of rabbits administered torcetrapib effluxed 48% more cholesterol from Fu5AH cells than did sera from control animals, possibly explaining the reduced aortic cholesterol content. Regression analyses indicated that lesion area in the torcetrapib-treated group was strongly correlated with the ratio of total plasma cholesterol to HDL-C but not with changes in other lipid or lipoprotein levels. CETP inhibition with torcetrapib retards atherosclerosis in rabbits, and the reduced lesion area is associated with increased levels of HDL-C.**—Morehouse, L. A., E. D. Sugarman, P-A. Bourassa, T. M. Sand, F. Zimetti, F. Gao, G. H. Rothblat, and A. J. Milici. **Inhibition of CETP activity by torcetrapib reduces susceptibility to diet-induced atherosclerosis in New Zealand White rabbits.** ***J. Lipid Res.*** **2007.** 48: **1263–1272.**

Supplementary key words cholesteryl ester transfer protein • cholesterol efflux • reverse cholesterol transport

High levels of high density lipoprotein-cholesterol (HDL-C) have been associated with a decreased incidence of coronary heart disease in epidemiological studies (1–3), and HDL has a number of potentially antiatherogenic properties that may account for its atheroprotective effects. HDL is a key mediator of reverse cholesterol transport, the process by which excess peripheral tissue cholesterol is shunted back to the liver. However, HDL has antiinflammatory (4, 5), antioxidative (6, 7), and antithrombotic activities (8, 9) that may also contribute to its antiatherogenic effects. Because our understanding of exactly how HDL protects against atherosclerosis is not complete, and because HDL speciation, metabolism, and function are complex, perhaps not all mechanisms for increasing HDL-C will ultimately be shown to have equivalent effects on the atherosclerotic process. One HDL-increasing target that has already triggered debate in this regard is cholesteryl ester transfer protein (CETP).

CETP is a plasma glycoprotein that transfers cholesteryl esters (CEs), triglycerides, and phospholipids among circulating lipoproteins (10, 11). CETP transfers neutral lipids down concentration gradients; as such, the physiologically relevant direction of the transfer of CE is from the CE-enriched HDL fraction to non-HDL lipoproteins, with retrograde transfer of triglycerides. In the context of reverse cholesterol transport, the transfer of CE via CETP can divert HDL-CE from the direct, hepatic, specific uptake pathway to an indirect pathway for hepatic CE delivery involving the receptor-mediated uptake of apolipoprotein B (apoB)-containing lipoproteins. Under conditions in which hepatic apoB uptake is downregulated, CETP action results in a CE enrichment of non-HDL lipoproteins, which could contribute to atherogenesis. Perhaps because of these complexities, the introduction of the CETP transgene into mice of various genetic backgrounds has not yielded a consistent effect on athero-

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Abbreviations: apoB, apolipoprotein B; AUC, area under the curve; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; FPLC, fast protein liquid chromatography; HDL-C, high density lipoprotein-cholesterol; TPC, total plasma cholesterol.

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sclerosis (12–16), adding uncertainty to the role of CETP in atherogenesis.

Interest in CETP as a pharmacologic target for increasing HDL-C was buoyed by the identification of individuals deficient in CETP who exhibit severalfold increases of HDL-C (17). The HDL particles that accumulate in CETP deficiency are CE-enriched and cleared more slowly (18), underscoring the importance of CETP in HDL lipoprotein remodeling and perhaps suggesting that these individuals, despite their greater HDL levels, may have impaired reverse cholesterol transport. There are not sufficient numbers of these CETP-deficient subjects to definitively determine whether the complete absence of CETP is antiatherogenic. A number of clinical and experimental studies have established a clear inverse correlation between CETP activity and levels of HDL-C (19–24). But as with the conflicting transgenic mouse data, the clinical data are split regarding whether reduced CETP activity is antiatherogenic (25–28).

Therefore, although higher levels of HDL-C are associated with reduced atherosclerotic disease epidemiologically and HDL levels are increased in individuals with low CETP activity, some of the data associated with CETP deficiency suggest that it might not be associated with reduced atherosclerosis. To examine this experimentally, several groups of investigators have used the cholesterol-fed rabbit, a species that naturally expresses CETP, and have modulated CE transfer activity via antisense oligonucleotides (29), vaccine (30), or small molecule inhibitors (31, 32). In general, decreasing CETP expression or activity has been reported to be antiatherogenic, but taken as a whole, the results have not been convincing, especially with regard to whether HDL increase played a significant role in the reported antiatherogenic effect. In the majority of these studies, there were greater absolute decreases in non-HDL-C fractions than increases in HDL-C. In the only report claiming no effect of CETP inhibition on lesion formation, non-HDL-C levels were significantly higher than in the other studies and not significantly decreased in the CETP inhibitor group (31), prompting the question of whether the reduction of atherosclerosis with CETP inhibition was attributable to the increase of HDL-C or the reduction of non-HDL-C levels (33). To address this question, we tested torcetrapib, a robust CETP inhibitor, in the cholesterol-fed rabbit, achieving a multiple-fold increase in HDL-C without a significant decrease in non-HDL-C levels.

