Evacetrapib is a novel, potent, and selective inhibitor of cholesteryl ester transfer protein that elevates HDL cholesterol without inducing aldosterone or increasing blood pressure

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Abstract  Cholesteryl ester transfer protein (CETP) catalyzes the exchange of cholesteryl ester and triglyceride between HDL and apoB-containing lipoprotein particles. The role of CETP in modulating plasma HDL cholesterol levels in humans is well established and there have been significant efforts to develop CETP inhibitors to increase HDL cholesterol for the treatment of coronary artery disease. These efforts, however, have been hampered by the fact that most CETP inhibitors either have low potency or have undesirable side effects. In this study, we describe a novel benzazepine compound evacetrapib (LY2484595), which is a potent and selective inhibitor of CETP both in vitro and in vivo. Evacetrapib inhibited human recombinant CETP protein (5.5 nM IC₅₀) and CETP activity in human plasma (36 nM IC₅₀) in vitro. In double transgenic mice expressing human CETP and apoAI, evacetrapib exhibited an ex vivo CETP inhibition ED₅₀ of less than 5 mg/kg at 8 h post oral dose and significantly elevated HDL cholesterol. Importantly, no blood pressure elevation was observed in rats dosed with evacetrapib at high exposure multiples compared with the positive control, torcetrapib. In addition, in a human adrenal cortical carcinoma cell line (H295R cells), evacetrapib did not induce aldosterone or cortisol biosynthesis whereas torcetrapib dramatically induced aldosterone and cortisol biosynthesis. Our data indicate that evacetrapib is a potent and selective CETP inhibitor without torcetrapib-like off-target liabilities. Evacetrapib is currently in phase II clinical development.—Cao, G., T. P. Beyer, Y. Zhang, R. J. Schmidt, Y. Q. Chen, S. L. Cockerham, K. M. Zimmerman, S. K. Karathanasis, E. A. Cannady, T. Fields, and N. B. Mantlo. Evacetrapib is a novel, potent, and selective inhibitor of cholesteryl ester transfer protein that elevates HDL cholesterol without inducing aldosterone or increasing blood pressure. J. Lipid Res. 2011. 52: 2169–2176.

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Although statins have proven to be effective in reducing coronary artery disease through plasma LDL cholesterol reduction, residual risks of developing cardiovascular disease remain. Epidemiological studies suggest that beyond reducing LDL cholesterol, the inverse correlation of plasma HDL cholesterol to coronary artery disease may provide additional opportunities for further intervention. It is estimated that an elevation of 1 mg/dl plasma HDL cholesterol results in 2–3% reduction in cardiovascular risk (1, 2). Potential mechanisms for HDL cholesterol protection include its involvement in reverse cholesterol transport (3), anti-inflammatory (4), anti-oxidative (5), and anti-thrombotic processes, and vessel relaxation (6). The relative quantitative contribution of each mechanism to coronary artery disease protection remains to be fully elucidated.

CETP is a 74 kDa glycoprotein that is primarily synthesized in human liver and adipose tissues and is secreted into the circulation, where it becomes associated with HDL particles. It catalyzes the reciprocal neutral lipid exchange (cholesteryl ester and triglyceride) between HDL and apoB-containing lipoprotein particles, and as a result, plasma HDL cholesterol is reduced (7). Although plasma CETP activity is inversely correlated to HDL cholesterol levels (8), the role of CETP in coronary artery disease has not been conclusively established. Recent studies in humans suggest that CETP may function as a pro-atherogenic molecule (9). The atherogenicity of CETP in animal models appears to be dependent on the background of the animal models. In most atherosclerosis models, CETP functions as a pro-atherogenic molecule.

