# Description of the torcetrapib series of cholesteryl ester transfer protein inhibitors, including mechanism of action[S]

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**Abstract We have identified a series of potent cholesteryl ester transfer protein (CETP) inhibitors, one member of which, torcetrapib, is undergoing phase 3 clinical trials. In this report, we demonstrate that these inhibitors bind specifically to CETP with 1:1 stoichiometry and block both neutral lipid and phospholipid (PL) transfer activities. CETP preincubated with inhibitor subsequently bound both cholesteryl ester and PL normally; however, binding of triglyceride (TG) appeared partially reduced. Inhibition by torcetrapib could be reversed by titration with both native and synthetic lipid substrates, especially TG-rich substrates, and occurred to an equal extent after long or short preincubations. The reversal of TG transfer inhibition using substrates containing TG as the only neutral lipid was noncompetitive, suggesting that the effect on TG binding was indirect. Analysis of the CETP distribution in plasma demonstrated increased binding to HDL in the presence of inhibitor. Furthermore, the degree to which plasma CETP shifted from a free to an HDL-bound state was tightly correlated to the percentage inhibition of CE transfer activity. The finding by surface plasmon resonance that torcetrapib increases the affinity of CETP for HDL by ~5-fold likely represents a shift to a binding state that is nonpermissive for lipid transfer. In summary, these data are consistent with a mechanism whereby this series of inhibitors block all of the major lipid transfer functions of plasma CETP by inducing a nonproductive complex between the transfer protein and HDL.**—Clark, R. W., R. B. Ruggeri, D. Cunningham, and M. J. Bamberger. **Description of the torcetrapib series of cholesteryl ester transfer protein inhibitors, including mechanism of action. *J. Lipid Res.* 2006.** 47: **537–552.**

**Supplementary key words** lipid transfer • high density lipoprotein • atherosclerosis • cardiovascular disease

Despite the demonstration of the atheroprotective effects of HDL over the past several decades (1–4), no current therapy exists that is effective and well tolerated for increasing the levels of this lipoprotein (5). Although the use of extended-release niacin (Niaspan) at daily doses of 2 g or less has served to minimize the high incidence of vasodilatory effects, such as flushing and itching (6), toleration issues continue to limit compliance. Also, at 2 g/day, niacin increases high density lipoprotein cholesterol (HDL-C) by <30% (7). The high levels of HDL associated with human cholesteryl ester transfer protein (CETP) deficiency (8) have suggested CETP inhibition as a means of increasing HDL. Although expression of human CETP in transgenic mice has produced mixed results regarding its atherogenicity, more consistent antiatherogenic effects have resulted from the inhibition of endogenous CETP in rabbits (8). In the wake of the beneficial effects observed through CETP inhibition in rabbits by induction of autoantibodies (9) and by administration of a synthetic inhibitor (10), these interventions have progressed to trials aimed at increasing HDL in humans. Although the use of the CETP vaccine has yet to demonstrate sufficient anti-CETP response to increase HDL (11), 900 mg/day of the inhibitor JTT-705 increased HDL-C by 34% and decreased low density lipoprotein cholesterol (LDL-C) by 7% (12).

We have identified a new series of CETP inhibitors culminating in the development of torcetrapib, which is currently undergoing phase 3 clinical trials. In the initial 2 week phase 1 study, torcetrapib at doses of 120 mg once per day and 120 mg twice per day raised HDL-C by 73% and 91%, respectively, and decreased LDL-C by 21% and 42%, respectively (13). In a subsequent trial, the combination of 120 mg/day torcetrapib with 20 mg/day atorvastatin over 4 weeks reduced LDL-C an additional

Abbreviations: apoA-I, apolipoprotein A-I BPI, bactericidal/permeability-increasing protein; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FC, free cholesterol; FPLC, fast-protein liquid chromatography; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; Mr, apparent molecular weight; PC, phosphatidylcholine; PL, phospholipid; PLTP, phospholipid transfer protein; SPR, surface plasmon resonance; TC, total cholesterol; TG, triglyceride; WT, wild-type.

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17% beyond that achieved with atorvastatin alone while increasing HDL-C by 58% (14). Torcetrapib has also been shown to prevent atherosclerosis in rabbits (15), consistent with previous studies demonstrating an antiatherogenic effect for CETP inhibition (8–10). Given the degree of inhibition achieved by different doses of torcetrapib, the magnitude of these changes in plasma lipids are as expected based on the known effects of partial and complete CETP deficiency. That torcetrapib successfully reproduces the phenotypes associated with CETP deficiency derives both from its specificity and a mode of action that blocks all of the major lipid transfer functions of the protein. CETP not only catalyzes the exchange of cholesteryl ester (CE) between plasma lipoproteins but also transfers triglyceride (TG) and phospholipid (PL). In vivo CETP activity typically results in the net mass transfer of CE from the CE-rich HDL fraction to TG-rich lipoproteins (VLDLs, chylomicrons, and their remnants) and the reciprocal transfer of TG from these particles to HDL (8). Decreased CETP activity, due to either CETP deficiency or the use of inhibitors, results in an HDL population with decreased TG and increased CE and a non-LDL fraction with increased TG and decreased CE. These compositional changes lead to increased plasma HDL levels and decreased LDL, attributable in large part to the delayed clearance of HDL (16, 17) and the increased clearance of LDL (18). Therefore, although the screens used in the search for inhibitors of CETP have generally relied on measuring CE transfer, the reciprocal effect of increased HDL and decreased LDL likely requires the inhibition of both CE and TG transfer.

Consistent with the multiple lipid transfer functions of CETP is the presence of binding sites for both neutral lipids and PL. CETP has been shown to extract and bind labeled CE, TG, and PL from donor liposomes, and when reisolated the CETP was able to donate this bound lipid to LDL (19) or liposomes (20) acting as acceptors. This ability as well as the kinetics of transfer (20–22) demonstrate a carrier mechanism requiring both the reversible binding of lipid to CETP and the transient association of CETP to donor and acceptor lipoproteins. Therefore, inhibition of CETP activity could be achieved by several classes of agents, including those that *a*) block lipid binding or prevent its release once bound, *b*) cause the excessive or insufficient binding of CETP to its lipoprotein substrates, and *c*) induce gross structural modifications that inactivate the protein.