MATERIALS AND METHODS

Study design

Male New Zealand White rabbits (1.5–1.75 kg; Covance, Denver, PA) were housed at Pfizer, an American Association of Laboratory Animal Care-accredited facility, and an in-house committee reviewed all experimental procedures for adherence to ethical treatment standards. A scheme of the design of the atherosclerosis study is shown in **Fig. 1**. Rabbits (n = 47) were fed an atherogenic diet (0.2% cholesterol, 10% coconut oil, and 1.2% ethyl lactate) (Harlan-Teklad, Madison, WI) for 5 days and assigned to one of the treatment groups based on their total plasma cholesterol (TPC) responses (n = 23 or 24 per group). Control animals continued consuming the cholesterol-containing diet for an additional 16 weeks; the treated group was fed the same diet but with increasing amounts of torcetrapib [0.15, 0.3, and 0.6% (w/w), 1 week at each dose] to identify the dose necessary to achieve at least 3-fold increases of HDL. Despite the fact that the targeted level of HDL-C increase was achieved at the 0.15% dose level, the dose-escalation phase of the experiment was completed as initially planned. Because of the known variability of rabbits to an atherogenic diet, we were unsure that the starting dose of torcetrapib would maintain HDL-C levels at greater than three times control levels throughout the study. After dose escalation, rabbits were returned to the 0.15% dose level for the remaining 13 weeks of the study. This level of dietary feeding equated to ~90 mg/kg/day torcetrapib at the start of the study, gradually declining as the rabbits gained weight to 60–65 mg/kg/day by the end of the study. There was no difference in body weight or food consumption in torcetrapib-treated rabbits relative to controls (data not shown).

In preliminary studies to gauge the extent of atherosclerosis in rabbits fed this diet, the non-HDL-C levels in ~10–15% of the

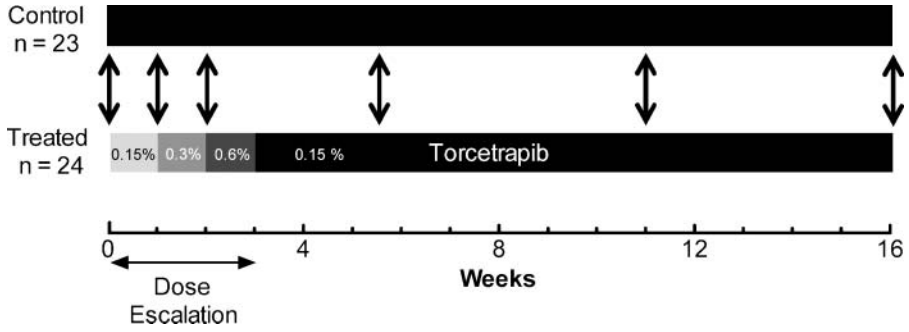


Fig. 1. Atherosclerosis study design. New Zealand White rabbits were acclimated for 1 week in a house and then fed a 0.2% cholesterol, 10% coconut oil diet for 5 days to identify rabbits hyporesponsive and hyperresponsive to dietary cholesterol feeding. After assignment to one of two treatment groups, rabbits were fed the atherogenic diet with or without torcetrapib for an additional 16 weeks. The torcetrapib-treated group was subjected to an initial 3 week dose escalation to determine the dose of torcetrapib that would maintain high density lipoprotein-cholesterol (HDL-C) levels at three times control levels. Animals were bled at the indicated time points, and plasma was analyzed for plasma lipids and lipoproteins, cholesteryl ester transfer protein (CETP) activity, and plasma torcetrapib levels, as described in Materials and Methods.

rabbits began declining after 8–10 weeks, and those animals with the lowest non-HDL-C (<150 mg/dl) had no aortic lesions at 16 weeks. It was not possible to identify these abnormal responders before the start of the study, because their initial response to an atherogenic diet was not predictive of subsequent hyporesponsiveness. Because the goal of this study was to determine whether CETP inhibition was antiatherogenic, including animals with no aortic atherosclerosis in either treatment group would only serve to increase the variability in each group. Thus, before the start of the study, we chose to prospectively identify these rabbits by measuring non-HDL-C at 14 weeks and to exclude any animals whose non-HDL-C did not exceed this 150 mg/dl threshold. Torcetrapib treatment did not affect the number of animals exhibiting this atypical response (four treated vs. three controls). One control animal expired at 6 weeks as a result of a congenital heart valve defect, so the total number of rabbits completing the study was 39 (19 controls and 20 torcetrapib-treated).

Lesion analysis

Rabbits were anesthetized with intravenous pentobarbital (50 mg/kg), and aortas were perfused in situ with PBS followed by 10% formalin, excised, and stored in 1% gum arabic and 30% sucrose at 4°C overnight before returning them to 10% formalin. After removal of adventitia and connective tissue, aortic lesion area was quantitated en face by individuals blinded to treatment. Aortas were opened longitudinally and mounted on black foam boards, and sequential aortic images were captured using a Spot Camera (Diagnostic Instruments, Sterling Heights, MI). Digital images were merged using AnalySIS software (Soft Imaging Systems, Lakewood, CO). A preliminary test evaluating the aortic lesion areas in the same aortas had revealed no differences in lesion quantitation regardless of whether the aortas were analyzed unstained or stained with Sudan IV, so aortas were analyzed unstained, because this also enabled subsequent aortic lipid analyses. Unstained lesions were opaque, raised, and easily distinguishable from surrounding unlesioned areas.

