Evacetrapib Is a Novel, Potent, and Selective Inhibitor of Cholesteryl Ester Transfer Protein that Elevates High-Density Lipoprotein Cholesterol without Inducing Aldosterone or Increasing Blood Pressure

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Running title: A novel CETP inhibitor without blood pressure or aldosterone effect
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Abstract

Cholesteryl ester transfer protein (CETP) catalyses the exchange of cholesteryl ester and triglyceride between high-density lipoproteins (HDL) and apolipoprotein B (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 apolipoprotein AI (apoAI), evacetrapib exhibited an ex vivo CETP inhibition ED₅₀ of less than 5 mg/kg at 8 hours 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 to the positive control, torcetrapib. In addition, in a human adrenal cortical carcinoma cell line (H295R cells), evacetrapib did not induce aldosterone or cortisol biosynthesis while 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.
**Introduction**

While 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 KD 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). While 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.
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 since 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). While the exact reasons for this observation 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, evacetrapib, 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 analogue that self quenches its fluorescent signal was prepared as reported previously (32). The particle contained 15 mole percent BODIPY-CE analogue (cholesteryl 4,4-difluoro-5-(2-pyrrolyl)-4-bora-3a,4a-diaza-s-indacene- 3-undecanoate, Molecular Probes C-12681), 33 mole percent cholesteryl oleate (Sigma C-9253), 8 mole percent triolein (glyceryl trioleate, Sigma T-7140), and 44 mole percent POPC (1-palmitoyl-2-oleoyl-sn-glycero-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 26s 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 minutes. 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 minutes. 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 analogues to apoB-containing lipoproteins. IC\textsubscript{50} values (concentration of compound causing a 50% inhibition of CETP activity) were determined by non-linear 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/\mu l) was quantified by an ELISA kit. The medium was then aliquoted in 0.2% 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 \mu l diluted CETP protein (final concentration 7 nM) and 2.5 \mu l of compound stock. After a 30 minute incubation at 37°C, 5 \mu l of substrate stock (the same stock used in the human plasma CETP assay), 0.16 \mu l of VLDL stock (2.5 mg/ml, Intracel) and 145 \mu l of CETP buffer was added, and the incubation was continued for another 4 hours. 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
DMEM/F12 (3:1) (Invitrogen) with 2.5% Nu-serum (BD Biosciences) and 1x ITS Premix culture supplement (BD Biosciences). Cells were seeded and cultured in 24-well plates. When reaching about 80% confluence, cells were treated with designated compounds for 20 hours and the RNAs were purified from the cells using RNeasy Mini Kit (Qiagen, Valencia, CA). One μg of RNA was used for Reverse transcription (RT) to synthesize the first strand cDNA using SuperScript III First-Strand Synthesis kit (Invitrogen). The RT reaction was diluted with H₂O (1:10) and used to perform the TaqMan real-time quantitative PCR with CYP11B2 and CYP11B1 primer/probes and TaqMan Universal PCR Master Mix by an ABI Prism 7900 sequence detector. GAPDH was used as an internal control. The relative expression of CYP11B2 and CYP11B1 was determined by the Ct method (Applied Biosystems).

**Aldosterone EIA Assay.** Aldosterone levels were determined utilizing an Aldosterone EIA kit (Cayman Chemicals) according to the manufacturer’s recommendations. All reagents were prepared according to the protocol. Briefly, aldosterone standards, control and conditioned media (50 μL) were mixed with 50 μL aldosterone AChE tracer (aldosterone-acetylcholinesterase conjugate) and 50 μL of anti-aldosterone monoclonal antibody. The mixture was then transferred to a 96-well dish coated with goat polyclonal antibody to IgG. The plate was then incubated for 18 hours at 4°C. After incubation, the plates were washed 5 times in wash buffer, and 200 μL of Ellman’s Reagent was added to each well. The plate was developed in the dark for 90 to 120 minutes at room temperature with shaking. The developed plate was read at a wavelength of 412 nm by SpectraMax (Molecular Devices). Aldosterone concentrations were determined by
comparing the absorbance of the conditioned media to the aldosterone standards using a four parameter curve fit in Sigma Plot.

