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Short Communication

The *in Vitro* Plasma Distribution of a Novel Cholesteryl Ester Transfer Protein Inhibitor, Torcetrapib, Is Influenced by Differences in Plasma Lipid Concentrations

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Purpose. To determine the lipoprotein distribution of Torcetrapib in normolipidemic or hyperlipidemic human plasma and assess any changes in distribution due to lipid profile.
Methods. Torcetrapib was incubated with human plasma samples, and the distribution was measured across four fractions: triglyceride-rich lipoprotein (TRL), low-density lipoprotein, high-density lipoprotein, and lipoprotein-deficient plasma fraction. Two stocks of human plasma were used, one considered normolipidemic (total cholesterol concentration = 164 mg/dL, triglycerides concentration = 139 mg/dL, protein concentration = 912 mg/dL), the other hyperlipidemic (total cholesterol = 260 mg/dL, triglycerides = 775 mg/dL, protein = 917 mg/dL). The plasma samples were incubated with Torcetrapib at 37°C, and the incubation was stopped with the addition of sodium bromide and cooling to 4°C. The plasma samples were then separated by density gradient ultracentrifugation to their lipoprotein fractions. The resulting lipoprotein fractions and an aliquot of incubated plasma were analyzed by a validated gas chromatography/tandem mass spectrometry analytical method. The distribution of Torcetrapib was determined first with varying incubation times, then with several concentrations.
Results. At concentrations of 250 and 500 ng/mL, Torcetrapib distributed evenly across the four fractions in normolipidemic plasma. At the same concentrations in hyperlipidemic plasma, approximately 84% of Torcetrapib was found in the TRL fraction, with the remaining 16% evenly partitioned between the low-density lipoprotein, high-density lipoprotein, and lipoprotein-deficient plasma fractions.
Conclusions. The results suggest that lipid profile affects the distribution of Torcetrapib in hyperlipidemic human plasma lipoprotein fractions. The preferential distribution of Torcetrapib into the TRL fraction in hyperlipidemic plasma needs to be investigated to see if it will affect the pharmacological effect of Torcetrapib *in vivo*.

KEY WORDS: CP-529,414; distribution; lipid profile; plasma lipoproteins; Torcetrapib.

INTRODUCTION

Lipoproteins, commonly involved in the transport of lipids throughout the bloodstream, seem to have a wider biological role than simply the movement of water-soluble lipids from the systemic circulation to tissues (1). They are also involved in the binding and subsequent transport of a number of water-insoluble compounds, including amphotericin B (AmpB) and cyclosporine A (CSA). Current research further suggests that changes in the lipoprotein binding of drug compounds may have a major impact on the efficacy and safety of the aforementioned compounds, particularly because they are often administered to patients with abnormal lipid metabolism.

Cholesteryl ester transfer protein (CETP) is a 476-amino-acid hydrophobic glycoprotein with a molecular weight of 74 kDa (2) that is secreted primarily from the liver (3). CETP expression between mammalian species is variable, with undetectable levels in rats and mice, moderate levels in humans, and high levels in rabbits (4). The majority of CETP in humans is synthesized in the liver, with lower levels produced in the adipose tissue, kidney, heart, and spleen (5).

CETP facilitates the transfer of cholesteryl esters from high-density lipoprotein (HDL) to apo-B-containing lipoproteins [very low density lipoprotein (VLDL) and low-density lipoprotein (LDL)] with a reciprocal transfer of triglyceride (6,7). CETP plays an important role in the metabolism and remodeling of plasma lipoproteins (8). CETP may also play a role in certain disease processes such as atherosclerosis by redistributing cholesterol from the antiatherogenic HDL particles to the proatherogenic LDL particles. Thus the

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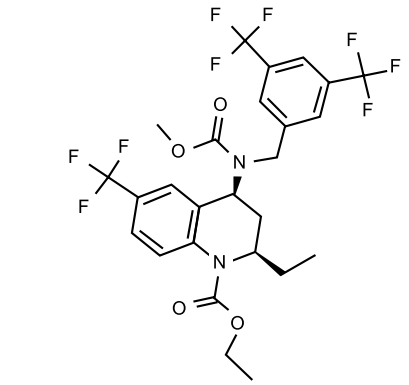
development of an inhibitor to prevent the redistribution of cholesterol and inhibit this process of atherosclerosis has been the subject of much research activity (9–17).

