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Assessing the mechanisms of cholesteryl ester transfer protein inhibitors

Meng Zhang[a,b], Dongsheng Lei[a], Bo Peng[a], Mickey Yang[a], Lei Zhang[a], M. Art Charles[c], Kerry-Anne Rye[d], Ronald M. Krauss[e], Douglas G. Johns[f], Gang Ren[a,*]

[a] Molecular Foundry, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA
[b] Department of Applied Science & Technology, University of California, Berkeley, CA 94720, USA
[c] School of Medicine, University of California–San Francisco, San Francisco, CA 94110, USA
[d] School of Medical Sciences, Faculty of Medicine, University of New South Wales, Sydney, NSW 2052, Australia
[e] Children's Hospital Oakland Research Institute, Oakland, CA 94609, USA
[f] Merck Research Laboratories, Rahway, NJ 07065, USA

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ABSTRACT

Cholesteryl ester transfer protein (CETP) inhibitors are a new class of therapeutics for dyslipidemia that simultaneously improve two major cardiovascular disease (CVD) risk factors: elevated low-density lipoprotein (LDL) cholesterol and decreased high-density lipoprotein (HDL) cholesterol. However, the detailed molecular mechanisms underlying their efficacy are poorly understood, as are any potential mechanistic differences among the drugs in this class. Herein, we used electron microscopy (EM) to investigate the effects of three of these agents (Torcetrapib, Dalcetrapib and Anacetrapib) on CETP structure, CETP-lipoprotein complex formation and CETP-mediated cholesteryl ester (CE) transfer. We found that although none of these inhibitors altered the structure of CETP or the conformation of CETP-lipoprotein binary complexes, all inhibitors, especially Torcetrapib and Anacetrapib, increased the binding ratios of the binary complexes (e.g., HDL-CETP and LDL-CETP) and decreased the binding ratios of the HDL-CETP-LDL ternary complexes. The findings of more binary complexes and fewer ternary complexes reflect a new mechanism of inhibition: one distal end of CETP bound to the first lipoprotein would trigger a conformational change at the other distal end, thus resulting in a decreased binding ratio to the second lipoprotein and a degraded CE transfer rate among lipoproteins. Thus, we suggest a new inhibitor design that should decrease the formation of both binary and ternary complexes. Decreased concentrations of the binary complex may prevent the inhibitor was induced into cell by the tight binding of binary complexes during lipoprotein metabolism in the treatment of CVD.

1. Introduction

Elevated plasma low-density lipoprotein cholesterol (LDL-C) and decreased high-density lipoprotein cholesterol (HDL-C) levels are two major risk factors for cardiovascular disease (CVD) [1]. Drugs that decrease LDL-C levels (for example, statins) have consistently been shown to decrease the incidence of CVD. In contrast, drug-induced increases in HDL-C levels have not yet been clearly shown to decrease CVD events [2].

Cholesteryl ester transfer protein (CETP) plays a key role in the transfer of neutral lipids between HDL and LDL particles and contributes to the transfer of cholesteryl ester into atherogenic LDL particles. Previous genetic studies of a family with a well-established inherited CETP deficiency have revealed that mutations in CETP can lead to a splicing defect and are associated with elevated HDL-C levels [3,4]. To date, numerous CETP inhibitors have been identified and assessed in clinical trials. The CETP inhibitors that have been previously studied or are currently in phase III outcome studies include Torcetrapib [5], Dalcetrapib [6], Anacetrapib [7], Evacetrapib [8] and TA-8995 [9]. Despite the clinical interest in CETP inhibitors, their detailed mechanisms of action affecting CETP function and neutral lipid transfer remain poorly understood.

Human CETP is a plasma glycoprotein composed of 476 amino acids and has a molecular mass of ~53 kDa before post-translational modification (fully glycosylated CETP has a molecular weight of ~74 kDa) [10]. On the basis of its crystal structure, CETP has a banana-like shape with four structural components: an N-terminal β-barrel domain, a C-terminal β-barrel domain, a central β-sheet, and a C-terminal extension (a distorted amphipathic helix (i.e., helix X) involving Glu465-Ser476 at the C-terminus) [11].