**Cpy11B1 and Cyp11B2 bDNA Assay.** The bDNA assay was developed and performed according to QuantiGene Reagent System (Panomics, Inc. Fremont, CA) (33). The Cyp11B1 and Cyp11B2 oligonucleotides for the bDNA assay were designed based on the NM_000497.2 (human Cyp11B1) and NM_000498 (human Cyp11B2) with the Probedesigner software (Bayer). The Cpy11B1 and Cyp11B2 oligonucleotides of capture extenders (CEs), label extenders (LEs), and blockers (BLs) were designed using the software. H295R cells were seeded at the density of 8 x 10^4 cells/well in 96-well plates and treated with compounds 5 hours after seeding the cells. Torcetrapib was added to each plate as a positive control, and DMSO wells were used as the baseline to calculate the fold inductions of Cyp11B1 and Cyp11B2 by the respective compounds. H295R cells were treated with compounds for 48 hours, and the EC50 values (concentration of compound causing a 50% activation of Cyp11B1 & Cyp11B2) were determined by non-linear regression.

**Human CETP and ApoAI double transgenic model.** A line of transgenic mice to screen CETP inhibitors was generated by crossing human CETP transgenic mice and human apoAI mice at Taconic Farms. A homozygous line of mice with hepatic expression of human CETP, which was under the control of a human AI promoter (34), was mated to the homozygous line of mice with hepatic expression of human AI under the control of its own promoter (35) yielding offspring mice that were heterozygous for both human AI and human CETP. These mice were treated orally with compound at various doses in a vehicle containing 79.5% corn oil, 20% oleic acid, and 0.5% labrafil.
The mice were bled from the retro-orbital sinus at 4 and 8 hours post single oral dose. The mice were euthanized by CO₂ asphyxiation and terminal blood was collected by cardiac puncture after a 24-hour treatment. HDL-C was measured in serum from the 8 and 24 hour time-points using a Hitachi clinical analyzer HDL-C enzymatic kit. FPLC was used to evaluate HDL-C elevation.

An assay to measure \textit{ex vivo} CETP activity was used to monitor the activity of compound inhibition of human CETP. The same particles containing the self-quenching CE-BODIPY analogue used in the 96% human plasma assay were used in the \textit{ex vivo} assay. The 5 \(\mu\)l substrate particles were mixed with 2.5 \(\mu\)l of human VLDL (Intracel) in 237.5 \(\mu\)l buffer (Tris/NaCl/EDTA, pH7.4) along with 5 \(\mu\)l of serum from mice treated with either compound or vehicle. The CETP activity in the serum from the mice treated with compound relative to the CETP activity in the serum from the mice treated with vehicle was determined and expressed as the percent inhibition of CETP activity. HDL cholesterol was analyzed on a Hitachi clinical chemistry analyzer. ED\textsubscript{50} values were determined using SigmaPlot.

\textbf{Blood pressure model.}

The blood pressure study was carried out using telemetered, male, obese Zucker Diabetic rats (ZDF fa/fa rats, Charles River Labs, 8 weeks of age on arrival; \(n=4\)). All rats underwent surgical implantation of a telemetry transmitter for continuous monitoring of hemodynamic parameters throughout the study. The rats were acclimated on Purina 5008 chow and house water \textit{ad libitum} until 11 weeks of age. Mean daily blood pressure for the 24-hour period immediately prior to administration of compound was taken as the baseline blood pressure. On the day of an experiment, a single 160 mg/kg dose (in 10% acacia) of evacetrapib as the lysine salt was administered by oral gavage, and the drug effect was taken as the average daily mean
arterial pressure (MAP) during the 24-hour period following the dose. Data were expressed as the change in MAP from baseline. Following the last day of blood pressure monitoring, samples were collected from the orbital sinus at 1, 2, 4, 8, and 24 hours post dose into tubes containing EDTA and processed into plasma. Plasma concentrations of evacetrapib were measured using liquid chromatography tandem mass spectrometry.

**Statistics** Statistical analyses of *in vivo* data were performed using JMP software (version 5.1, SAS, Cary, NC). The significance of differences among groups in baseline values was determined using a one-way analysis of variance (ANOVA). The significance of the change from baseline within each group was performed using Student’s *t* test for paired samples. Drug group mean values were compared to the vehicle group using ANOVA/t-test for paired samples. A *P*-value of less than 0.05 was considered to indicate statistical significance.
Results