Recently, a new CETP inhibitor, Torcetrapib (CP-529,414), was developed and tested in two studies in human subjects. In the first, the effect of CETP inhibition on plasma HDL levels was studied in healthy subjects (13). With the highest dose of 120 mg administered orally twice daily for 14 days, CETP activity decreased by 80%, although the CETP mass increased, apparently because the mechanism of action of Torcetrapib represents the shift of free CETP to HDL-bound form. With the above treatment, plasma LDL decreased by 42% and HDL cholesterol (HDL-C) increased by 91%, as did apo Al and apo E, by 27 and 66%, respectively (13). In the second study, Torcetrapib was given to 19 normal subjects with low plasma HDL-C levels (<40 mg/dL), nine of whom received 20 mg atorvastatin daily (11). In subjects who received only 120 mg/day Torcetrapib for 4 weeks, HDL was increased by 46%; in those treated also with atorvastatin, the increase was 61%. A much higher rise in HDL-C, 106%, was observed when the drug was given twice daily, but with no atorvastatin (11). In six subjects treated with the two drugs, there was also a 17% decrease in LDL cholesterol (LDL-C). In addition, Torcetrapib decreased the levels of the proatherogenic small dense LDL particles and increased the concentration of large HDL to values seen in subjects with normolipidemia (11). However, to date, limited information about the plasma distribution of Torcetrapib following administration has been reported.

The primary objective of this study was to determine the plasma distribution of Torcetrapib in normolipidemic and hyperlipidemic human plasma. The specific aim was to assess whether the changes to lipid and protein profiles would impact the drug's *in vitro* distribution.



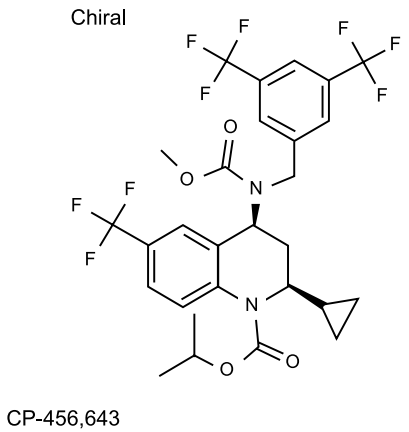


Fig. 1. Chemical structures of Torcetrapib (CP-529,414; analyte) and CP-456,643 (internal standard).

MATERIALS AND METHODS

Chemicals and Reagents

Torcetrapib (CP-529,414; aqueous solubility <40 ng/mL; cLog P = 7.6) and an internal standard, CP-456,643, were obtained from Pfizer's sample bank in Groton, CT, USA. Structures are shown in Fig. 1. Pooled human plasma was purchased from Bioreclamation (Hicksville, NY, USA). Acetonitrile and isopropanol [high-performance liquid chromatography (HPLC) grade] were purchased from Fisher Scientific (Toronto, ON, Canada). Sodium chloride, sodium bromide, and sodium hydroxide were purchased from Sigma Aldrich (St. Louis, MO, USA). Cholesterol standard was purchased from StanBio Laboratory (Boerne, TX, USA), and triglyceride standard was purchased from Thermo Trace (Waltham, MA, USA). Cholesterol reagent and triglyceride reagent were purchased from Roche Diagnostics (Laval, QC, Canada). The BCA Protein Assay Kit used to characterize protein levels was obtained from Pierce Biotechnology (Rockford, IL, USA). Protein, cholesterol, and triglyceride level characterizations and preparation of normolipidemic and hyperlipidemic plasma samples with Torcetrapib were performed at the University of British Columbia, Vancouver, BC, Canada. Samples were then sent to Tandem Labs (Salt Lake City, UT, USA) for quantitation of Torcetrapib.

Prescreening and Characterization of Human Plasma Pools: Selection of Patients

Human plasma was derived from whole blood donations obtained in the United States from donations at FDA-registered centers. All blood was treated with sodium EDTA as an anticoagulant and plasma was obtained. The normolipidemic plasma, lot # BRH22805, was obtained from Bioreclamation's normal human plasma pool, comprised of five subjects who were classified as normal by an examining physician prior to blood donations. The hyperlipidemic plasma, lot # BRH33510, was obtained from three donors prescreened to have total cholesterol levels of 300 mg/dL or greater and triglyceride levels greater than 300 mg/dL.