Evacetrapib is a 74 kDa glycoprotein that is primarily synthesized in human liver and adipose tissues and is secreted into the circulation, where it becomes associated with HDL particles. It catalyzes the reciprocal neutral lipid exchange (cholesteryl ester and triglyceride) between HDL and apoB-containing lipoprotein particles, and as a result, plasma HDL cholesterol is reduced (7). Although plasma CETP activity is inversely correlated to HDL cholesterol levels (8), the role of CETP in coronary artery disease has not been conclusively established. Recent studies in humans suggest that CETP may function as a pro-atherogenic molecule (9). The atherogenicity of CETP in animal models appears to be dependent on the background of the animal models. In most atherosclerosis models, CETP functions as a pro-atherogenic molecule.
(10–15), and in an LCAT transgenic background CETP activity reduces the development of atherosclerosis (16).

Development of CETP inhibitors has been reported in recent years, namely dalcetrapib (JTT-705), torcetrapib, and anacetrapib. Dalcetrapib is a thiol agent, which acts as an irreversible inhibitor and appears to modify one of the 13 cysteine residues within the CETP protein that may be involved in either lipid binding or lipid transfer activities (14, 17). The CETP inhibitory mechanisms of torcetrapib and anacetrapib remain to be elucidated, and recent data suggest that both compounds increase the association of CETP with HDL particles (17, 18). Both dalcetrapib and torcetrapib demonstrated anti-atherosclerotic activity in hypercholesterolemic rabbits (14, 15). More importantly, torcetrapib inhibited lesion development in this model without reducing LDL cholesterol levels, suggesting that HDL particles derived from CETP inhibition is functional in vessel protection (15). These molecules have been studied in humans and have been shown to significantly increase HDL cholesterol (19–25). Significant LDL cholesterol reduction has also been observed for torcetrapib and anacetrapib. In addition, the size of both HDL and LDL particles is increased (18, 26). The latter observation suggests a potential further benefit of CETP inhibition because small, dense LDL particles are considered to be an important risk factor in the development of atherosclerosis.

Torcetrapib was studied in a phase III clinical trial (the Investigation of Lipid Level Management to Understand its Impact in Atherosclerotic Events, ILLUMINATE) as a fixed dose combination with atorvastatin. However, this phase III study was prematurely discontinued because of findings of increased cardiovascular events and total mortality (27). Although the exact reasons for these observations remain to be clarified, torcetrapib has been found to increase blood pressure and plasma aldosterone levels and to alter electrolyte concentrations in humans (27). These effects are believed to be off-target as suggested by recent studies (25, 28, 29). In addition, subsequent analysis of the ILLUSTRATE (The Investigation of Lipid Level Management Using Coronary Ultrasound to Assess Reduction of Atherosclerosis by CETP Inhibition and HDL Elevation) and ILLUMINATE trial data suggests a significant inverse relationship between changes in HDL cholesterol and coronary atheroma volume and cardiovascular events (30, 31). Thus, a potent, selective CETP inhibitor without off-target side effects may be viable as a way to increase HDL cholesterol for the purpose of preventing and treating cardiovascular disease.

In this study, we describe such a compound, evace-trapib, which is a novel, selective CETP inhibitor that is currently in phase 2 clinical development. Specifically, we describe its inhibition of CETP activity both in human plasma and in a human CETP transgenic mouse model. Importantly, the inhibition of CETP in the animal model was associated with significant HDL cholesterol elevation without increases in aldosterone or blood pressure.

METHODS

Human plasma CETP BODIPY assay

Human plasma obtained from the Central Indiana Regional Blood Center was filtered, pooled, aliquoted, and frozen at −80°C. The concentration of CETP in the plasma was measured by ELISA and found to be in the range of 1–4 μg/ml. A substrate micro-emulsion particle containing a fluorescent cholesterol ester analog that self quenches its fluorescent signal was prepared as reported previously (32). The particle contained 15 mol percent BODIPY-CE analog (cholesteryl 4,4-difluoro-5-(2-pyryl)pyrrolyl) 4-bora-3a,4a-diaza-s-indacene-3-undecanoate, Molecular Probes C-12681), 33 mol percent cholesteryl oleate (Sigma C-9253), 8 mol percent triolein (glyceryl trioleate, Sigma-T-7140), and 44 mol percent POPC (1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine, Avanti Polar Lipids 850457). The components were mixed and the solvents were evaporated under N2 and then dissolved in dioxane (Allied Signal 087-1). The components were injected through a 26g gauge-needle on a Hamilton syringe into buffer (7.4 pH Tris, NaCl, EDTA) in a 37°C ultrasonic water bath. The substrate was aliquoted and stored at 4°C.