Assay for CE transfer

Animals were bled from the marginal ear vein weekly during the first 2 weeks of dose escalation and approximately monthly thereafter. CE transfer activity was estimated using a fluorescent transfer assay. It was necessary to use this assay instead of the typical radiolabel transfer assays because the non-HDL fractions in the extremely hyperlipidemic samples did not precipitate after low-spin centrifugation; thus, it was difficult to determine the level of CE transfer activity by conventional methods. BODIPY-CE and apoA-I-containing emulsion particles were generated by a direct sonication procedure (34). Briefly, 7 mg of phosphatidylcholine and 0.75 mg of triolein (Avanti Polar Lipids, Alabaster, AL) were mixed with 3 mg of BODIPY-CE (Invitrogen, Carlsbad, CA) in chloroform and vacuum-dried at 60°C. Lipids were solubilized at 65°C in phosphate-buffered saline by sonication for 2 min under a stream of nitrogen. The preparation was cooled to 45°C, and 5 mg of apoA-I (Biodesign, Saco, ME) was added. The preparation was resonicated (at 25% of full power) for 20 min at 45°C, pausing after each minute to cool the probe. The sonicate was spun for 30 min at 3,000 *g*, adjusted to 1.12 g/ml using NaBr, and layered below a solution of 1.10 g/ml NaBr. After spinning for 48 h at 100,000 *g*, any unemulsified lipid, unincorporated protein, and small dense particles were discarded. The fluorescently labeled particles were dialyzed in phosphate-buffered saline with 0.02% (w/v) sodium azide. To assay for CETP activity in isolated plasma samples, fluorescent donor particles were incubated in PBS with 20% plasma at 37°C, and the transfer of BODIPY-CE to endogenous lipoproteins was monitored at 485 nm excitation and 520 nm emission in a Gemini plate reader (Molecular Devices, Sunnyvale, CA). Inhibition of transfer was calculated by comparing the plasma CE transfer rate from each treated rabbit to the mean CE transfer rate observed in the control group. This assay had been characterized in numerous experiments, including those in which the biotinylated, fluorescent donor particles were incubated with human plasma and the appearance of BODIPY-CE in the endogenous lipoprotein fraction was monitored. After precipitation of biotinylated donor particles with streptavidin beads, BODIPY-CE was present in all plasma lipoprotein classes, and its accumulation in endogenous lipoproteins was both time- and CETP-dependent. Side-by-side comparisons of this fluorometric assay with a radiolabeled assay using human and rabbit plasma yielded excellent correlations between the two methods (r = 0.98 and 0.91 for humans and rabbits, respectively).

Lipid, lipoprotein, and torcetrapib analyses

Lipoprotein cholesterol content was determined using gel filtration on a fast protein liquid chromatography (FPLC) unit (Gilson, Middleton, WI) equipped with in-line, postcolumn cholesterol detection (35). ApoA-I was determined using an immunoturbidity assay (Sigma-Aldrich, St. Louis, MO) with human apoA-I standards on a Roche-Hitachi autoanalyzer (Roche Diagnostics, Indianapolis, IN). Aortic free cholesterol (FC) and CE contents were determined by Lipomics (West Sacramento, CA) (36). Triglycerides were measured using a kit (Wako Chemical, Richmond, VA) adapted to a microtiter plate format. Plasma levels of torcetrapib were measured as described previously (37).

Cholesterol efflux

Sera were isolated from separate groups of rabbits fed the atherogenic diet with or without torcetrapib for 2 weeks. The cholesterol efflux potential of the sera was determined using an in vitro assay similar to one that has been described previously (38). Fu5AH hepatoma cells were maintained on MEM (Mediatech Cellgro, Herndon, VA) containing 5% calf serum (Sigma-Aldrich) and gentamycin. Cells at 90% confluence were trypsinized and plated at 0.6×10^6 cells/well on 12-well plates. Cell number was determined using a Z1 Coulter Particle Counter (Hialeah, FL). The medium was supplemented with the ACAT inhibitor CP-113,818 (2 μg/ml) during labeling, equilibration, and the flux stages of the experiment. Cells were labeled for 24 h with [^{3}H]cholesterol (Perkin-Elmer Analytical Sciences, Boston, MA) in medium supplemented with 2.5% calf serum. After an equilibration period in 0.2% BSA-containing medium, efflux was measured by incubating the cells with medium containing 2.5% rabbit sera for 8 h. To inhibit de novo cholesterol synthesis, mevinolin (5 μg/ml) was added to the efflux medium. Cholesterol efflux was measured by the release of [^{3}H]cholesterol into the medium and expressed as a percentage of that previously incorporated into cell monolayers. Cholesterol efflux in this cell line is predominantly via a scavenger receptor class B type I-dependent mechanism (39).

Statistical analysis

Statistical analyses were done using LabStats, a Microsoft Excel add-in software package developed at Pfizer. The results were checked for consistency using a commercially available software package (MathSoft, Inc., Cambridge, MA). Area under the curve (AUC) values for lipid and lipoprotein parameters were calculated for each animal and used in linear regression analyses.

TABLE 1. Cholesteryl ester transfer protein activity and lipid, lipoprotein, and apolipoprotein values

Group	Week Number	Plasma Torcetrapib	CE Transfer	TPC	HDL	Non-HDL	TPC/HDL Ratio	Triglyceride	ApoA-I
Control (n = 19)	0	ND	1.16 ± 0.11	122 ± 11					
	1	ND	1.67 ± 0.05	204 ± 19	57 ± 4	148 ± 18	3.7 ± 0.3	53 ± 3	33 ± 2
	2	ND	1.69 ± 0.04	285 ± 31	49 ± 3	237 ± 31	6.3 ± 0.8	50 ± 5	34 ± 2
	5.5	ND	3.68 ± 0.13	430 ± 49	48 ± 3	382 ± 48	9.2 ± 1.0	65 ± 10	30 ± 5
	11	ND	2.60 ± 0.32	524 ± 72	55 ± 4	469 ± 69	9.3 ± 1.1	114 ± 14	26 ± 4
	16	ND	3.37 ± 0.12	703 ± 109	57 ± 6	645 ± 104	12.0 ± 1.3	95 ± 16	27 ± 5
AUC				6,240 ± 600	700 ± 35	5,540 ± 580	120 ± 10	1,100 ± 120	420 ± 50
Torcetrapib (n = 20)	0	ND	1.32 ± 0.10	116 ± 10					
	1	0.85 ± 0.04	0.33 ± 0.01	280 ± 18	200 ± 15	80 ± 9	1.4 ± 0.1	22 ± 2	46 ± 3
	2	2.05 ± 0.24	0.34 ± 0.01	401 ± 32	255 ± 22	145 ± 17	1.6 ± 0.1	27 ± 2	49 ± 2
	5.5	1.81 ± 0.27	0.67 ± 0.05	500 ± 66	270 ± 35	230 ± 39	2.0 ± 0.2	35 ± 3	71 ± 8
	11	1.42 ± 0.10	0.58 ± 0.05	788 ± 113	323 ± 51	465 ± 70	2.8 ± 0.2	84 ± 11	79 ± 9
	16	1.47 ± 0.11	0.99 ± 0.10	897 ± 122	207 ± 32	690 ± 104	5.3 ± 0.7	81 ± 17	75 ± 12
AUC				8,530 ± 1,020	3,750 ± 470	4,770 ± 630	36 ± 2	740 ± 80	1,050 ± 90
P			<0.0001	0.096	<0.0001	0.15	<0.0001	0.015	<0.0001