Characterization of Triglyceride Levels

The plasma was assayed for total triglycerides as well as a sample of the plasma fractions. For analysis of the lipoprotein fractions, a standard curve was prepared using a 200-mg/dL triglyceride standard solution (Thermo Electron, Boston, MA, USA) diluted with distilled water to 100, 50, 25, and 12.5 mg/dL. The standards and the samples were analyzed in a microplate-based assay. Briefly, 2 μL of blank, standard, or sample was added in duplicate to a 96-well plate

(Corning Costar, Cambridge, MA, USA); 200 μL of triglyceride reagent was added; and the plate was shaken for 10 s and incubated for 10 min at 37°C. The plate was read using a Labsystems Multiskan Ascent plate reader (Thermo, Waltham, MA, USA) with a 492-nm filter.

Characterization of Cholesterol Levels

The plasma samples and lipoprotein fractions were analyzed for cholesterol using a standard curve analysis technique. Briefly 2 μL of blank, standard, or sample was added to a well of a 96-well microplate in duplicate; 200 μL of cholesterol reagent was added; and the plate was shaken and allowed to incubate for 5 min at 37°C. The plate was read using a Labsystems Multiskan Ascent microplate reader with a 492-nm filter.

Characterization of Protein Levels

The plasma samples and the lipoprotein fractions were analyzed for protein content using the BCA protein assay (Pierce Biotechnology) following the protocol suggested in the product literature. Briefly, 25 μL of blank, standard, or sample was added to a well of a 96-well plate in duplicate; 200 μL of working reagent was added to each well; and the plate was shaken for 30 s The plate was then incubated for 30 min at 37°C and then read using a Labsystems Multiskan Ascent microplate reader using a 540-nm filter.

Preparation of Torcetrapib

Torcetrapib was dissolved in acetonitrile and diluted to an appropriate concentration for spiking into plasma: 200 ng/μL for the 1000-ng/mL experiments, 100 ng/μL for the 500-ng/mL experiments, and 50 ng/μL for the 250-ng/mL experiments.

Lipoprotein Separation by Density Gradient Ultracentrifugation

Treatment of the Plasma Samples

Pooled human plasma was added to test tubes in 4-mL aliquots. Twenty microliters of the appropriate Torcetrapib solution was added, and the tubes were mixed thoroughly using a vortex mixer. The plasma was incubated at 37°C for the required amount of time. The incubation was stopped by adding and dissolving 1.360 g of sodium bromide and cooled rapidly on ice to 4°C for a minimum of 2 h. The concentrations for experiment 2 was 1000 ng/mL; the concentrations for experiment 3 were 250 and 500 ng/mL.

Separation of Lipoprotein Components

Normolipidemic plasma (BRH22805) was thawed and aliquoted into 12 clean disposable glass test tubes, 4 mL per test tube. Twenty microliters of the appropriate Torcetrapib solution was added to six of the test tubes for each concentration and incubation time tested. Each test tube was covered with parafilm and mixed using a vortex. The test tubes were incubated at 37°C for the required length of time. The test tubes were then removed from the water bath, and incubation was halted by cooling to 4°C and by adjusting the density of each solution to 1.25 g/mL with the addition of 1.36 g of sodium bromide. The tubes were placed in an ice bucket and placed in a fridge for a minimum of 2 h to cool the solution. The plasma was separated into its lipoprotein and lipoprotein-deficient fractions by step-gradient ultracentrifugation (18,19). Briefly, plasma samples (3.0 mL) were placed into centrifuge tubes, and their solvent densities were adjusted to 1.25 g/mL by the addition of solid sodium bromide (0.34 g/mL of plasma). Once the sodium bromide had dissolved into the plasma, 2.8 mL of the highest-density sodium bromide solution (density of 1.21 g/mL) was layered on top of the plasma solution. Then, 2.8 mL of the second sodium bromide solution (density of 1.063 g/mL) was layered on top of the sample, followed by 2.8 mL of the third sodium bromide solution (density of 1.006 g/mL). Upon completion of layering with the sodium bromide density solutions, four distinct regions of progressively greater densities (from top to bottom of the tube) were observed (19). All sodium bromide solutions were kept at 4°C prior to the layering of the density gradient. The centrifuge tubes were placed in an SW 41 Ti swinging bucket rotor (Beckman, Mississauga, ON, Canada) and centrifuged at 40,000 rpm (288,000 × *g*; *k* factor = 128) at a temperature of 15°C for 18 h (L8-80 M; Beckman). Following UC, each density layer was removed using a Pasteur pipette, and the volume of each lipoprotein fraction was measured.