The data in this report indicate that the increased binding of plasma CETP to HDL induced by torcetrapib results in a nonproductive complex unable to engage in further rounds of lipoprotein binding and lipid loading and unloading. This mechanism provides sufficient explanation for how these low molecular weight inhibitors are able to block both neutral and PL transfer activities of CETP even though the binding of these lipids is thought to occur at different sites on the protein (23, 24). In this report, a similar mechanism is also described for another, previously described inhibitor series, suggesting that it may emerge as a common mode of action for this class of agents.

## MATERIALS AND METHODS

### Materials

Routine chemicals and nonlabeled lipids were purchased from Sigma. Enzymes and kits for the analysis of free and total cholesterol, and for TGs, as well as lipid calibrators were from Roche Diagnostics; the PL B assay kit was from Wako. The EZ-Link NHS-LC biotin and Sulfolink kits, as well as bis(sulfosuccinimidyl) suberate and disuccinimidyl suberate cross-linking agents, were obtained from Pierce. Mouse and goat anti-human apolipoprotein A-I (apoA-I) antibodies were purchased Chemicon. $^{3}$H-labeled cholesteryl oleate, [$^{3}$H]dipalmitoyl phosphatidylcholine (PC), [$^{14}$C] cholesteryl oleate, and [$^{14}$C]triolein were from New England Nuclear; PC (1-palmitoyl-2-[1-[$^{14}$C]oleoyl) was from Amersham. Sephadex and Q-Sepharose resins were purchased from Pharmacia, and Toyopearl was from Tosohaas. CHO cells stably transfected with either wild-type (WT) human CETP or the residues 470–475 C-terminal deletion mutant cDNA were kindly provided by Dr. Alan Tall (Columbia University). Purified phospholipid transfer protein (PLTP) was a gift from Dr. Christian Ehnholm (National Public Health Institute, Helsinki, Finland).

### Purification of CETP

Both WT and C-terminal deletion mutant CETP were purified from medium conditioned by CETP expressing CHO cells as described previously (25). Where delipidated purified CETP was required, the CHO medium was first lyophilized and then extracted using isopropanol/$CHCl_3$ (11:7) by the method of Rose and Oklander (26). The purified delipidated CETP demonstrated the same specific activity for the transfer of [$^{3}$H]cholesteryl oleate to HDL as nondelipidated purified CETP in side-by-side assays (data not shown).

### Preparation of lipoprotein and liposome substrates

[$^{3}$H]- and [$^{14}$C]-labeled and nonlabeled lipoproteins were prepared essentially as described previously (25). VLDL/intermediate density lipoprotein, LDL, and HDL were isolated from the $d < 1.019$ g/ml, the $d = 1.019–1.063$ g/ml, and the $d = 1.10–1.21$ g/ml ranges, respectively. Labeled unilamellar liposome substrates used in CETP lipid binding assays were produced by sonication as described previously (25).

### Assay of CETP, PLTP, LCAT, and lipoprotein and hepatic lipase activities

For the determination of in vitro CETP activity in whole human plasma, the bidirectional transfer of $^{3}$H-labeled cholesteryl oleate from HDL to the non-HDL plasma fraction and of [$^{14}$C]-labeled triolein from LDL, or VLDL, to HDL was determined simultaneously, as described previously (13).

For studies on the effects of preincubation time or lipoprotein titration on inhibition, isolated donor and acceptor lipoprotein as well as purified CETP were used (see Fig. 9 below). The incubation period for transfer assay was 30 min for the preincubation experiments and ranged from 45 min (high acceptor) to 3 h (low acceptor) for the titration studies. In all cases, the fraction of donor lipid transferred by the end of the assay was ≤20% and within the linear range for the assay. The transfer assays for Fig. 9A–C were terminated by precipitating non-HDL lipoprotein with $PO_4$/Mn (100 mM/8–12 mM) and counting aliquots of the HDL-containing supernatant by liquid scintillation.

PLTP activity was assayed essentially by the protocol of Jauhiainen et al. (27). Briefly, [$^{3}$H]PC (dipalmitoyl)-labeled liposomes and HDL (1.10–1.21 g/ml density cut) were incubated for 1.5 h with and without 35 ng of purified PLTP per 175 μl assay.

The reaction was stopped by adding 0.75 volume of precipitating solution containing 215 mM $MnCl_2$, 500 mM NaCl, and 450 U/ml heparin. The sample was centrifuged, and an aliquot of the HDL-containing supernatant was counted by liquid scintillation. Counts present in the supernatant for (−)PLTP controls were subtracted from those in (+)PLTP samples to correct for nonspecific transfer. For comparison, purified CETP replaced PLTP in parallel assays using either the same [$^3$H]PC donor liposomes or liposomes labeled with [$^3$H]cholesteryl oleate (0.25 mol%) to determine the inhibition by torcetrapib of CETP-dependent PL or CE transfer, respectively, under similar conditions.

For LCAT activity, a pool of native human plasma with or without torcetrapib was incubated at 37°C for 2–8 h. Plasma free and total cholesterol were determined enzymatically for each time point, including a nonincubated sample kept on ice. CE was calculated by the difference between TC and FC. LCAT activity, represented by the change in CE/free cholesterol ratio over time, was compared in the absence and presence of increasing concentrations of inhibitor.

HL and LPL activities were determined by measuring the generation of FFA from the hydrolysis of human VLDL TG. Assays were performed in 10 mM Tris, pH 8.0, 150 mM NaCl, and 1.0 mM EDTA containing 1.5 mg/ml (HL) or 3.0 mg/ml (LPL) fatty acid-free BSA at 37°C for the indicated times.

