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Evacetrapib Reduces Pre β -1 HDL in Patients with Atherosclerotic Cardiovascular Disease or Diabetes

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Abstract

BACKGROUND AND AIMS—To investigate the effect of cholesteryl ester transfer protein (CETP) inhibitor on subclasses of HDL in patients with atherosclerotic cardiovascular disease or diabetes. CETP inhibitor-mediated a dramatic induction of HDL-cholesterol has no effect on the protection of cardiovascular disease (CVD). However, the mechanism is still unknown. Data on the effects of this class of drugs on subclasses of HDL are either limited or insufficient.

METHODS—Baseline and 3-month post-treatment samples from the atorvastatin 40 mg plus evacetrapib 130 mg (n=70) and atorvastatin 40 mg plus placebo (n=30) arms were utilized for this purpose. Four subclasses of HDL (large HDL1, medium HDL2, small HDL3, and Pre β -1 HDL) were separated according to their size and quantified by densitometry using a recently developed native polyacrylamide gel electrophoresis (PAGE) system.

RESULTS—Relative to placebo, while evacetrapib treatment dramatically increased HDL1 and HDL2 subclasses, it significantly reduced small dense HDL3 (27%) as well as Pre β -1 HDL (36%) particles. Evacetrapib treatment reduced total LDL, but also resulted in polydisperse LDL with LDL particles larger and smaller than the LDL subclasses of the placebo group.

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Author contributions: Y.C. and J.D. designed the study and performed most of experiments, and they contributed equally to this work; X.Z. and X.C. did some experiments and statistical analysis; L.W., H.C., and J.G. involved in designing the study and edited the paper; X.C.J. designed the study and wrote the manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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CONCLUSION—Evacetrapib reduced Pre β -1 HDL and small dense HDL3 in patients with ASCVD or diabetes on statin. Pre β -1 HDL and HDL2 are negatively interrelated. The results could give a clue to understand the effect of CETP inhibitors on cardiovascular outcomes.

TRIAL REGISTRATION—[ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02227784) NCT02227784.

Graphical Abstract

Keywords

CETP inhibitor; HDL-cholesterol; HDL subclasses; Pre β -1 HDL; Pre β -1 HDL measurement; Atherosclerotic cardiovascular disease or diabetes

Introduction

Epidemiological studies have shown high-density lipoprotein cholesterol (HDL-C) levels to be independently and inversely correlated to cardiovascular disease (CVD)¹. This relationship is thought to be largely mediated by the ability of HDL to transport excess cholesterol from peripheral tissues back to the liver for excretion, a process known as reverse cholesterol transport². Potent cholesteryl ester transfer protein (CETP) inhibitors, like torcetrapib, evacetrapib and anacetrapib, significantly increase HDL-C and decrease LDL-C levels, as monotherapy and in combination with statins^{3–6}. However, except for anacetrapib⁷, these lipoprotein changes have not resulted in reduction of major cardiovascular events (MACE)^{8–10}. It has been hypothesized that small increase in blood pressure and hsCRP, as well as qualitative changes in HDL subclasses observed with CETP inhibitors could partly offset the beneficial effect of LDL-C reduction¹¹.

It is well known that HDL particles are very heterogeneous¹², and many HDL subclasses have been described depending on the analytical technique used for separation. One of these subclasses, pre β 1-HDL, plays a critical role in reverse cholesterol transport but it is particularly challenging to quantify. Two-dimensional nondenaturing linear gel electrophoresis followed by apoA-I immunoblotting and image analysis were first utilized to identify pre β 1-HDL, pre β 2-HDL, and α HDL¹³. Subsequently, a further separation of lipid-free apoA-I from pre β 1-HDL using the same technique was described¹⁴. However, other investigators have not been able to distinguish lipid-free monomolecular apoA-I from pre β 1-

HDL using an in-house sandwich enzyme immunoassay^{15, 16}. A modified two-dimensional gel system has also been used to define 12 subfractions of HDL, including pre β 1-HDL and pre β 2-HDL¹⁷. We have recently established a native polyacrylamide gel electrophoresis (PAGE) system with lipid pre-staining for quantification in human serum of pre β 1-HDL and other HDL subclasses, as well as LDL¹⁸. In the present study, we utilized this method to evaluate the effect of evacetrapib on HDL subclasses in patients with atherosclerotic cardiovascular disease (ASCVD) or diabetes on background of atorvastatin 40 mg daily enrolled in the ACCENTUATE trial¹⁹.