Preparation of the Lipoprotein Fractions for Transport and Analysis

The fractions were split into two vials; one vial contained sufficient 2-propanol for a final concentration of 10% (v/v), the other contained no additive. The vials containing 2-propanol were shipped at 4°C to Tandem Labs for quantification of the Torcetrapib concentrations by gas chromatography/tandem mass spectrometry (GC/MS/MS). The other vial was stored at −20°C until the lipid and protein analyses could be completed. The 1 mL of plasma remaining was similarly separated into two vials for analyses.

Quantitation of Torcetrapib by GC/MS/MS Method

The concentrations of Torcetrapib in plasma and different lipoprotein fractions were determined using a non-GLP GC (Thermo Finnigan Trace GC)/MS (Finnigan MA T TSQ 7000)/MS method.

Briefly, 50-μL aliquots of plasma sample were subjected to protein precipitation and then analyzed by GC/MS/MS. An internal standard, CP-456,643, was added to all samples except blanks. The dynamic range of the curve was 0.500 to 100 ng/mL. Quality control (QC) samples were run in human plasma in duplicate at low (1.5 ng/mL), medium (40.0 ng/mL), and high (80.0 ng/mL) concentrations with each analytical run. All QC samples prepared in human plasma were within 15% of the nominal value. In addition, diluted QC samples were prepared in plasma in duplicate with dilution factors of 2, 10, and 25. QC samples were also prepared in duplicate at 7.5, 200, and 400 ng/mL in LDL, HDL, VLDL, and lipoprotein-deficient plasma (LPDP) fractions; lipoprotein QC samples were then diluted 1:5. All QC

Table I. Cholesterol, Triglycerides, and Protein Concentrations of the Normolipidemic and Hyperlipidemic Human Plasma Pools

Sample	Concentration (mg/dL)		
	Cholesterol	Triglycerides	Protein
Normolipidemic	164 ± 6	139 ± 25	912 ± 172
Hyperlipidemic	260 ± 22	775 ± 68	917 ± 172

samples prepared in lipoprotein were within 20% of the nominal value.

Determination of the Lipoprotein Distribution of Torcetrapib

Tandem Labs provided Pfizer, Inc. with the Torcetrapib concentration in each of the plasma samples and lipoprotein fractions transported. The mass of Torcetrapib in each sample was calculated using the volumes measured at the time of separation and the concentrations obtained. There were two recovery values obtained for each experiment. The recovery after incubation was determined by comparing the Torcetrapib concentration in the plasma samples with the theoretical concentration for the experiment. This value represents the loss of compound to the glass test tube during incubation. The second recovery statistic was obtained by adding the mass recovered in each of the fractions and comparing to the concentration of compound found in the appropriate plasma sample. This recovery statistic represents the loss of the compound due to separation and not due to incubation. For comparison between experiments, the distributions were normalized for mass recovered.

Data and Statistical Analysis

Differences in drug distribution between different plasmas were determined by a one-way analysis of variance (ANOVA) (GraphPad InStat; GraphPad Software, San Diego, CA, USA). Critical differences were assessed by Student–Newman–Keuls post hoc tests. Differences were considered significant if the p value was less than 0.05. All data are expressed as mean ± standard deviation. CP-529,414 and CP-456,643 chromatographic peaks were integrated using Finnigan Xcalibur (v1.1). Analyte concentrations were calculated using Watson DMLIMS (Wayne, PA, USA) software (v 6.4.0.02;) with a quadratic regression analysis of calibration curves (weighted $1/x^2$).

RESULTS AND DISCUSSION

The lipid profiles of the normolipidemic and hyperlipidemic human plasma used in this experiment were determined, and the concentrations of cholesterol, triglycerides, and protein in the two plasma pools are shown in Table I. The normolipidemic plasma pool had cholesterol, triglycerides, and protein concentrations of 164, 139, and 912 mg/dL, respectively. The hyperlipidemic plasma pool had cholesterol, triglycerides, and protein concentrations of 260, 775, and 917 mg/dL, respectively. The concentrations of cholesterol, triglyceride, and protein were also determined in randomly selected samples of the four fractions [triglyceride-rich lipoprotein (TRL), LDL, HDL, and LPDP] prepared from both normolipidemic and hyperlipidemic samples and are shown in Table II.