### Binding of inhibitors to CETP

The association of $^3$H-inhibitor to CETP in purified form as well as in whole human plasma was determined. Purified CETP or control proteins were incubated with labeled inhibitor in PBS, pH 7.4, at the concentrations indicated. Free inhibitor was then removed by centrifuging the samples through G-50F Sephadex spin columns. After initial experiments demonstrated the lack of binding of the inhibitors to human IgG, in one experiment in which binding at lower CETP concentrations was tested, IgG was included as a carrier protein to improve the recovery of CETP off the columns (see Fig. 3A below). The distribution of $^3$H-inhibitor among plasma components was examined by adding inhibitor to human plasma, incubating for 10 min at 37°C, followed by fast-protein liquid chromatography (FPLC) fractionation at 4°C using dual Superose 6 HR10/30 columns connected in tandem. Isotonic 10 mM Tris, pH 8.0, 150 mM NaCl, 1.0 mM EDTA, and 0.02% $NaN_3$ (isotonic Tris) served as mobile phase at a flow rate of 0.2 ml/min.

### Association of CETP with lipoprotein

The ability of this series of inhibitors to alter the interaction of CETP with lipoprotein was determined by several methods, including FPLC fractionation and native PAGE/Western analysis of shifts in the apparent molecular weight of CETP, plasma CETP distribution by sequential ultracentrifugation, immunoaffinity extraction of CETP from plasma with or without inhibitor, and examination of CETP binding to isolated HDL using surface plasmon resonance (SPR). For FPLC analysis, human plasma with or without inhibitor was incubated for 10 min at 37°C. A 500 μl aliquot was injected in-line to dual Superose 6 columns as described above. As described previously (25), to ensure good recovery of CETP from plasma fractionated by FPLC, a low ionic strength buffer was used, in this case 65 mM sucrose, 225 mM mannitol, 10 mM Tris, pH 7.4 at 4°C, and 1.0 mM EDTA. In the plasma distribution studies using $^3$H-inhibitor described above, it was found that addition of inhibitor, particularly at higher concentrations, enhanced the recovery of CETP after FPLC when using isotonic buffer containing 150 mM NaCl. The converse also appeared true. Plasma to which exogenous CETP was added, as well as $^3$H-inhibitor, resulted in greater recovery of inhibitor than did native plasma alone. These observations suggest that the inhibitor-CETP complex is less likely to bind nonspecifically to the column matrix than either the inhibitor or CETP alone.

The effect of torcetrapib on the electrophoretic mobility of CETP in plasma or samples containing isolated lipoprotein was studied by native gel electrophoresis followed by Western blotting using capture and $^{125}$I-labeled detection anti-CETP monoclonal antibodies as described previously (25). Electrophoresis was performed at 120–150 V for 2.5 h using 4–12% (Novex) or 4–15% (Bio-Rad, Criterion) Tris-HCl minigels. High molecular weight native standards run in parallel lanes were used to calculate the apparent molecular weights of CETP-containing species. Transfer to nitrocellulose was accomplished using 25 mM Tris, 192 mM glycine containing 0.5% BSA, and 0.05% Tween 20. This sandwich blot protocol enabled both nontreated and torcetrapib-treated (HDL-bound) plasma CETP to be detected to an equal degree. For selected experiments, after the addition of torcetrapib and before electrophoresis, plasma was treated with a combination of water-soluble [bis(sulfosuccinimidyl) suberate] and water-insoluble (disuccinimidyl suberate) cross-linking agents (at 0.6 and 1.0 mM, respectively) for 1 h at room temperature. The distribution of CETP between free and HDL-bound states was determined by phosphor screen autoradiography of the $^{125}$I-labeled Western blots using a Molecular Dynamics Storm 860 scanner and ImageQuant software. To size different fractions of HDL used in CETP-HDL binding experiments, a combination of Sudan black staining and apo-A-I Western blotting was used. For apoA-I Western blotting, Chemicon anti-human apoA-I mouse monoclonal (MAB011) and $^{125}$I-goat polyclonal (AB740) antibodies were used for capture and detection, respectively.

For CETP immunoaffinity, a mouse anti-human CETP monoclonal antibody, 28B6, was covalently bound to beaded agarose via sulfhydryl linkage according to the protocol from Pierce (SulfoLink kit). This monoclonal antibody, with little or no inhibitory activity against CETP, binds both free and HDL-bound forms of CETP in the absence of detergent (data not shown). For the extraction of CETP, 10 ml of plasma with or without torcetrapib was diluted with an equal volume of isotonic Tris followed by the addition of 1 ml of 28B6 agarose. The samples were mixed at 4°C for 2.5 h and then transferred to columns. The eluted plasma was saved, and the columns were washed sequentially with 10 ml of isotonic Tris and 6 ml of 10 mM Tris, pH 7.8, 1 mM EDTA, and 0.02% $NaN_3$ (low ionic Tris) collected as 1.0 ml fractions. CETP was then eluted from the columns with 6 ml of 100 mM glycine, pH 3.25, and 1–2 ml of glycine, pH 3.0, into tubes containing Tris to neutralize the pH. The columns were washed with 2 ml of PBS. All fractions were assayed for both CETP and apoA-I mass by RIA and ELISA, respectively.

The effects of torcetrapib on CETP binding to immobilized HDL were determined by SPR using a Biacore 3000 instrument. HDL (d = 1.10–1.21 g/ml density fraction) was biotinylated through amide bond formation using the EZ-Link NHS-LC reagent (Pierce). For each experiment, ~1,200 resonance units of biotinyl-HDL was immobilized on a Biacore streptavidin chip. Purified CETP was incubated with or without torcetrapib in 10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% DMSO, and 1 mg/ml BSA, pH 7.4, then injected for the specified time period over the HDL at a flow rate of 100 μl/min at 25°C. All binding data were double referenced against an unmodified streptavidin surface and buffer blank injections. Data fitting was performed using Biacore Biaevaluation Software.