Biochemical studies have revealed that CETP interacts with surface phospholipids of HDL particles via a hydrophilic/hydrophobic

* Corresponding auhtor at: Lawrence Berkeley National Laboratory, Molecular Foundry Rm 2220, 1 Cyclotron Road, MS 67R2206, Berkeley, CA 94720, USA.
E-mail address: gren@lbl.gov (G. Ren).

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interaction [12,13]. Although a protein-lipid-involved binding system can be detected and analyzed [14], usually with co-sedimentation assays [15] and micro-calorimetry [16], the particular domains of CETP involved in binding lipoproteins and the detailed binding mechanisms remain elusive. The difficulty in studying the mechanism of CETP lies in the heterogeneity of its lipoprotein substrates and in the softness and high flexibility of their complex three-dimensional (3D) structure [17–19]. These properties limit the application of the other experimental procedures. For example, gel shift studies are challenging for the separation of molecules with large variations in molecular mass (the molecular mass of LDL is ~10 times that of HDL and ~250 times that of CETP). Gel filtration is challenging for isolating soft molecules, especially the one containing lipids. Ultracentrifugal separation may cause detachment of CETP from the lipoprotein [20]. Fluorescence resonance energy transfer (FRET) studies are limited by difficulties in expressing the full-length apolipoprotein B 100 (an LDL-containing protein of ~500 kDa, among one of the largest proteins) and determining a method to produce the reconstituted LDL. Immunological quantification of lipoproteins may also be inaccurate because of different combinations of polypeptides and modifications of HDL [21,22]. Most importantly, the binding of CETP is not a stationary process. HDL can alter the shape and components along with time in an HDL-CETP mixture [23], thus causing difficulty in producing accurate quantitative measurements.

Electron microscopy (EM) has an advantage over traditional biochemistry assays in studying lipoproteins, because of the large variety of lipoprotein subclasses [24]. Our early EM studies have shown that CETP bridges HDL and LDL together, thereby forming a ternary complex [25] in which the N-terminal β-barrel domain inserts into the surface lipid monolayer of HDL. The observation of a ternary complex supports the "tunnel mechanism" of CETP for the transfer of neutral lipids between different lipoproteins. Our EM study has revealed that the binding between CETP and HDL is mediated by a protein-lipid interaction [26]. This protein-lipid interaction makes it possible for five or more CETPs to share one HDL substrate (more than the number of HDL-containing proteins). Recently, molecular dynamics (MD) simulations in a CETP study demonstrate that the N-terminal β-barrel domain is flexible [27,28] and can penetrate into the HDL surface, thereby facilitating the uptake of cholesteryl ester [29]. The latest all-atomic MD simulation shows that CEs can be transferred through the CETP tunnel under a series of driving forces [30]. A parallel study using coarse-grained MD simulation on a microsecond scale has also suggested that CETP possesses a high degree of conformational flexibility and can form a continuous tunnel traversing its long axis [28], through which CEs and triglycerides (TGs) can be directionally transferred in the absence of an additional driving force.

Although MD simulations have predicted several underlying CETP mechanisms in CE transfer [27,29–32], experimental mechanistic studies of CETP inhibition at the molecular level remain to be performed. Herein, we used EM techniques, including cryo-electron microscopy (cryo-EM) and optimized negative staining (OpNS), to investigate the effects of CETP inhibitors on the CETP-lipoprotein structure and their conformations under various incubation times.

2. Results

Effects of CETP inhibitors (Torcetrapib, Anacetrapib and Dalcetrapib) on CETP structure—Cryo-EM is a commonly used method to study protein structures under near-native conditions because it prevents possible artifacts induced by fixatives and stains, such as lipid stacking and flatness. However, images of small proteins (< 100 kDa) generally are of very low contrast, thus making their visualization and 3D reconstruction a challenging process. Given that CETP is an approximately 53 kDa asymmetric molecule (~74 kDa for fully glycosylated CETP) that is too small for cryo-EM, optimized negative staining (OpNS) [33,34] was used to investigate how CETP inhibitors influence the CETP structure.