A time-course experiment was conducted to determine how long samples should be incubated for Torcetrapib distribution to reach equilibrium. Normolipidemic and hyperlipidemic plasmas were fortified with Torcetrapib at a concentration of 1000 ng/mL and analyzed after 15-, 30-, and 60-min incubations. Normolipidemic plasma reached equilibrium after a 15-min incubation, whereas hyperlipidemic plasma reached equilibrium after 60 min. Fig. 2 contains a plot of the time-course distribution data for both normolipidemic and hyperlipidemic plasmas. The statistical analysis of each of the time-course data sets revealed that the differences in distribution between normolipidemic and hyperlipidemic plasmas for each time point were statistically significant with a p value less than 0.01 by one-way ANOVA. The results of the time-course experiments indicated that 60 min was required for the hyperlipidemic plasma to reach equilibrium. The normolipidemic samples seemed to reach equilibrium in as little as 15 min. For continuity, 60 min was chosen as the incubation time for both plasma types in the concentration-dependence experiment.

The distribution of Torcetrapib in normolipidemic and hyperlipidemic human plasma samples was examined at two concentrations: 250 and 500 ng/mL. Although the normolipidemic plasma reached equilibrium after 15 min, both pools of spiked plasma samples were incubated for 60 min to allow the hyperlipidemic plasma to fully reach equilibrium.

Table II. Concentrations of Cholesterol, Triglycerides, and Protein in Randomly Selected Samples of TRL, LDL, HDL, and LPDP Fractions of Normolipidemic and Hyperlipidemic Human Plasma

Sample	Plasma Pool	Concentration (mg/dL) in Each Fraction			
		TRL	LDL	HDL	LPDP
Cholesterol	Normolipidemic	30.4	97.0	27.6	3.1
	Hyperlipidemic	184.0	110.0	10.7	10.2
Triglycerides	Normolipidemic	56.8	25.7	9.2	12.3
	Hyperlipidemic	673.0	76.7	16.2	29.5
Protein	Normolipidemic	2.7	6.0	9.1	677.0
	Hyperlipidemic	18.7	8.0	9.6	580.0

$N = 6$; mean data.

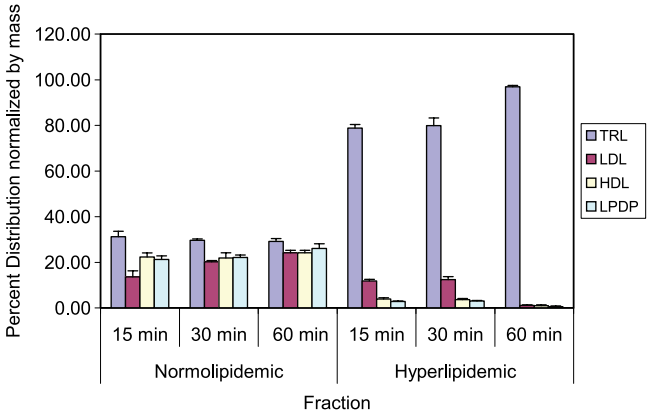


Fig. 2. Plot of the time-course distribution changes after a 1000-ng/mL dose of Torcetrapib in normolipidemic and hyperlipidemic plasma organized by plasma type and incubation time. The values are the mean percentages in a normalized distribution; the error bars are one standard deviation; $n = 6$ for all groups except 60 min, normolipidemic, which is $n = 5$. Abbreviations: TRL, triglyceride-rich lipoproteins (which include very low density lipoproteins and chylomicrons); LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein-deficient plasma (which contains albumin and alpha-1 glycoprotein).

The distribution of Torcetrapib was normalized for total mass recovered to allow comparison between plasma pools. At both concentrations of 250 and 500 ng/mL, Torcetrapib distributed evenly across the four fractions in normolipidemic plasma as shown in Fig. 3. At the same concentrations in hyperlipidemic plasma, approximately 84% of Torcetrapib was found in the TRL fraction, with the remaining 16% evenly partitioned between the LDL, HDL, and LPDP fractions. The distribution of Torcetrapib across the lipoprotein fractions was independent of concentration. The statistical analysis of the concentration-dependence experiment revealed the following observations:

- The difference in distribution between normolipidemic and hyperlipidemic data sets for every fraction was significant with a p value less than 0.001.
- There were no differences between the data sets 250 and 500 ng/mL for any given fraction.
- There is a significant difference between the HDL-normolipidemic data sets ($p < 0.01$) but no statistically significant differences between the normolipidemic LDL or LPDP normolipidemic data sets.