In vitro pharmacology

Evacetrapib (LY2484595) is a novel benzazepine-based CETP inhibitor that has been developed at Lilly Research Laboratories. As shown in Figure 1 for comparison, anacetrapib is a substituted oxazolidinone, torcetrapib is a substituted dihydroquinoline, and dalcetrapib is a substituted thioproprionate. 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 causing half-maximum inhibition of CETP activity in this assay was 5.5 nM. This compares to 25.2 nM for torcetrapib and 21.5 nM for anacetrapib in the same assay. The compound was also tested in a human plasma CETP assay, in which human plasma CETP was used as the source for protein activity. The compound IC50 value in this assay was 26.0 nM, compared to an IC50 of 39.5 nM for torcetrapib and 46.3 nM for anacetrapib (Table 1). In the CEREP cell surface receptor (CSRMiniPanel) screening, no significant inhibition was observed against the receptors and transporters when evacetrapib was tested at 1 μM. There was also no significant activity against the nuclear receptor panel when the compound was tested at the same concentration (data not shown). Collectively, these data indicate that evacetrapib represents a novel, selective and potent CETP inhibitor in vitro.
**In vivo pharmacology**

To evaluate the *in vivo* CETP inhibitory efficacy, we developed an animal model of human CETP and human ApoAI double transgenic mice. It is known that mice do not possess CETP activity, and accordingly, human CETP transgenic mice were developed. The effect of human CETP activity on mouse HDL cholesterol level was not as significant as expected, presumably because mouse HDL particles are not good substrates for human CETP (36). In contrast, human ApoAI transgenic mice generate human-like HDL particles, and therefore a human CETP/ApoAI 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 hours post dose respectively. Evacetrapib dosed orally at 30 mg/kg resulted in 129.7% increase in HDL-C 8 hours after oral administration (Figure 2A and 2B). 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 to torcetrapib, evacetrapib was further evaluated in the human CETP/ApoAI double transgenic mice at multiple doses. The ED$_{50}$ values of CETP inhibitory activity 8 hours 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 Figure 3A) compared to ED$_{50}$ 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 (Figures 3B), and this was
further proven by fast pressure liquid chromatography (FPLC) analysis of lipoproteins (Figure 3C). These data indicated that evacetrapib is a potent CETP inhibitor in vivo that results in significant HDL-C 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 screened several male rat strains (including wild type Sprague-Dawley rats, ZDF rats, Dahl salt sensitive rats, fructose fed rats and Zucker fatty rats) in which 60 mg/kg of torcetrapib was dosed daily orally for 6 days and the blood pressure was measured hourly in the rats via telemetry on day 6 during the study. ZDF rats appeared to have the most significant increase in blood pressure resulting from torcetrapib treatment (data not shown), and thus the blood pressure elevation effect of torcetrapib was further evaluated in a dose response study in which 20, 60 and 200 mg/kg doses were used in ZDF rats. As shown in Figure 4A, torcetrapib dose-dependently increased blood pressure in ZDF rats with a maximum increase in mean arterial blood pressure (MAP) of 14 mmHg in the first two hours post oral dosing. The effect of evacetrapib on the mean arterial blood pressure was then evaluated with vehicle, torcetrapib, or evacetrapib in this model. Torcetrapib significantly elevated MAP (7.6 mmHg) at 60 mg/kg while evacetrapib did not demonstrate any significant change in MAP (Figure 4B). Torcetrapib achieved a 64-fold exposure multiple while 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.

**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 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 (Figure 5). Compared to 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). While 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 volume was regressed in patients that had the most significant HDL cholesterol elevation in spite of the cardiovascular toxicity from torcetrapib (30). These data point to the possibility that inhibition of CETP may be a
viable mechanism for CAD prevention, but a potent and selective compound without off-target toxicity is needed to explore such a hypothesis.

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 IC₅₀ 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 ED₅₀ 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 while 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, while 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.
Acknowledgement

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Figure legends

**Figure 1.** Chemical structures of evacetrapib (LY2484595), dalcetrapib, torcetrapib and anacetrapib.

**Figure 2.** CETP inhibitory activity of evacetrapib in human ApoAI and CETP double transgenic mice. A. Evacetrapib was orally dosed, and the CETP inhibitory activity 8 hours post oral dose was evaluated by the *ex vivo* CETP activity assay as described in Methods. * P<0.05 compared to vehicle control. B. HDL cholesterol elevation resulting from the corresponding CETP inhibition. * P<0.05 compared to vehicle control.

**Figure 3.** *In vivo* CETP inhibitory activity of evacetrapib: dose response and ED$_{50}$ evaluation. A. Dose-dependent inhibition of CETP activity in human ApoAI and CETP double transgenic mice. 30 mg/kg torcetrapib was used as a control. B. Corresponding HDL cholesterol elevation resulting from CETP inhibition. * P<0.05 compared to vehicle control. C. HDL cholesterol elevation as evaluated by FPLC analysis.