Methods

Study design

Patient disposition and characteristics, as well as baseline and post-treatment laboratory value for the ACCENTUATE trial ([ClinicalTrials.gov NCT02227784](https://clinicaltrials.gov/ct2/show/study/NCT02227784)) have previously been reported¹⁹. For this research question, samples were available for 30 patients on atorvastatin 40 mg daily and 70 patients on atorvastatin 40 mg plus evacetrapib 130 mg daily, at baseline and after 90 days of treatment. Eli Lilly and Company provided Xian-Cheng Jiang's laboratory at SUNY Downstate Medical Center with the samples and certain information from the ACCENTUATE study for the analyses under a Material Transfer Agreement between Lilly and SUNY Downstate Medical Center in 2017. Submission of this manuscript for publication has followed the terms of that Agreement. The measurement in the study was in a blind fashion.

Study Procedure

The measurement was in a blind fashion. We followed an established a system¹⁸ for the measurement of subclasses of HDL. Briefly 1) Solution A: Acrylamide (9.75 g; Fluca) and 0.25 g N,N'-methylenebisacrylamide (Fluca) in deionized water to 100 ml final volume. 2) Solution B: Tris (18.30 g) to 24 ml of 1 N HCl and diluted to 100 ml final volume with deionized water. 3) Solution C: Acrylamide (19.60 g) and 0.40 g N,N'-methylenebisacrylamide in deionized water to 100 ml final volume. 4) Solution D: Tris (6.06 g) and 1.17 g EDTA-Na₂ in deionized water to 100 ml final volume. 5) Staining solution: Sudan Black-B (0.125 g; Sigma) to 25 ml isopropanol:ethylene glycol (4:1, V/V), mixed well, and incubated in a water bath at 37°C overnight. 6) Running buffer: Tris (6.0 g) and 28.8 g glycine in deionized water to 1000 ml final volume, and then diluted 10-fold with deionized water before use.

The gradient gels in 6 × 100 mm glass tubes were prepared as indicated in previously¹⁸. Each tube gel comprised three layers differing in polyacrylamide concentration (7.0%, 3.6%, and 3.0%) and height (45 mm, 40 mm, and 7 mm, respectively). Each gel tube was inserted vertically into an electrophoretic cell (DYY-III 27B Model, Liuyi Inc., Beijing). The upper and lower chambers of the electrophoretic cell contained ~400 ml and ~600 ml running buffer, respectively. Serum (100 μ l) was mixed with 10 μ l of staining solution, and 50 μ l was loaded on the top of the tube gel; electrophoresis was carried out for 3.0 h at 100 V. Gel image was acquired by a customized scanner (Model BeneScan-1000 from BENEFI and

MICROTEK, Inc. China). The lipid-staining area under the curve (AUC) was calculated automatically by the densitometer.

Statistical analysis

Continuous variables were reported as means $\pm$ standard deviation (SD) and were compared by paired Student's t-test between before treatment and after treatment. parameter (Pre β 1-HDL, HDL1, HDL2, HDL3 and LDL) was the difference between the parameter value after treatment and that before treatment. For univariate analyses, Pearson's correlation was used to determine the correlation of Pre β 1-HDL with other lipid parameters. P values < 0.05 were considered statistically significant. All analyses were done using SPSS version 16 analytical software (SPSS Inc., Chicago).

Results

As shown in Figure 1A, our tube native PAGE system could separate HDL (HDL total) into four fractions, pre β 1-HDL, HDL1, HDL2, and HDL3. The percentage of each HDL subclass in total HDL are pre β 1-HDL (about 5%), HDL1 (about 10 %), HDL2 (about 50%), and HDL3 (about 35%) (Fig. 1B). HDL1, HDL2, and HDL3 were confirmed by their densities, as previously described¹⁸. Pre β 1-HDL was also confirmed by the treatment of 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), an LCAT inhibitor. Serum was incubated in the absence or presence of 2 mM DTNB, pre β 1-HDL further conversion could be blocked after DTNB treatment (Fig. 1C).

Figure 2A shows typical results from three patients treated with atorvastatin plus evacetrapib and three treated with atorvastatin plus placebo. Evacetrapib treatment reduced pre β 1-HDL, HDL3, and LDL, and dramatically increased HDL2 and HDL1, with no changes observed in the patients on placebo. In Figure 2A, results from 2 patients with CETP homozygous deficiency (the G $\rightarrow$ A mutation in the first position of intron 14 of the CETP gene)²⁰ are shown for comparison. Like patients treated with evacetrapib, these 2 patients had no detectable pre β 1-HDL, reduced HDL3, and major increase in larger HDL.

We next sought to quantify the changes in lipoproteins. Evacetrapib treatment dramatically increased total HDL by 2.7 folds, HDL1 by 3.9 folds, and HDL2 by 4.6 folds, and reduced pre β 1-HDL by 36% and HDL3 by 27% (Table 1, and Fig. 3). Moreover, evacetrapib treatment reduced LDL by 36% (Table 1), with appearance of larger and smaller LDL particles (polydisperse LDL) as compared to placebo treated patients.