The analysis of the plasma pools used in this experiment indicates that the levels of cholesterol and triglycerides increased in each of the hyperlipidemic fractions when compared to the corresponding normolipidemic fractions, 5.6-fold, 9-fold, and 7-fold respectively. The protein levels in LDL, HDL, and LPDP were relatively unchanged. The total mass of protein was highest in the LPDP fraction, as this is the fraction where unbound plasma proteins such as albumin and α_1-glycoprotein localize.

Our laboratory has been investigating the importance of drug–lipoprotein interactions for a number of years. Preliminary studies from our group and others have suggested that the association of therapeutic compounds with lipoproteins can alter the pharmacokinetics, efficacy, and toxicological profile of the compound (20,21). Lipoproteins have been found to be carriers of many hydrophobic drugs, and the lipid profile of the patient can alter the lipoprotein distribution of the therapeutic compound with significant clinical implications (20).

In the present study, our findings support the hypothesis that the lipid profile of the plasma alters the lipoprotein distribution of Torcetrapib. The predominant association of Torcetrapib with the TRL fraction in all the hyperlipidemic experiments could be a result of the increased cholesterol, triglyceride, or protein concentrations. The mass of cholesterol present in the hyperlipidemic TRL fraction is less than the mass of cholesterol in the LDL fraction; this result is probably indicative that the association of Torcetrapib is not directly correlated to cholesterol concentration. Further studies will be required to determine whether the partitioning of the compound is due to the increased triglycerides present or whether it is an association with a specific protein that is overexpressed in hyperlipidemic patients and localized to the TRL fraction. The protein assays that were run indicate that there is increased protein concentration in the hyperlipidemic TRL fraction relative to the normolipidemic, but the specific proteins that are increased are unknown. Torcetrapib has a high affinity for CETP, and the altered distribution could be a function of altered CETP plasma distribution in hypertriglyceridemia.

The results of these experiments offer conclusive support to the hypothesis that that *in vitro* lipoprotein distribution of the novel Pfizer compound, Torcetrapib, is altered depending on the lipid and protein profile of the plasma. Future studies as a result of these findings should include the identification of the factor(s) responsible for the strong association of Torcetrapib with TRL in hyperlipidemic plasma and the therapeutic implications of such an association. These studies could be of importance because Torcetrapib is intended for patients that are hypercholesterolemic with low plasma HDL-C levels.

In summary, the distribution of Torcetrapib in human plasma is dependent upon the lipid profile of the plasma.

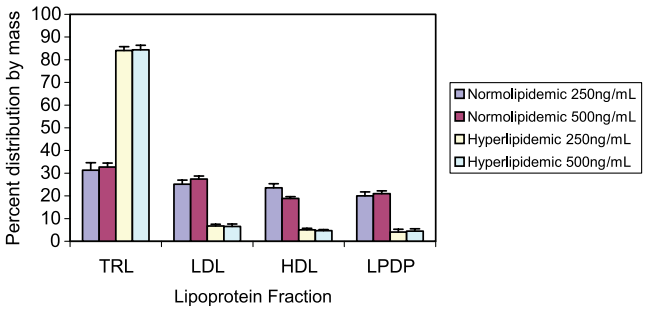


Fig. 3. Lipoprotein distribution of Torcetrapib at concentrations of 250 and 500 ng/mL in normolipidemic and hyperlipidemic plasma. The values represent the mean percent distribution normalized for recovered mass of Torcetrapib. The error bars represent one standard deviation; $n = 6$ for each group. Abbreviations: TRL, triglyceride-rich lipoproteins (which include very low density lipoproteins and chylomicrons); LDL, low-density lipoproteins; HDL, high-density lipoproteins; LPDP, lipoprotein-deficient plasma (which contains albumin and alpha-1-glycoprotein).

Torcetrapib highly partitioned into the TRL fraction. However, at concentrations of 250 and 500 ng/mL, Torcetrapib concentration did not affect the distribution across fractions. The results suggest that lipid profile affects the distribution of Torcetrapib in hyperlipidemic human plasma lipoprotein fractions. The preferential distribution of Torcetrapib into the TRL fraction in hyperlipidemic plasma is not expected to affect the pharmacological effect of Torcetrapib *in vivo* due to the low concentration of CETP in human plasma and high affinity of Torcetrapib toward CETP.

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