ApoA-I, apolipoprotein A-I; AUC, area under the curve; CE, cholesteryl ester; ND, not detected; TPC, total plasma cholesterol. Values at week 0 represent the data in the two groups of animals after 5 d of feeding the atherogenic diet. Values at 1 and 2 weeks represent the data in groups of rabbits fed the atherogenic diet with or without torcetrapib added at the 0.15% and 0.3% levels, respectively. All values are means ± SEM. Plasma torcetrapib levels are expressed as μg/ml; torcetrapib was not detected in the sera of any control animals at any time point or in treated animals at baseline. CE transfer rates are expressed in relative fluorescence units/min. Lipid and lipoprotein values are expressed as mg/dl. ApoA-I values are expressed as human apoA-I equivalents in mg/dl. Lipid and lipoprotein AUC values (with the exception of TPC/HDL ratio) are expressed as mg/week/dl. *P* values for treatment were calculated using a Student's *t*-test of log-transformed AUC values or repeated-measures ANOVA (CE transfer).

Differences between the mean AUC values of lipid and lipoproteins in control and torcetrapib-treated groups were evaluated using Student's *t*-test on log-transformed data, except where noted.

RESULTS

Torcetrapib administration results in sustained inhibition of CETP

The first 3 weeks of this 16 week study consisted of a dose-escalation phase to select the dose of torcetrapib that resulted in a multiple-fold increase of HDL-C. This was achieved at the 0.15% dose level (~90 mg/kg/day); the mean HDL-C in rabbits given 0.15% torcetrapib during the first week was 200 mg/dl, compared with a mean of ~60 mg/dl for control rabbits. After completion of the dose-escalation phase, the treated rabbits were fed the 0.15% diet for the remaining 13 weeks of the experiment. This dose of torcetrapib resulted in a sustained inhibition of CE transfer throughout the experiment (**Table 1**); CE transfer was at least 70% inhibited throughout the duration of the study. Because blood was obtained just before the feeding the daily food ration, these transfer inhibition values in all likelihood represented a minimum, as plasma levels of torcetrapib and the resulting inhibition would be expected to have been greater throughout the remainder of the day.

Lipoprotein changes and FPLC profiles

Plasma lipid and lipoprotein levels in the two groups of rabbits are summarized in Table 1. Inhibition of plasma CETP activity by torcetrapib resulted in mean HDL-C levels that were >3-fold greater than the mean values in control rabbits at 1 week. This treatment-related increase in HDL-C was maintained throughout the study, although HDL-C levels at each time point examined were variable. Similarly, apoA-I levels were increased 2.5-fold by torcetrapib treatment. Non-HDL-C levels increased progressively in both groups of animals fed the atherogenic diet. Non-HDL-C levels in torcetrapib-treated rabbits were lower than in controls during the initial 5.5 weeks of the study (Table 1) but were equivalent to those of the control group at 11 and 16 weeks. The averaged plasma lipoprotein FPLC profiles of all control and torcetrapib-treated rabbits at 5.5 and 16 weeks are shown in **Fig. 2**. At both time points, levels of HDL-C were significantly greater in the torcetrapib-treated group than in controls, and the HDL-C peak was shifted to the left, indicating a larger average size of the HDL particles in treated animals relative to controls (Fig. 2), as has been observed previously in CETP-deficient individuals and subjects treated with torcetrapib. The major change in the FPLC profiles at 16 weeks relative to 5.5 weeks was the increase in non-HDL-C observed in both control and treated rabbits.

The AUCs of lipid and lipoprotein values from individual animals over the duration of the study were calculated and used in subsequent regression analyses. The rationale for comparing lipid and lipoprotein AUCs

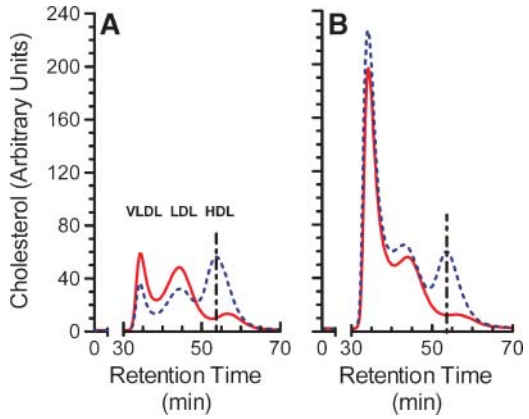


Fig. 2. Fast protein liquid chromatography (FPLC) gel filtration profiles of plasma from control and torcetrapib-treated rabbits. Plasma lipoproteins were fractionated by gel filtration with in-line, postcolumn cholesterol detection. The traces show mean detector responses from all of the individual plasma samples (n = 19 control rabbits and 20 torcetrapib-treated rabbits) at 5.5 weeks (A) or 16 weeks (B) of treatment. Error bars are excluded for legibility. The variability of the individual lipoprotein data are shown in Table 1. The FPLC profile for the control group is shown as a solid red line, and that for the torcetrapib-treated group is shown as a dotted blue line. The perpendicular lines are drawn to illustrate the larger average size of HDL particles in torcetrapib-treated rabbits relative to those from control animals.

with lesion area was that the extent of aortic atherosclerosis in each rabbit should be at least partially dependent upon its plasma lipid and lipoprotein levels. There were highly significant differences in HDL-C AUC and the TPC AUC/HDL AUC ratio in torcetrapib-treated rabbits compared with controls (Table 1). However, despite differences between plasma non-HDL-C values of the two groups at early time points, there was no statistically significant difference in the plasma non-HDL-C AUC between control and torcetrapib-treated groups. Triglyceride levels were modestly but consistently lower in torcetrapib-treated rabbits relative to controls.