Univariate correlations of pre β 1-HDL (after treatment – before treatment) with other lipoprotein subclasses are shown in Table 2. pre β 1-HDL positively correlated with HDL3 and LDL, and negatively correlated with HDL2 only in the evacetrapib treated group.

DISCUSSION

The novel finding of the present study is the reduction of Pre β -1 HDL particles with evacetrapib treatment in patients with ASCVD or diabetes on atorvastatin 40 mg per day, when a recently developed native PAGE system was used to quantify this particular HDL

species. Moreover, we found that Pre β -1 HDL and HDL2 are negatively interrelated. As previously shown by other authors, here we also observe a reduction of highly functional small HDL particles with evacetrapib, in addition to the dramatic increase of large HDL^{21–24}. Finally, our native PAGE system clearly shows that evacetrapib treatment results in qualitative changes in the distribution of LDL species with prevalence of very large and very small particles, thus recapitulating the polydisperse LDL phenotype in CETP-deficient subjects²⁵.

Previous studies have shown that pre β 1-HDL particles increased after treatment with CETP inhibitors, such as evacetrapib and TA-8995, in subjects with mild dyslipidemia on atorvastatin 20 mg/d or rosuvastatin 10 mg/d, respectively^{23, 24}. These studies used nondenaturing two-dimensional gel electrophoresis (2DGE)²⁴ and/or immunofixation²³ to quantify pre β 1-HDL particles. Here, we report a reduction of Pre β -1 HDL particles with evacetrapib in a different population (ASCVD or diabetes) on higher atorvastatin dose (40 mg/d), using a different method to measure this HDL class.

Our native PAGE system and the traditional 2DGE clearly have a different ability to detect and quantify pre β 1-HDL, with the latter appearing less suitable for this measurement. Traditional 2DGE has been used to show that serum of patients with Tangier disease contains pre β 1-HDL, but not mature HDL²⁶. However, this technique could have limitations in distinguishing pre β 1-HDL from free apoA-I¹⁷. In contrast, our native PAGE system does not detect any pre β 1-HDL in serum of Tangier patients, in support of the concept that the absence of ABCA1 in these patients results in lack of free apoA-I lipidation and subsequent pre β 1-HDL formation¹⁸. Like Tangier patients, also those treated with evacetrapib in the present study had very low levels of pre β 1-HDL. It is well known that “pre β 1-HDL-like” lipid-poor apoA-I or nascent HDL is formed when lipid-free apoA-I retrieves phospholipids and cholesterol from cells in a process mediated by ABCA1^{27, 28}. Thus, the levels of pre β 1-HDL should be an indicator of the capacity for nascent HDL formation and initial reverse cholesterol transport.

Another key finding of this study is that even though evacetrapib treatment increases the number of large HDL, it actually reduces small dense HDL3 particles, like other CETP inhibitors. A decrease of small HDL particles has been observed by NMR with dalcetrapib at the highest dose (900 mg/d)²². Evacetrapib and TA-8995 also decreased small α -3 and pre α -3 HDL as measured by 2DGE, in both monotherapy and combination therapy^{23, 24}. Small, dense, and protein-rich HDL are currently believed to possess potent biological and atheroprotective activities^{29, 30}. For instance, HDL3 particles are enriched with specific sphingolipids, such as sphingomyelin and sphingosine-1-phosphate²⁹, which are involved in nitric oxide generation and protection against oxidative stress-associated endothelium dysfunction³¹. Very recent studies suggested that age acts as a confounding factor associated with both low HDL3 cholesterol and increased carotid atherosclerosis^{32, 33}. Moreover, HDL3 particles displayed greater efficacy in removing cellular cholesterol as compared with other HDL subpopulations²⁹.

The reverse cholesterol transport should compose two pathways: 1) HDL-mediated pathway³⁴; and 2) LDL-receptor-mediated pathway³⁴. CETP inhibitor blocks CE transfer from HDL

to LDL and blocks the second pathway. Thus, HDL2 particles could be associated with an abnormal reverse cholesterol transport. The high inter-relationship between pre β 1-HDL and HDL2 could reflect a situation that CETP inhibition-mediated accumulation of HDL2 can block the formation of pre β 1-HDL as well as HDL3 or promote its degradation.

Pre β 1-HDL and small HDL3 particles play an important role in cholesterol efflux, the first step of reverse cholesterol transport^{35–37}, and oxidative stress-associated endothelium dysfunction³¹. What we need to do should be promoting the formation of pre β 1-HDL and HDL3, for instance from triglyceride-rich lipoproteins-HDL pathway^{38, 39} or cholesterol efflux pathway⁴⁰.