### Lipid binding to CETP

The effects of inhibitor on the ability of CETP to bind CE, TG, or PL were examined in dual-label experiments using $^3$H-

inhibitor and [$^{14}$C]-labeled lipid. Purified CETP was preincubated with or without $^{3}$H-inhibitor in isotonic Tris, pH 7.4, containing 6 mg/ml BSA for 5–10 min at 37°C. PC liposomes with the [$^{14}$C]-labeled test lipid incorporated were then added, and incubation was continued for 1.5 or 4 h as specified. The liposomes were separated from free CETP by FPLC at 30°C using low ionic strength buffer (10 mM Tris and 1.0 mM EDTA, pH 7.8). For cholesteryl oleate and PC binding studies, a single 30 cm × 1.0 cm diameter Toyopearl HW55F sizing column was used. For the triolein binding experiments, in some instances dual 30 cm Sephadex G150 columns connected in tandem were used (see Fig. 8C below). The inhibitor and test lipid concentrations of the resulting fractions were determined by liquid scintillation counting, PL was determined by the enzymatic method, and CETP was determined by RIA (25). The concentrations in the incubation sample of CETP, [$^{14}$C]-lipid, PC, and $^{3}$H-inhibitor were as follows: for [$^{14}$C]cholesteryl oleate as test lipid, 1.5, 7.5, 300, and 4.5 μM, respectively; for [$^{14}$C]PC as test lipid, 9, 300, and 30 μM; and for [$^{14}$C]triolein as test lipid, 3, 7.5, 375, and 5 μM.

## RESULTS

### Potent and selective inhibition of CETP

For the purposes of describing the torcetrapib series of CETP inhibitors, both torcetrapib itself (**Fig. 1A**) and a close analog, Pfizer A (Fig. 1B), were used. Both potently block CE and TG transfer in whole plasma with approximate $IC_{50}$ values of 50 nM. A typical inhibition curve for torcetrapib is shown in Fig. 1C. In this case, for a pool of fasted human plasma having a CETP concentration of 34 nM, the $IC_{50}$ for CE and TG transfer was 47 and 51 nM, respectively. In parallel assays, the two inhibitors were equal in potency (Fig. 1D).

Torcetrapib not only inhibits CETP-mediated neutral lipid transfer but PL transfer as well. A comparison of torcetrapib's ability to inhibit CETP-dependent transfer of CE and PL transfer versus PLTP-dependent PL transfer, under similar conditions, is detailed in **Fig. 2A**. Clear inhibition of CE and PL transfer by CETP is achieved with 10–30 nM torcetrapib, whereas concentrations up to 10 μM have no effect on PLTP. Further evidence of selectivity is shown by the lack of effect of torcetrapib on LCAT (Fig. 2B) and on both hepatic and lipoprotein lipases (Fig. 2C).

### Inhibitors bind with 1:1 stoichiometry to CETP

Because the concentration of torcetrapib and its analogs required for the inhibition of CETP is close to that of plasma CETP and far less than that of the lipoprotein

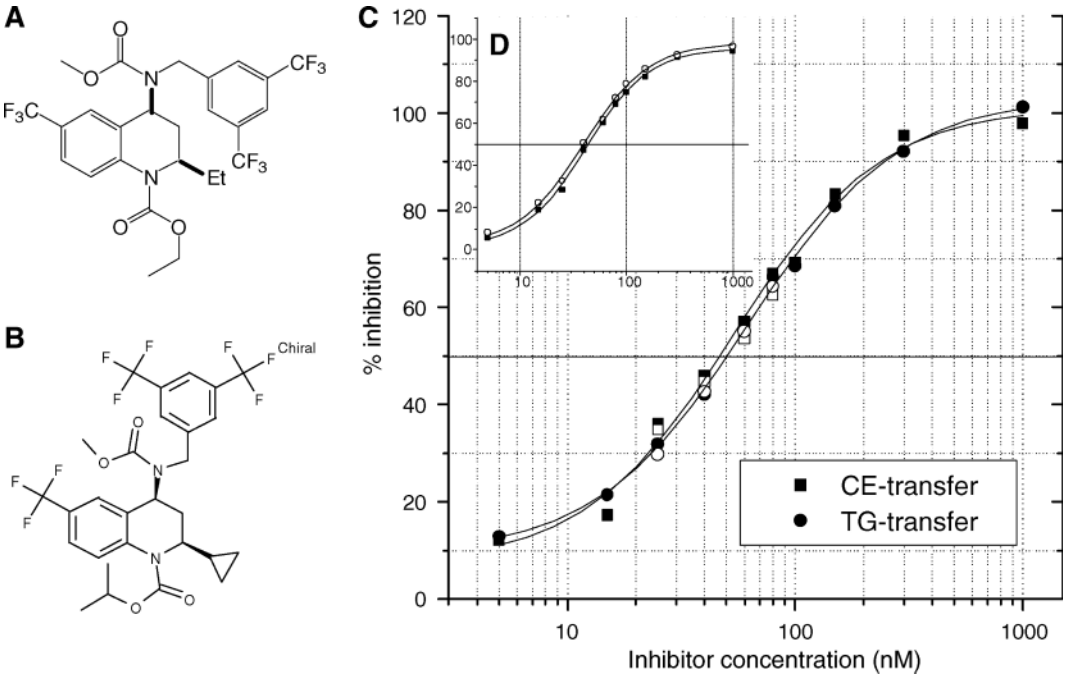


**Fig. 1.** A: Torcetrapib (CP529,414). B: Pfizer A, a close analog of torcetrapib. C: Inhibition by torcetrapib of cholesteryl ester transfer protein (CETP) activity in whole human plasma [CETP = 1.8 μg/ml (34 nM); total plasma choleterol (TPC), high density lipoprotein cholesterol (HDL-C), and triglyceride (TG) = 212, 41, and 124 mg/dl, respectively]. The bidirectional transfer of triolein from LDL to HDL and of cholesteryl oleate from HDL to the non-HDL plasma fraction was determined simultaneously using [$^{14}$C]triolein-labeled LDL and [$^{3}$H]cholesteryl oleate-labeled HDL as donor substrates. The percentage inhibition relative to control was determined from the linear portions of the transfer curves: 2.25 h for [$^{14}$C]triolein transfer and 1.5 h for [$^{3}$H]cholesteryl oleate. For selected samples [controls and 25–>80 nM inhibitor (open symbols)], plasma and HDL free and total cholesterol were measured and cholesteryl ester (CE) was determined by the difference. This allowed transfer rates to be calculated using actual specific activities for HDL and non-HDL CE and triolein at each time point. D: Comparison of potency for torcetrapib and Pfizer A in whole plasma determined by transfer of [$^{3}$H]cholesteryl oleate from HDL to LDL and of [$^{14}$C]cholesteryl oleate from LDL to HDL (closed symbols, torcetrapib; open symbols, Pfizer A).