**Figure 4.** Evacetrapib does not induce blood pressure elevation in ZDF rats. Compound dosing and blood pressure measurement were performed as described in Methods. A. Dose dependent induction of blood pressure by torcetrapib in ZDF rats. B. Lack of blood pressure induction in ZDF rats by evacetrapib. Torcetrapib was used as a control in the same study. * P<0.05 compared to vehicle control.

**Figure 5.** Evacetrapib does not induce aldosterone or cortisol synthase mRNA in a human cortical carcinoma H295R cells. The bDNA method was used as described in Methods, and the fold induction of the mRNA of aldosterone or cortisol synthase was indicated. Torcetrapib was used as a control. Similar data was observed in more than 3 separate experiments.
Table 1. *In vitro* potency of three CETP inhibitors in buffer and plasma CETP assays.

<table>
<thead>
<tr>
<th><em>in vitro</em> CETP Activity Assay</th>
<th>Human recombinant CETP assay IC$_{50}$ (nM)</th>
<th>Human plasma CETP assay IC$_{50}$ (nM)</th>
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References:


Figure 1

evacetrapib

anacetrapib

dalcetrapib

torcetrapib
Figure 2A

The figure shows a bar graph comparing CETP activity across different treatments: Vehicle, torcetrapib, and evacetrapib. The y-axis represents CETP activity (Relative) ranging from 0 to 120, and the x-axis lists the treatments. The Vehicle treatment shows the highest activity, followed by torcetrapib and evacetrapib, which have significantly lower activity levels indicated by asterisks (*) on the graph.
Figure 2B

The bar chart shows the comparison of HDLc (mg/dl) levels across different treatments.

- **Vehicle**
- **torcetrapib**
- **evacetrapib**

The chart indicates a significant increase in HDLc levels for both torcetrapib and evacetrapib compared to the vehicle group. The asterisk (*) denotes statistical significance.
Figure 3A

CETP Activity (Relative)

30 mg/kg torcetrapib
Evacetrapib (ED50 +/- SE = 4.1 +/- 0.8 mg/kg)
Figure 3B

![Graph showing HDLc levels with different mg/kg of evacetrapib and torcetrapib](image)

- HDLc (mg/dl)
- mg/kg evacetrapib
- Vehicle 0.3 1 3 10 30 torcetrapib

* indicates a significant difference from the Vehicle group.
Figure 3C

The graph shows the cholesterol response (in mv) over time (in minutes) for different doses of torcetrapib and evacetrapib. The x-axis represents time in minutes ranging from 15 to 45, and the y-axis represents cholesterol response in mv ranging from 0 to 400.

- **Vehicle** (black line)
- **30mg/kg torcetrapib** (blue line)
- **1mg/kg evacetrapib** (green line)
- **3mg/kg evacetrapib** (purple line)
- **10mg/kg evacetrapib** (cyan line)
- **30mg/kg evacetrapib** (red line)

The graph indicates a peak response at approximately 35 minutes for most treatments, with the vehicle showing a slight increase but maintaining a lower response compared to the treatments.
Figure 4A

![Graph showing average daily mean arterial blood pressure (mmHg) over time (days) for different treatment groups: vehicle, torcetrapib 20mg/kg, torcetrapib 60mg/kg, and torcetrapib 200mg/kg. The graph indicates a rise in blood pressure over time for the treatment groups compared to the vehicle group, with statistically significant differences marked by asterisks (*) at various time points.](Image)
Figure 4B

Δ MAP (mm Hg) from baseline

- Vehicle A
- 60 mg/kg torcetrapib
- Vehicle B
- 160 mg/kg evacetrapib

* Indicates significant difference from baseline.
Figure 5

The graph shows the fold induction of torcetrapib and evacetrapib at various concentrations ([µM]) as follows:

- Torcetrapib:
  - At 0.001 [µM], fold induction is approximately 0.5.
  - At 0.01 [µM], fold induction increases to about 2.
  - At 0.1 [µM], fold induction reaches a peak at around 8.
  - At 1 [µM], fold induction decreases to about 4.
  - At 10 [µM], fold induction stabilizes at around 1.5.

- Evacetrapib:
  - Throughout the range of concentrations, evacetrapib shows a relatively flat profile with fold induction close to 1.5.

The graph indicates that torcetrapib has a significant fold induction at lower concentrations, peaking at 8 before decreasing, while evacetrapib maintains a consistent fold induction across the range of concentrations.