We used the same samples as the ACCENTUATE trial which indicated an increase in cholesterol efflux after the treatment of the CETP inhibitor¹⁹. We believe that CETP inhibitor-mediated increasing of cholesterol efflux capacity is due to a dramatic induction of total HDLs and is not related with promoting reverse cholesterol transport. Similar study was conducted by Dr. Daniel J. Radar's group at University of Pennsylvania. They reported that both phospholipid transfer protein (PLTP) transgenic and PLTP knockout mice have significantly reduced HDL level in the circulation and both mice also have significantly reduced cholesterol efflux capacity. However, both overexpression and deficiency have no effect on macrophage reverse cholesterol transport⁴¹. Thus, plasma cholesterol efflux capacity measured by incubation of macrophages or cells overexpressed ABCA1 or ABCG1 with human or mouse plasma does not reflect the real situation of reverse cholesterol transport. The measured cholesterol efflux capacity should be greatly influenced by the HDL in the plasma used in the experiment.

There is a limitation of current study. We have only demonstrated a reduction in pre β 1-HDL and HDL3 but not a reduction in HDL functionality. We speculated that reduction of pre β 1-HDL could be a reason why CETP inhibitor-mediated HDL-cholesterol raising has no effect on CVD prevention. Further work should be done as according to a previous reports^{42,43}.

In summary, CETP inhibitor, evacetrapib, reduces Pre β -1 HDL and small dense HDL3 in patients with ASCVD or diabetes on statin. Pre β -1 HDL and HDL2 are negatively interrelated. Other CETP inhibitors could have similar effects. These results could give a clue to understand the effect of CETP inhibitors on cardiovascular outcomes. Moreover, we have to be aware of the limitation of the methods previously used for Pre β -1 HDL quantification and, in fact, we should re-evaluate these measurements. We believe Pre β -1 HDL is an anti-atherogenic^{18, 44} instead of pro-atherogenic^{45–47} lipoprotein.

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Figure 1. Native PAGE system to separate HDL subclasses. Human serum pre-stained with Sudan Black. A. Three representative samples: LDL and HDL1, HDL2, HDL3, and pre β 1-HDL bands are indicated. B. Corresponding densitometric scans. C. Human serum treated with or without DTNB (2.0 mM) treatment, 37°C for 12 hours, then pre-stained with Sudan Black. Gel image was acquired by a customized scanner. The lipid-staining area under the curve (AUC) was calculated automatically by the densitometer.

Figure 2. Representative samples showing the effect of evacetrapib on LDL and HDL subclasses. A. Three representative samples of patients on evacetrapib and three on placebo. B. Two representative samples of healthy subjects and two of patients with CETP homozygous deficiency (the G→A mutation in the first position of intron 14 of the CETP gene).

Figure 3. Evacetrapib treatment significantly decreased Preβ-1 HDL and HDL3.
Dot plotting for all the samples.

Table 1.

Effect of evacetrapib on human serum lipoproteins

	Variable	Before	After	p value
Placebo	Total HDL	6642 ± 2375	6815 ± 2830	0.40
	Pre β -1 HDL	370 ± 90	384 ± 104	0.39
	HDL3	2805 ± 922	2888 ± 1025	0.46
	HDL2	2942 ± 1176	3089 ± 1462	0.42
	HDL1	425 ± 186	455 ± 239	0.24
	LDL	6163 ± 2101	6913 ± 2699	0.07
Evacetrapib	Total HDL	5953 ± 2202	15818 ± 8216	<0.0001
	Pre β -1 HDL	394 ± 101	254 ± 97	<0.0001
	HDL3	2517 ± 817	1832 ± 635	<0.0001
	HDL2	2631 ± 1126	12175 ± 6034	<0.0001
	HDL1	411 ± 158	1589 ± 1451	<0.0001
	LDL	6003 ± 1910	3866 ± 939	<0.0001

After electrophoresis, each tube was subjected to densitometry (604 nm; Model CS-9301, Shimadzu, Inc. Japan) and peak area for each lipoprotein was obtained and then compared. Value (Arbitrary Unit) for variables are given as means $\pm$ SD and evaluated using paired t-test.

Table 2.
Univariate correlates of pre β -1 HDL among total cohort, placebo group and evacetrapib group:

Pearson's correlation was used to determine correlation of Pre β -1 HDL with other lipid parameters.

Variable	Total cohort (n=100)		Placebo (n=30)		Evacetrapib (n=70)	
	r	p	r	p	r	p
HDL3	0.474	<0.0001	0.156	0.411	0.367	0.002
HDL2	-0.618	<0.0001	-0.050	0.793	-0.378	0.001
HDL1	-0.307	<0.0001	0.096	0.614	-0.084	0.489
LDL	0.518	<0.0001	-0.001	0.995	0.376	0.001