Aortic lesion areas and lipid content

The proximal aortic lesion areas of all animals are shown in **Fig. 3A**. Mean aortic lesion area was ~40% in the control group compared with ~16% in the treated group, a highly significant 60% reduction ($P = 0.001$). Aortic images from several pairs of rabbits matched for non-HDL-C AUC values are shown in **Fig. 4**. In each instance, the torcetrapib-treated animal had a multiple-fold increase in HDL-C AUC compared with its matched control and a corresponding reduction in aortic lesion area. Because aortic lesion area was determined on unstained aortic tissue, the same tissue could be analyzed subsequently for FC and CE content. Subsets of aortas (n = 10 per group) with the same mean lesion areas as their respective treatment groups were analyzed for cholesterol content. Levels of both aortic FC and CE were decreased by ~60% in the torcetrapib-treated groups compared with the controls (Fig. 3B), and the total aortic cholesterol content was significantly and highly correlated with lesion area, irrespective of treatment (Fig. 3C).

Correlation of aortic lesion area with lipoprotein levels

The relative contributions of changes in lipid and lipoprotein levels to aortic lesion area were assessed using linear regression analyses. The slopes of the regression lines of lesion area versus non-HDL AUC were significantly different in the two groups of rabbits (**Fig. 5A**), and only the slope of the line fitted to the control group data was significantly different from zero. This indicated that non-HDL-C levels were positively associated with the extent of

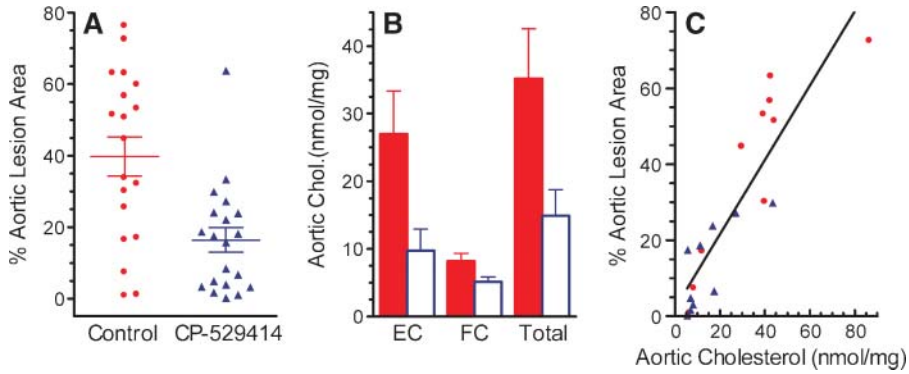
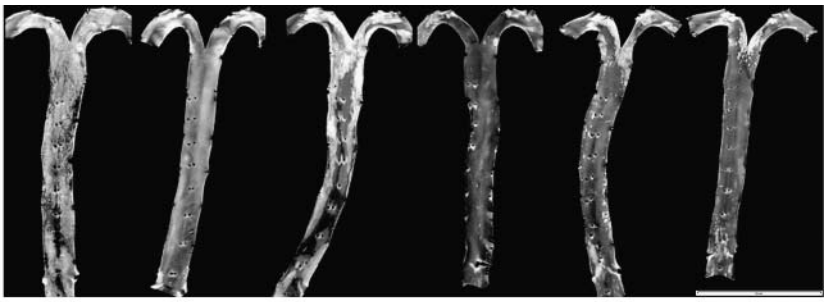


Fig. 3. Aortic lesion area and cholesterol content. Data from control rabbits (n = 19) are shown in red circles or solid bars, and data from torcetrapib-treated animals (n = 20) are shown in blue triangles or open bars. A: Scatterplot of the total lesion area in the proximal aortas from control and torcetrapib-treated rabbits showing a significant reduction in lesion area after 16 weeks. Error bars indicate mean lesion areas ± SEM (39.8 ± 5.4% vs. 16.4 ± 3.4% for control and torcetrapib-treated groups, respectively; $P = 0.001$). B: Aortic cholesterol content from a subgroup of control and torcetrapib-treated rabbits (n = 10/group; mean lesion areas ± SEM of 39.7 ± 7.8% and 13.3 ± 3.6%, respectively). Aortic free cholesterol (FC), cholesteryl ester (EC), and total cholesterol contents in torcetrapib-treated rabbits were significantly lower than their respective levels in aortas from control animals ($P < 0.001$). C: Aortic lesion area was highly correlated with total aortic cholesterol content ($P < 0.0001$).



ID	Control	Treated	Control	Treated	Control	Treated
Non-HDL	5400	5200	8300	8400	5400	5600
HDL	660	6000	780	4500	660	2000
% Lesion	76.2	3.9	63.0	15.7	33.7	18.2

Fig. 4. Comparison of aortic lesions in control and torcetrapib-treated rabbits. Aortic lesions from several pairs of rabbits matched for non-HDL area under the curve (AUC) showing reduced aortic atherosclerosis in torcetrapib-treated rabbits after 16 weeks of treatment. The contrast of the original black and white images has been enhanced for improved reproduction. Lipoprotein AUC values are in mg/week/dl. Bar = 50 mm.

atherosclerosis in only the control group of rabbits, suggesting that torcetrapib treatment mitigated the proatherogenic effect of non-HDL lipoproteins. Because the mean non-HDL-C AUC values did not differ between the two groups of rabbits, other factors presumably accounted for the reduced aortic atherosclerosis observed in torcetrapib-treated animals.