Plasma was thawed and warmed to 37°C for use in the assay. Compound dilutions were made in 100% DMSO. Plasma was mixed with compound and allowed to incubate at 37°C for 30 min. Substrate particles were then added to plasma and compound mixture. Final volume percentages in the reaction were: plasma 96%, substrate particle 3%, and DMSO (with compound) 1%. The reaction was allowed to incubate at 37°C for 240 min. The fluorescent signal was measured in a fluorescent plate reader with 544 nm excitation and 595 nm emission upon the transfer of fluorescent cholesterol ester analogs to apoB-containing lipoproteins. IC50 values (concentration of compound causing a 50% inhibition of CETP activity) were determined by nonlinear regression.

Buffer CETP BODIPY assay

Human CETP cDNA was amplified from a human liver cDNA library and the sequence was confirmed to be identical to the published sequence. The cDNA was subcloned into a pcDNA3.1 vector, under the control of CMV promoter. A stable line was established in CV1 cells in which the above-mentioned construct was used to express the recombinant human CETP. The medium contained the secreted recombinant CETP protein and the amount (19 ng/ml) was quantified by an ELISA kit. The medium was then aliquoted in 4% BSA and stored at −80°C. The stock CETP protein was diluted 150-fold in CETP buffer (10 mM Tris, 150 mM NaCl, and 2 mM EDTA) before use. The assay was set up in a 96-well plate. Each well received 97.5 μl of CETP buffer (final concentration 7 nM) and 2.5 μl of compound stock. After a 30 min incubation at 37°C, 5 μl of substrate stock (the same stock used in the human plasma CETP assay), 0.16 μl of VLDL stock (2.5 mg/ml, Intracel) and 145 μl of CETP buffer were added, and the incubation was continued for another 4 h. Signal was read as described above for the human plasma CETP assay.

Real-time PCR detection of CYP11B2 and CYP11B1 mRNA

CYP11B2 and CYP11B1 gene expression as evaluated by real-time PCR was performed according to TaqMan Gene Expression Assay guidelines (Applied Biosystems, Foster City, CA). The TaqMan probes and primers were purchased from Applied Biosystems (CYP11B1 ID: Hs01596404_m1 and CYP11B2 ID: Hs01597732_m1). The human adrenal cortical carcinoma cell line H295R was purchased from ATCC, and cells were grown in
A novel CETP inhibitor without blood pressure or aldosterone effect

RESULTS

In vitro pharmacology

Evacetrapib (LY2484595) is a novel benzazepine-based CETP inhibitor that has been developed at Lilly Research Laboratories. As shown in Fig. 1 for comparison, anacetrapib is a substituted oxazolidinone, torcetrapib is a substituted dihydroquinoline, and dalcetrapib is a substituted thiopropriionate. The in vitro activity of evacetrapib against CETP was first tested in the buffer CETP assay, in which human recombinant CETP protein was used as the source for the protein activity. The concentration of the compound
A double transgenic mouse line was generated by breeding the two transgenic models (37). The desired in vivo activities of human CETP inhibitors included the specific inhibition of human CETP activity and the elevation of HDL cholesterol. Evacetrapib administered orally at 30 mg/kg resulted in 98.4%, 98.6%, and 18.4% inhibition of CETP activity at 4, 8 and 24 h post dose respectively. Evacetrapib dosed orally at 30 mg/kg resulted in 129.7% increase in HDL-C 8 h after oral administration (Fig. 2A, B). The efficacy of evacetrapib was comparable to that of torcetrapib.