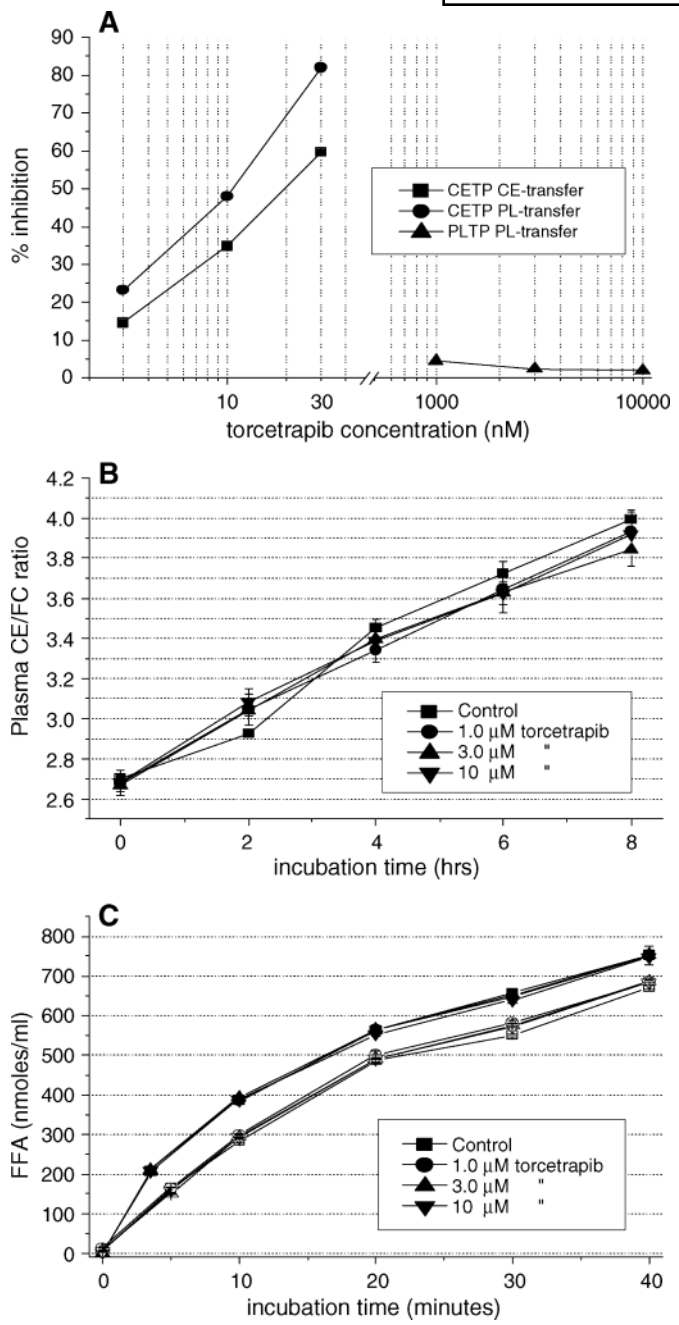


**Fig. 2.** A: Comparison of inhibition by torcetrapib of CETP-dependent CE and phospholipid (PL) transfer versus phospholipid transfer protein (PLTP)-dependent PL transfer. Assays, performed in triplicate, contained labeled donor liposomes and acceptor HDL, both at 310 μM PL. CETP-dependent CE and PL transfer assays contained CETP at 4 and 150 ng/175 μl assay. The PLTP assay contained 35 ng PLTP/175 μl. B: A pool of human plasma from four individuals was examined for the effects of torcetrapib on LCAT activity. Free and total cholesterol contents of plasma, assayed in quadruplicate, were determined at the indicated times. CE was calculated by subtracting free cholesterol (FC) from total cholesterol. LCAT activity is reflected by the increase in the plasma CE/free cholesterol ratio in the absence or presence of increasing torcetrapib concentrations. C: The effects of torcetrapib on hepatic lipase (closed symbols) and LPL (open symbols) activities were determined by measuring the release of free fatty acid from human VLDL in the absence or presence of inhibitor. Error bars represent standard deviation.

substrates, it was expected that these inhibitors exerted a direct effect on CETP. The results of a direct binding experiment, using Pfizer A, are shown in **Fig. 3A**. At preincubation ratios of inhibitor/CETP < 1.0, essentially all inhibitor was recovered with CETP in the collected sample, indicating that all drug was in the CETP-bound state. At preincubation ratios of 1–2 mol of inhibitor per CETP, the resulting ratio of bound inhibitor to CETP was close to 1. At higher preincubation ratios, the stoichiometry of bound inhibitor to CETP increased slightly, and then at ratios > 4:1, the resulting postspin column sample exhibited an increase in bound inhibitor up to 7 mol torcetrapib/CETP as a result of nonspecific aggregation of CETP and inhibitor (Fig. 3A, inset). The appearance of the plateau in the binding curve at ~1 mol inhibitor/mol CETP suggested a single high-affinity binding site on CETP. In similar experiments, the binding of [$^3$H]torcetrapib to purified CETP, both the wild type and a mutant form with residues 470–475 deleted, was also examined (**Table 1**). The mutant form of CETP was previously reported to have greatly reduced binding and transfer of CE and TG compared with WT CETP (23, 28). A mean of 1.1 mol of torcetrapib was bound per mole of WT CETP, and 1.0 mol/mol mutant CETP. The lack of a significant difference in inhibitor binding to WT versus mutant CETP as well as the fact that the residual CE transfer activity of the mutant was blocked by torcetrapib (data not shown) demonstrated that residues 470–475 were not involved in the action of the inhibitor. Human albumin and IgG tested under the same conditions bound little or no inhibitor.