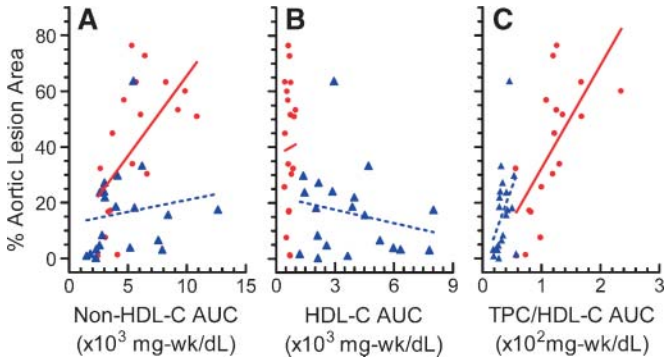


Fig. 5. Inhibitory effect of torcetrapib on aortic atherosclerosis correlates with HDL-C increase. Linear regression analyses of percentage aortic lesion area versus lipoprotein levels in control and torcetrapib-treated rabbits. Data from control rabbits are shown in red circles, and data from torcetrapib-treated rabbits are shown in blue triangles. A: Non-HDL AUC was positively correlated with aortic lesion area in control but not in torcetrapib-treated rabbits. The slope and intercept ± SEM of the regression line for the control group were 0.00082 ± 0.00023 and 8.0 ± 9.8, and the corresponding parameters for the regression analysis of the torcetrapib-treated group were 0.00012 ± 0.0002 and 12.0 ± 7.6. B: HDL AUC was negatively correlated with aortic lesion area in torcetrapib-treated rabbits but not in controls. The slope and intercept ± SEM of the regression line for the control group were 0.00060 ± 0.0044 and 37.0 ± 22.2, and the corresponding parameters for the torcetrapib-treated group were −0.00023 ± 0.00032 and 22.0 ± 9.6. C: Total plasma cholesterol (TPC)/HDL ratio was correlated with lesion area in both groups of rabbits. The slope and intercept ± SEM of the regression line for the control group were 0.053 ± 0.012 and −5.0 ± 11.1, and the corresponding parameters for the regression analysis of the torcetrapib-treated group were 0.083 ± 0.049 and −4.0 ± 12.3. Because the individual lines were not different, a single line was fit to all of the data and the result was highly significant ($P < 0.001$).

A similar analysis of the HDL AUC data revealed a trend toward an inverse correlation between aortic lesion area and HDL-C in torcetrapib-treated animals, but it did not reach statistical significance (Fig. 5B). In contrast to the variability of the HDL AUC values in torcetrapib-treated rabbits, the HDL-AUC values of the control group were all clustered at the low end of the scale and thus did not show a significant correlation with lesion area either.

In contrast, the AUC of TPC/HDL, a single term capturing both the proatherogenic effect of non-HDL lipoproteins and the potential antiatherogenic effects of HDL, resulted in regression lines with highly significant positive slopes that were not statistically different (Fig. 5C). The non-HDL/HDL ratio was similarly highly correlated with aortic lesion area, but additional regression analyses of other lipid and lipoprotein variables did not yield additional insights. For example, despite a significant treatment effect on plasma triglycerides (Table 1), the slopes of the regression lines of lesion area versus plasma triglyceride AUC were not statistically different from zero. These data indicate that the antiatherogenic effect of torcetrapib is closely related to the increased levels of HDL-C.

Cholesterol efflux

The finding of reduced cholesterol content in the aortas from torcetrapib-treated rabbits relative to controls despite similar levels of atherogenic non-HDL lipoproteins led us to examine the ability of sera from control or torcetrapib-treated rabbits to stimulate cholesterol efflux. We tested sera from separate groups of animals fed the cholesterol-containing diet for 2 weeks in a cholesterol efflux assay using Fu5AH cells. Sera obtained from torcetrapib-treated rabbits stimulated FC efflux to a significantly greater extent than did sera from control animals (**Fig. 6A**). There was also a highly significant correlation between HDL-C and FC efflux, regardless of treatment (Fig. 6B).

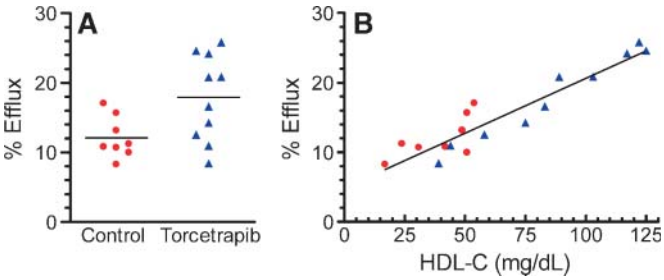


Fig. 6. Sera from rabbits treated with torcetrapib stimulate cholesterol efflux. Rabbits (n = 8 controls and n = 10 torcetrapib-treated) were fed the atherogenic diet for 2 weeks. In both panels, data from control animals are shown in red circles and data from torcetrapib-treated animals are shown in blue triangles. Data are means from a representative experiment in which sera samples were tested in triplicate. Values are expressed as the percentage of cellular radiolabeled FC recovered in the medium of Fu5AH cells after 8 h of incubation. A: Sera from torcetrapib-treated rabbits effluxed more cholesterol compared with sera isolated from control animals. The means ± SEM of the data from the control and treated groups are 12.1 ± 1.1 and 17.9 ± 2.0, respectively ($P = 0.02$ using an unpaired Student's *t*-test). B: Regression analysis of cholesterol efflux versus HDL levels ($P < 0.0001$).