To ensure the observation from the initial single dose in vivo efficacy study and also to define the relative in vivo potency and efficacy compared with torcetrapib, evacetrapib was further evaluated in the human CETP/ApoAI double transgenic mice at multiple doses. The ED50 values of CETP inhibitory activity 8 h post oral dosing for evacetrapib in two dose-response studies were calculated to be 3.5 and 4.1 mg/kg respectively (representative study shown in Fig. 3A) compared with ED50 values of 4.0, 2.3 and 1.3 mg/kg of torcetrapib in three separate studies (data not shown). Dose dependent HDL-C elevation was observed for evacetrapib (Figs. 3B), and this was further proven by FPLC analysis of lipoproteins (Fig. 3C). These data indicated that evacetrapib is a potent CETP inhibitor in vivo that results in significant HDL-cholesterol elevation in an appropriate animal model.

Evacetrapib does not increase blood pressure in Zucker diabetic fatty rats

In the ILLUMINATE trial, 60 mg of torcetrapib daily increased systolic blood pressure by 5.4 mmHg (27). This observation is believed to be an off-target effect of torcetrapib, as dalcetrapib and anacetrapib did not increase blood pressure in humans. It was thus essential for us to investigate whether evacetrapib would increase blood pressure at the preclinical stage. To do this, we initially...
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Evacetrapib does not induce aldosterone or cortisol synthesis in H295R cells

Besides the elevation in blood pressure, torcetrapib also resulted in changes in electrolytes in humans. It was found that the K+ and Na+ balance was changed most likely resulting from the corresponding CETP inhibition. To further evaluate the blood pressure elevation effect of torcetrapib, a dose response study was conducted in ZDF rats. As shown in Fig. 4A, torcetrapib dose-dependently increased blood pressure in ZDF rats with a maximum increase in MAP of 14 mmHg in the first 2 h post oral dosing. The effect of evacetrapib on the MAP was then evaluated with vehicle, torcetrapib, or evacetrapib in this model. Torcetrapib significantly elevated MAP (7.6 mmHg) at 60 mg/kg whereas evacetrapib did not demonstrate any significant change in MAP (Fig. 4B). Torcetrapib achieved a 64-fold exposure multiple and evacetrapib had a 142-fold exposure multiple. These results suggest that evacetrapib is a potent CETP inhibitor that will not likely induce blood pressure increases in humans.

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from the significant increase in plasma aldosterone in humans that received torcetrapib in the ILLUMINATE trial (27). Recent studies using a human adrenal cortical carcinoma H295R cell line suggested that torcetrapib directly induced aldosterone as well as cortisol synthesis and secretion through upregulation of two key genes involved in aldosterone and cortisol synthesis, Cyp11B1 and Cyp11B2, which encode aldosterone synthase and cortisol synthase, respectively (28). In our initial exploration of mechanisms through which torcetrapib induced aldosterone synthesis, we also observed that Cyp11B1 and Cyp11B2 mRNA were regulated by torcetrapib in H295R cells and that this was accompanied by increased aldosterone and cortisol levels in the culture medium (data not shown). A branched DNA (33) assay was then developed that took advantage of the sequence identities between the two genes. This assay detected Cyp11B1 or Cyp11B2 mRNA that was induced by torcetrapib (Fig. 5). Compared with torcetrapib, evacetrapib had no activity in the same experiment with compound concentrations up to 10 μM. These data indicated that evacetrapib is a potent CETP inhibitor that will not induce aldosterone or cortisol synthesis.