Further evidence for the presence of a single high-affinity binding site for the inhibitor on CETP is shown by the distribution profiles of $^3$H-inhibitor in human plasma (Fig. 3B). In native plasma, a single main peak of CETP coeluted with inhibitor at a stoichiometry of 1:1. Inhibitor in excess of that bound to CETP showed a preference for inclusion within TG-rich VLDL more so than the CE-rich LDL or HDL or the bulk protein fraction. In the case of plasma supplemented with purified CETP, inhibitor again coeluted with CETP with 1:1 stoichiometry. In this case, the increased portion of inhibitor associated with CETP corresponded with a decrease in the amount eluting with lipoprotein and especially the bulk protein fraction.

### Torcetrapib shifts plasma CETP to an HDL-bound state

In Fig. 3B, in the presence of inhibitor, CETP was shown to elute with an apparent size greater than that for HDL. The reason for this is revealed in **Fig. 4**. Human plasma CETP is a 476 amino acid glycoprotein that exhibits an apparent molecular weight (Mr) of 66,000–69,000 by SDS-PAGE (29). However, the protein separated by native PAGE appears as a broad band of 115–135 kDa (Fig. 4A). This higher apparent size, relative to spherical molecular weight standards, appears to be attributable to the elongated shape of CETP (20). When plasma was fractionated by FPLC (Fig. 4B), CETP eluted in a size range smaller than HDL, but with the addition of inhibitor, a large por-

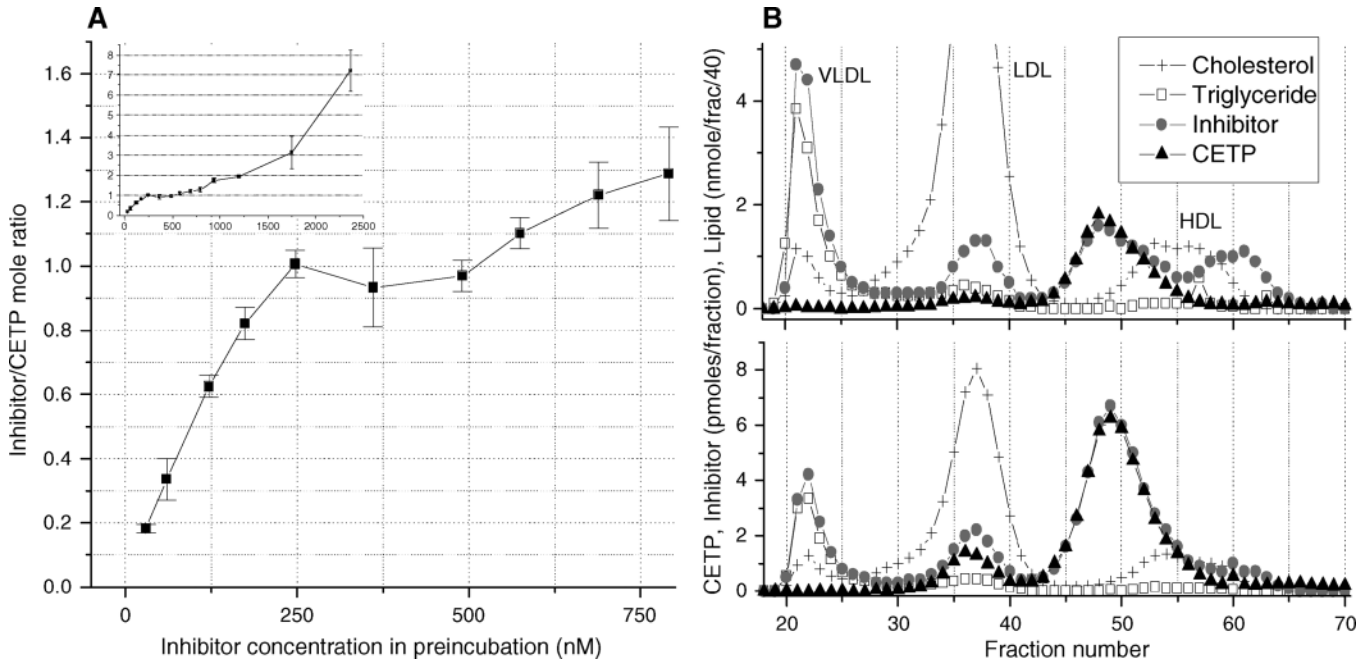


**Fig. 3.** A: Binding of [$^3$H]Pfizer A to purified CETP. CETP (240 nM) was incubated with varying concentrations of labeled inhibitor for 10 min at 37°C in quadruplicate. Human IgG was included as carrier protein at 500 μg/ml. Free drug was then removed by centrifuging aliquots, in duplicate for each sample, through G-50F Sephadex spin columns. The CETP and inhibitor concentrations of the collected sample were determined by CETP mass assay and scintillation counting (in duplicate/aliquot). Results are means for n = 4 measurements. Inset: Inhibitor-CETP binding resulting from preincubation ratios of inhibitor/CETP > 4. B: The distribution profile of $^3$H-inhibitor added to human plasma was determined by fast-protein liquid chromatography (FPLC) fractionation, followed by measurement of inhibitor, CETP, cholesterol, and TG content. The upper plot is for native plasma [1.95 μg CETP/ml (37 nM) and 34 mg/dl HDL-C], and the lower plot is for the same plasma supplemented with exogenous purified CETP to 10 μg/ml. Recoveries in combined fractions for cholesterol, TG, CETP, and inhibitor were 101, 102, 83, and 48%, respectively, for nonsupplemented plasma and 106, 91, 62, and 85% for supplemented plasma. The stoichiometries of CETP to HDL particles in the profiles shown for native and supplemented plasma are roughly 1:600 and 1:160, respectively. Error bars represent standard deviation.