OpNS is a negative staining method that has been refined from conventional NS protocols [35] by using cryo-EM images of apolipoprotein E_4 HDL as a control [33]. Notably, the OpNS protocol decreases the rouleaux artifact of lipoprotein particles [19,33]. OpNS has been validated through cryo-EM images of 84-base pair double-stranded DNA [36] and proteins with known structures, including GroEL and proteasomes [34]. The unique capability of OpNS to allow examination of small proteins has been documented with the 53 kDa cholesteryl ester transfer protein [25] and the IgG1 antibody and its peptide conjugates [37], which are challenging targets for cryo-EM imaging. For this reason, OpNS was chosen to examine the effects of CETP inhibitors on CETP structure.

Recombinant human CETP harboring an N341Q [14] mutation at a glycosylation site to enhance production yield yet exhibiting identical behavior to wild-type CETP in lipid transfer assays [38] was incubated with each inhibitor at its maximal inhibitory concentration (approximately 10 μM) [14]. After 1 h of incubation, samples were prepared using OpNS and examined by EM. As a control, CETP was incubated with inhibitor buffer only. On the basis of a survey micrograph and representative particle images, CETP appeared to have a banana-like shape, similar to its crystal structure [11]. No obvious polymerization, aggregation or conformational changes were observed under any of the experimental conditions (Fig. 1A–D, Supplemental Fig. 1–4). In the control sample (Fig. 1A), CETP measured 12.4 ± 1.9 nm in length and 4.2 ± 0.5 nm in width. The dimension was similar to that in crystals

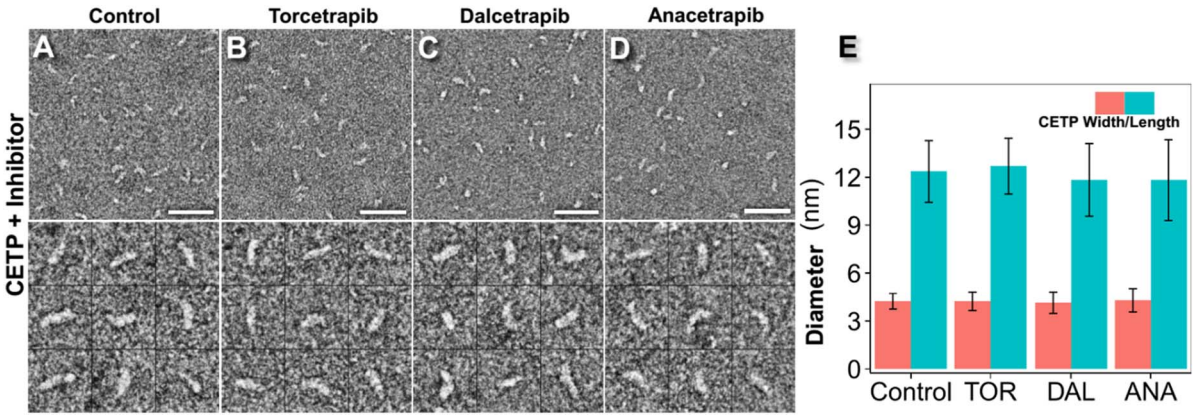

Fig. 1. Effects of inhibitors on CETP structure by OpNS EM. A) Survey view of optimized negative-staining EM images (top panel) and representative particle images of CETP (bottom panel), B) CETP incubated with Torcetrapib, C) CETP incubated with Dalcetrapib, D) CETP incubated with Anacetrapib, each at 37 °C for up to 1 h. E) Statistical analysis of CETP dimensions before and after treatment with inhibitors. *p*-values of 0.13, 0.06 and 0.06 were obtained for length and 0.91, 0.24 and 0.51 for width after treatment with Torcetrapib, Dalcetrapib and Anacetrapib, respectively (Student's *t*-test). Particle window size: A–D, 30 nm. Scale bars: 45 nm.