DISCUSSION

The role of CETP in atherogenesis has been controversial for more than a decade. Inhibition of CETP leads to increased steady-state levels of HDL-C, potentially augmenting its antiinflammatory, antithrombotic, and antioxidative potential. Conversely, some studies of human CETP deficiency and some animal studies have suggested that increased HDL levels as a result of reduced CETP activity may impede reverse cholesterol transport, thereby perhaps being proatherogenic. Larger HDL particles that tend to accumulate in CETP deficiency states may be less optimal acceptors of excess FC and/or may reflect impaired transfer of HDL-C to the liver.

We investigated the effect of robust CETP inhibition with torcetrapib on aortic lesion formation in cholesterol-fed rabbits. Our goal was not to mimic the clinical effects of torcetrapib but to achieve increases of HDL-C similar to those reported for homozygous, CETP-deficient individuals (17), with the goal of testing whether HDL-C increase as a result of CETP inhibition was proatherogenic or antiatherogenic.

Plasma torcetrapib levels reached an apparent steady-state level of ~1,500 ng/ml by 11 weeks. This level of systemic exposure was sufficient to inhibit CE transfer in the plasma from torcetrapib-treated animals by at least 70–80% throughout the duration of the study. These plasma concentrations of torcetrapib exceeded those reported in clinical studies of torcetrapib by 3- to 5-fold but did not provide greater inhibition than has been reported previously in humans. In light of the increase in non-HDL lipoproteins reported here, the induction of CETP expression by cholesterol feeding in rabbits (40, 41), and the more rapid clearance of torcetrapib in rabbits relative to humans (data not shown), it is not surprising that plasma torcetrapib concentrations greater than those reported to be therapeutic in humans were required to achieve robust CETP inhibition in this study.

Torcetrapib treatment increased HDL-C by >3-fold on average. The HDL-C levels at each time point were variable, perhaps a reflection of interanimal and/or day-to-day variation in the temporal consumption of the atherogenic diet that was provided ad libitum as well as of interanimal variability in the absorption and clearance of torcetrapib. The average CETP inhibition achieved decreased at 16 weeks relative to the magnitude of the effect observed at earlier time points, perhaps at least partially reflecting the decreasing mg/kg dose of compound as the animals gained weight. The addition of exogenous VLDL or LDL to human plasma reduced the apparent in vitro potency of torcetrapib (42), so the substantial increases in non-HDL-C at 11 and 16 weeks might have contributed to a less robust inhibition of CETP. However, the reason for the variable HDL-C levels is not clear. Non-HDL-C levels increased steadily throughout the experiment in both groups of rabbits, with a treatment difference being apparent only during the first phase of the study. This is in contrast to the reported effects of CETP inhibitors in humans, in which absolute increases in HDL-C levels and decreases of non-HDL-C levels were of similar magnitude (43).

Torcetrapib treatment resulted in a highly significant 60% reduction in proximal aortic lesion area. Aortic FC and CE contents in torcetrapib-treated rabbits were likewise reduced by ~60% compared with controls, and there was a direct and highly significant correlation between total cholesterol content and aortic lesion area irrespective of treatment.

These results are in general agreement with most of the published data on CETP inhibition in rabbits. Most investigators have reported that reduction in CETP mass or small molecule inhibition of CETP activity led to statistically significant reductions in lesion area. However, these reports have not been especially convincing, for several reasons. Pharmacological inhibition of CETP resulted in ~2-fold increases of HDL and an inhibition of atherosclerosis in one study (32) but had no effect in a second study with the same compound but with a diet slightly higher in cholesterol (31). Antisense (29) or vaccine (30) strategies for reducing CETP protein levels have also been reported to reduce aortic atherosclerosis by 30–40%, but a more modest reduction of CETP expression (30–50%) and correspondingly smaller changes in the lipoprotein profile were reported using these approaches.

In none of these four studies was there any attempt to correlate the magnitude of the lipoprotein changes with the resulting lesion area, thereby leaving unresolved the question of whether CETP inhibition was antiatherogenic by virtue of having increased HDL-C or decreased non-HDL-C (33). In attempting to address this question, we used an AUC calculation to combine all of the lipoprotein data into single terms and performed regression analyses to determine which of the measured lipoprotein changes correlated with the extent of lesion formation in this model. Of course the AUC measurement is an approximation of

lipid and lipoprotein changes occurring over the course of the study and does not necessarily capture changes in lipid or lipoprotein fluxes that may be important for the development of atherosclerosis. Nevertheless, non-HDL-C AUC was positively correlated with the extent of lesion area in control rabbits but not in torcetrapib-treated animals, suggesting that torcetrapib treatment somehow mitigated the proatherogenic effects of non-HDL. Because atherosclerotic lesions develop over time, it is certainly conceivable that the reduced non-HDL-C levels during the initial phase of this study may have partially contributed to the reduction in atherosclerosis observed after 16 weeks in the torcetrapib treatment group. However, the treatment difference in non-HDL-C levels apparent during the initial phase of the study was not apparent when comparing the non-HDL-C AUCs of the two groups.

A similar analysis of HDL AUC did not show a significant inverse correlation with lesion area in either treatment group. Because lesion formation in this model is driven by the consumption of an atherogenic diet and the associated increase in non-HDL-C levels, the lack of a significant inverse correlation with HDL-AUC alone was not unexpected, especially considering the variability in non-HDL AUC values observed in both groups of rabbits. However, the TPC AUC/HDL-C AUC ratio was highly correlated with the extent of atherosclerosis in both groups of rabbits. This term (or the non-HDL AUC/HDL AUC ratio) captures both the proatherogenic effects of non-HDL lipoproteins and the possible antiatherogenic effects of HDL lipoproteins in a single term. The slopes of the regression lines fit to data from control and torcetrapib-treated groups were not different, suggesting that the HDL-C increase observed with torcetrapib is an important contributor to the reduction of aortic atherosclerosis observed in this study.