tion of CETP shifted to an apparent size greater than HDL, as shown in Fig. 3B. This size shift induced by torcetrapib was also demonstrated by native PAGE/CETP Western analysis (Fig. 4C–E). In Fig. 4C, the Mr for purified CETP was compared with that for CETP in four individual whole plasma samples, in the absence or presence of torcetrapib. Compared with the 115–135 kDa value for purified CETP, the four plasmas, minus inhibitor, displayed CETP at mean values of 155–180 kDa. This modest increase in the apparent size of plasma CETP relative to purified CETP is consistent with the transient association of CETP with HDL particles during the initial stage of electrophoretic separation and has been observed previously for CETP incubated with isolated HDL (23). With the addition of torcetrapib, a shift in CETP to a much greater Mr was observed. For the plasmas with high HDL-C (predominantly $HDL_2$), the difference in Mr between that for purified CETP and plasma CETP was 230–270 kDa (Fig. 4C, lanes 6 and 12). For the plasma samples with low HDL-C (predominantly $HDL_3$), the shift in Mr from that for purified CETP was 170–200 kDa (Fig. 4C, lanes 3 and 9). Therefore, the magnitude for the shift in apparent size induced by torcetrapib was in the range expected for the tight association of CETP with an HDL particle.

For the experiment shown in Fig. 4D, plasma was fractionated into non-HDL lipoprotein, total HDL, and d > 1.25 g/ml subfractions by ultracentrifugation and then reconstituted with and without torcetrapib. Inhibitor added to either whole plasma (Fig. 4D, lane 2) or total reconstituted plasma (Fig. 4D, lane 4) induced a similar shift to a higher Mr for CETP. Torcetrapib added to the partial combination of non-HDL and d > 1.25 g/ml fraction (Fig. 4D, lane 6) showed a much reduced shift in Mr, indicating that the HDL fraction was responsible for the size change in plasma. This was also indicated by the large shift in size observed for torcetrapib added to the HDL

TABLE 1. Comparison of [$^3$H]torcetrapib binding to wild-type CETP, Δ470–475 deletion mutant CETP, and human albumin or IgG

| Test Protein | Number | Inhibitor-Protein Molar Ratio |
|---|---|---|
| Wild-type CETP | 10 | 1.06 ± 0.26 |
| Deletion mutant CETP | 8 | 1.00 ± 0.24 |
| Human albumin | 4 | 0.040 ± 0.018 |
| Human IgG | 4 | <0.001 |

CETP, cholesteryl ester transfer protein. Test proteins (2.0 μM) were incubated for 15 min at 37°C with [$^3$H]torcetrapib (3.0 μM), followed by removal of free inhibitor using G-50F Sephadex spin columns (duplicate aliquots/sample). The mean inhibitor-protein molar ratios ± SD for the collected material are shown. No significant difference for torcetrapib binding to wild-type versus mutant CETP was observed ($P = 0.625$ by Student's $t$-test).