**DISCUSSION**

The inverse relationship of HDL cholesterol to the incidence of coronary artery disease has served as a primary driving force in developing HDL-raising therapies. Recent studies on HDL structure and function favor the hypothesis that HDL plays an active role in vessel protection, potentially through a variety of different mechanisms (38). CETP has emerged as a prime target to modulate HDL cholesterol (7, 9). Several CETP inhibitors have been developed in recent years, and their clinical efficacy in raising HDL cholesterol has been proven (19–23, 25). Although the inhibitory mechanism may be different (17, 39), dalcetrapib (JTT-705), torcetrapib, and anacetrapib all significantly elevated HDL cholesterol in clinical studies. Significant LDL cholesterol lowering was also observed for torcetrapib and anacetrapib suggesting reduced cholesterol transfer from HDL to apoB-containing lipoprotein particles (25, 27). The phase III study on torcetrapib to evaluate compound efficacy and safety was prematurely terminated because of increased cardiovascular events and total mortality in the torcetrapib arm (27). The cardiovascular toxicity of torcetrapib is believed to be off-target, however, as dalcetrapib and anacetrapib do not increase blood pressure or change the electrolyte balance in humans (21, 25). In addition, the HDL cholesterol elevation resulting from CETP inhibition in the ILLUMINATE trial was a strong inverse predictor of cardiovascular events in the torcetrapib-treated group (31). Furthermore, analyses on the ILLUSTRATE trial suggested that the atheroma
Evacetrapib is a potent CETP inhibitor as was evaluated by two in vitro assays. In the buffer CETP assay, the absolute potency of the compound was 5.5 nM. In the human plasma CETP assay, the CETP concentration is about 2 μg/ml (25 nM) and the 36 nM IC_{50} value again indicates that evacetrapib is a potent CETP inhibitor against either the recombinant protein or CETP from human plasma. Evacetrapib is apparently much more potent than dalcetrapib. At a 600 mg dose, dalcetrapib only increases HDL cholesterol by about 30%, and no LDL cholesterol reduction was obvious at this dose (21). Thus, the efficacy of increasing HDL cholesterol and reducing LDL cholesterol by dalcetrapib is significantly limited by its potency. It is possible that part of the potential cardiovascular benefit from CETP inhibitors may derive from a significant LDL cholesterol reduction. It appears that a significant LDL cholesterol reduction is associated with a near complete inhibition of CETP, and in this regard, evacetrapib represents a significant advantage over dalcetrapib. The potency of evacetrapib appears greater than those of torcetrapib and anacetrapib, which achieved complete inhibition of CETP, resulting in a significant LDL cholesterol reduction and a dramatic HDL cholesterol elevation in clinical studies. As expected, evacetrapib dose-dependently inhibited human CETP activity in the human apoAI/CETP double transgenic mice and elevated HDL cholesterol levels. Comparable ED_{50} values were found for evacetrapib and torcetrapib. These data indicate that evacetrapib is a potent CETP inhibitor both in vitro and in vivo and that it will most likely significantly elevate HDL cholesterol and reduce LDL cholesterol in humans.

Contrary to the off-target effects of torcetrapib, evacetrapib is free of blood pressure and aldosterone induction. We first screened a variety of rat strains and identified the ZDF rat as a sensitive model to examine potential compound effect in raising blood pressure. Our findings in ZDF rats are consistent with other reports that the blood pressure induction effect of torcetrapib can be monitored in preclinical models (29). In the ZDF model, evacetrapib exceeded the exposure multiple more than 124-fold without blood pressure induction whereas significant and dose-dependent induction of blood pressure was observed for torcetrapib at comparable doses and exposure multiples. Our independent studies in H295R cells are also consistent with the previous report that torcetrapib induced aldosterone and cortisol directly from adrenal cortical cells (28). In H295R cells, the induction of Cyp11B1 and Cyp11B2 by torcetrapib was fairly potent and dramatic, whereas evacetrapib had no activity in the same assay.

Collectively, our data indicate that evacetrapib represents a novel, potent, and selective CETP inhibitor free of blood pressure and aldosterone induction off-target activities. Together, these data suggest that evacetrapib holds great promise as an agent to test whether CETP inhibition in humans provides cardiovascular protection. Evacetrapib is currently in phase II clinical trials.

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