The suggestion that inhibition of CETP and the resulting large increases in HDL-C could be proatherogenic has been partially based on data from individuals deficient in CETP. Pharmacological inhibition may be distinct from complete CETP deficiency in that residual CETP activity may mitigate the accumulation of atypical HDL particles observed in individuals completely deficient in CETP. In support of this concept, the lipid and lipoprotein phenotypes of subjects partially deficient in CETP activity more closely resemble those of individuals treated with CETP inhibitors (43). Likewise, the functionality of HDL lipoproteins in subjects administered CETP inhibitors may be better preserved than is reported in individuals completely devoid of CETP activity (44).

Although our data indicate that CETP inhibition is antiatherogenic, the mechanisms involved are not clear. The robust increase in HDL-C levels with torcetrapib and the strong correlation between aortic lesion area and TPC AUC/HDL AUC ratio indicate that increased levels of HDL-C appear to significantly contribute to the antiatherogenic effect of torcetrapib. HDL lipoproteins have antioxidant, antiinflammatory, and antithrombotic effects, one or more of which might contribute to the reduced lesion area observed in this study. The antiatherogenic activity most often cited for HDL is its participation in reverse cholesterol transport, of which the initial step is the efflux of excess FC from cells. Because there was not a significant reduction of non-HDL AUC in torcetrapib-treated rabbits that could have resulted in differential lipoprotein deposition, it is conceivable that the reduced aortic cholesterol content in the torcetrapib-treated rabbits at necropsy was the result of enhanced cholesterol efflux supported by the increased plasma HDL levels.

To determine the effects of torcetrapib administration on cholesterol efflux, we used plasma isolated from a separate group of rabbits fed an atherogenic diet with or without torcetrapib. Compared with sera from control rabbits, sera from torcetrapib-treated rabbits stimulated more FC efflux from Fu5AH cells. There was also a highly significant correlation between efflux and the HDL content of the sera, irrespective of treatment. The efflux data presented here are in agreement with those obtained with sera from torcetrapib-treated patients (45) and rabbits treated with a structurally unrelated CETP inhibitor (46), in contrast to what had been reported previously for HDL obtained from CETP-deficient subjects (47). An earlier study had reported that HDL isolated from CETP-deficient individuals was deficient in promoting cholesterol efflux (44). ApoE-containing HDL from CETP-deficient subjects was recently demonstrated to stimulate cholesterol efflux from macrophages via an ABCG1-dependent pathway (48), with the discrepancy between these results and the previous data presumably attributable to the exclusion of the large, apoE-enriched subfraction (44). Because Fu5AH cells efflux cholesterol primarily via a scavenger receptor class B type I-dependent pathway (39), additional efflux studies using whole serum and lipoprotein subfractions and other cell types such as macrophages will be necessary to more fully understand the potential contributions of increased HDL-C levels to enhanced FC efflux.

Despite the direct effect of CETP deficiency on HDL clearance and its subsequent accumulation in plasma, our data do not preclude the possibility that increased cholesterol efflux could contribute to the increased HDL-C levels observed in torcetrapib-treated rabbits. However, this possibility seems remote. A CETP inhibitor structurally similar to torcetrapib increased HDL-C by ~2-fold in chow-fed rabbits (49), indicating that substantial increases of HDL-C with CETP inhibition can occur without a substantial accumulation of tissue cholesterol. The liver and intestine are the primary organs of HDL biogenesis, at least in mice (50, 51), but the relative contributions of hepatic, intestinal, and peripheral sources of the cholesterol transported by HDL are not well understood. The contribution of cholesterol emanating from macrophages in the arterial wall to circulating HDL-C is envisaged to be very small relative to that from other tissue sources (52), so circulating levels of HDL-C are probably not a sensitive indicator of the removal of lesional cholesterol.

It has been hypothesized that changes in fecal sterol excretion could provide a more meaningful measurement of the flux of cholesterol undergoing reverse cholesterol transport, but this has yet to be demonstrated conclusively. To our knowledge, reduction in atherosclerotic lesion

area (or aortic cholesterol levels) in rabbits by increasing HDL and/or apoA-I and measurement of fecal sterol excretion have not been measured in the same study. Infusion of apoA-I (53) or HDL (54) reduced aortic cholesterol content and/or lesion area in rabbits to a similar extent as torcetrapib treatment did in this study, but fecal sterol excretion was not measured. In four subjects with heterozygous familial hypercholesterolemia, acute infusion of proapolipoprotein A-I in phosphatidylcholine liposomes resulted in a "surprisingly large" increase in fecal sterol excretion (55), but the fraction effluxing from atherosclerotic lesions is unknown. CETP inhibition results in a concomitant reduction in circulating LDL-C in humans that could potentially contribute to decreased deposition of cholesterol in the vessel wall, thereby reducing the cellular cholesterol available for efflux. For these reasons, the inhibition of lesion formation in rabbits reported here and the stimulation of cholesterol efflux by plasma from torcetrapib-treated rabbits or human subjects are not necessarily inconsistent with the lack of an increase in fecal sterol excretion reported previously in torcetrapib-treated subjects (56). Additional studies will be necessary to further clarify the effect of CETP inhibition on reverse cholesterol transport.

In conclusion, our results suggest that CETP inhibition with torcetrapib retards the progression of atherosclerosis in rabbits, perhaps as a result of the increase of HDL-C. It remains to be determined which of the antiatherogenic properties of HDL are important in inhibiting lesion formation in this model and whether this effect will translate to humans. While this article was in preparation, all clinical development of torcetrapib was halted after the independent Data and Safety Monitoring Board monitoring the ILLUMINATE morbidity and mortality study for torcetrapib recommended terminating the study because of a statistically significant imbalance in mortality between patients receiving torcetrapib/atorvastatin and those receiving atorvastatin alone.

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