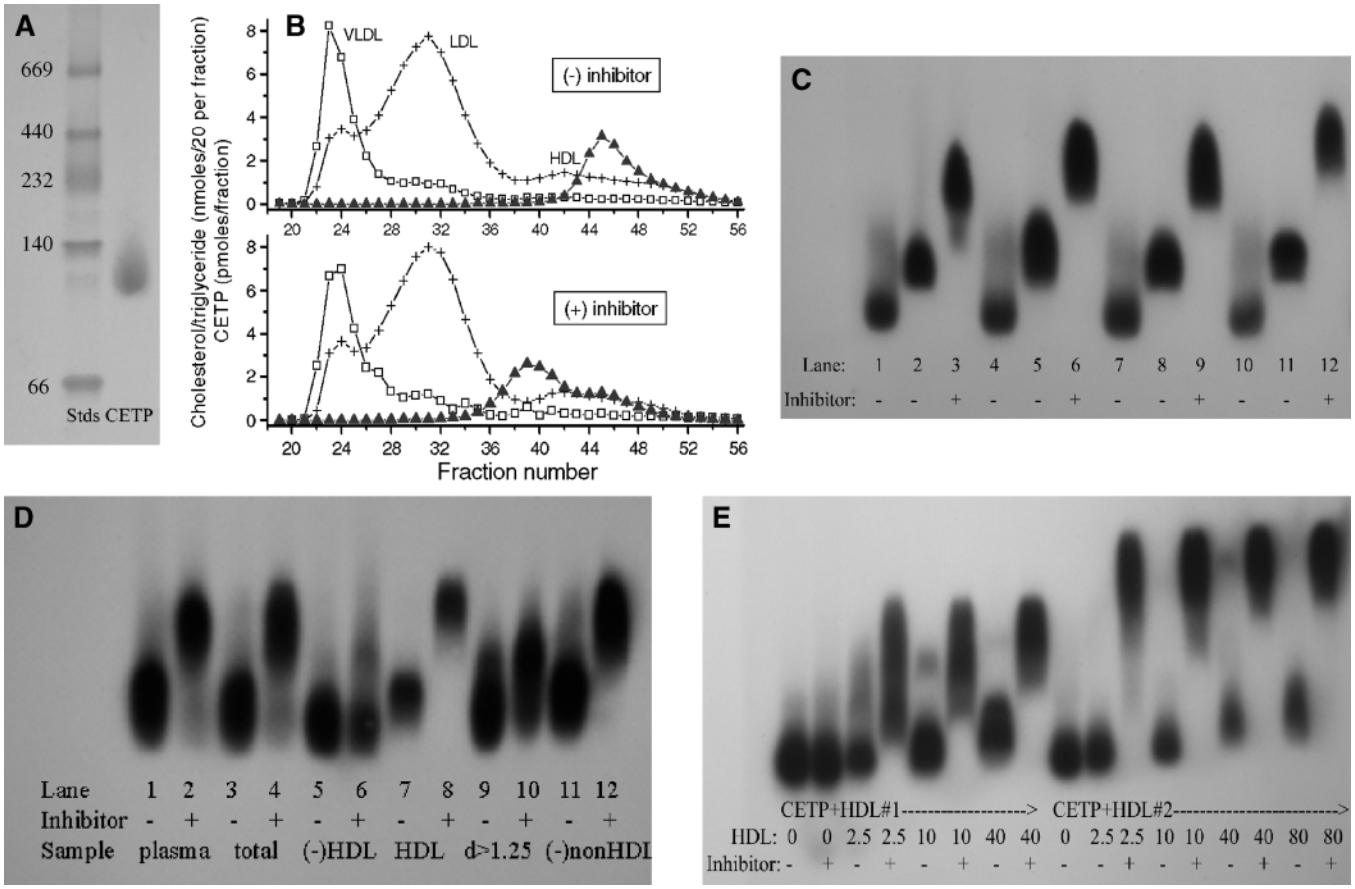


**Fig. 4.** A: Native PAGE of purified CETP relative to native molecular mass standards (Stds; from top, 669, 440, 232, 140, and 66 kDa). B: Effects of inhibitor on the apparent molecular weight (Mr) of CETP in human plasma. Pfizer A (500 nM) or DMSO was added to plasma and the sample was incubated for 10 min at 37°C, followed by FPLC fractionation and assay for CETP mass (triangles), cholesterol (crosses), and TG (squares). Recoveries of CETP within the combined fractions for minus and plus inhibitor samples were 94% and 109%, respectively. C: Native PAGE/anti-CETP Western blot analysis of purified CETP (lanes 1, 4, 7, and 10) compared with plasma CETP in the absence or presence of 500 nM torcetrapib. Plasma was obtained from male (lanes 2, 3) and female (lanes 8, 9) subjects with low HDL-C levels of 26 and 36 mg/dl, respectively, as well as from male (lanes 5, 6) and female (lanes 11, 12) subjects with high HDL-C of 58 and 55 mg/dl, respectively. D: Native-PAGE/CETP Western blot showing the effects of torcetrapib (250 nM) on the apparent size of the CETP-containing species in whole plasma (lanes 1, 2) and in total versus partially reconstituted plasma. Plasma from a single individual was fractionated by sequential ultracentrifugation into a $d < 1.063$ g/ml fraction, a $d = 1.063–1.25$ g/ml total HDL fraction, and a lipoprotein-depleted $d > 1.25$ g/ml fraction. Total reconstituted plasma (lanes 3, 4) consisted of all three fractions combined; lanes 5, 6, total minus HDL; lanes 7, 8, HDL only; lanes 9, 10, $d > 1.25$ g/ml only; lanes 11, 12, total minus $d < 1.063$ g/ml fraction. E: CETP Western blot showing the effect of torcetrapib (500 nM) on purified CETP in the presence of increasing concentrations of isolated HDL (expressed as mg/dl). HDL#1 and HDL#2 were obtained by FPLC subfractionation of plasma from a male (low HDL-C, 35 mg/dl) and female (high HDL-C, 87 mg/dl) subject, respectively. HDL#1 consisted predominantly of $HDL_{3b}$ particles (mean apparent molecular mass = 130 kDa, 8.0 nm diameter) but with smaller $HDL_{3c}$ species (85–115 kDa) present as well. HDL#2 contained both $HDL_{2b}$ (mean apparent molecular mass = 290 kDa, 10.9 nm) and $HDL_{2a}$ (mean apparent molecular mass = 215 kDa, 9.6 nm). Note: The doses of 250 and 500 nM inhibitor were chosen for these experiments because they produce nearly total inhibition of CETP activity (see Fig. 1C, D).

fraction alone (Fig. 4D, lane 8). Inhibitor added to the $d > 1.25$ g/ml fraction alone (Fig. 4D, lane 10) produced a partial shift in Mr, similar to that seen for a portion of CETP in lane 6 for the combined $d < 1.063$ g/ml + $d > 1.25$ g/ml fractions. Combination of HDL plus the $d > 1.25$ g/ml fraction produced results with torcetrapib (Fig. 4D, lane 12) similar to those of whole plasma and total reconstituted plasma, demonstrating that the non-HDL fraction was not required for the effect. In Fig. 4E, the effect of torcetrapib on the mobility of purified CETP in the presence of increasing concentrations of small HDL (HDL#1) or large HDL (HDL#2) was determined. In the absence of HDL, torcetrapib had no effect on the Mr of purified CETP. When HDL was added incrementally, from very low to physiological concentrations, there was a progressive increase in Mr for CETP. In the absence of torcetrapib, CETP reached maximum values of 165 and 180 kDa at physiological concentrations of HDL#1 and HDL#2, respectively. This change is similar in magnitude to the difference between purified CETP and plasma CETP in the absence of torcetrapib (Fig. 4C) and supports the interpretation that the higher Mr for plasma CETP versus purified CETP results from the delayed migration of CETP in the early stages of electrophoresis as a result of its

transient association with HDL. In contrast, when torcetrapib was added, CETP shifted to the much larger mean values of 325 kDa (~11.5 nm diameter) and 585 kDa (~15.25 nm diameter) with HDL#1 and HDL#2, respectively, consistent with the tight association of CETP with the different sized HDLs.

### The degree to which torcetrapib shifts CETP to an HDL-bound state correlates tightly with inhibition

Further evidence that torcetrapib induced complex formation between plasma CETP and HDL was provided by fractionating plasma by sequential ultracentrifugation into non-HDL, HDL, and d > 1.21 g/ml subfractions (**Fig. 5A**). CETP mass determination showed the non-HDL lipoprotein fraction to be devoid of CETP under all conditions. In the absence of torcetrapib, ~25% of total plasma CETP was found in the HDL fraction and 75% in the d > 1.21 g/ml fraction. With increasing torcetrapib concentrations, CETP shifted to the HDL fraction, and at 500 nM inhibitor, the distribution was reversed to 75% HDL-associated and 25% in the d > 1.21 g/ml fraction. The same plasma-torcetrapib series was also analyzed by native PAGE/CETP Western blotting (Fig. 5B). As was the case for density fractionation, increasing torcetrapib concentration progressively shifted plasma CETP to an HDL-bound state. However, the degree of CETP-HDL binding indicated by the Western analysis correlated more closely with the percentage inhibition of CETP activity than did the density distribution. Because the high salt and centrifugal forces used for density fractionation could alter the nature of the CETP-HDL complex, native PAGE/Western analysis was used to further study the correlation of HDL binding and inhibition. Twelve individual plasma samples were treated with 0–500 nM torcetrapib followed

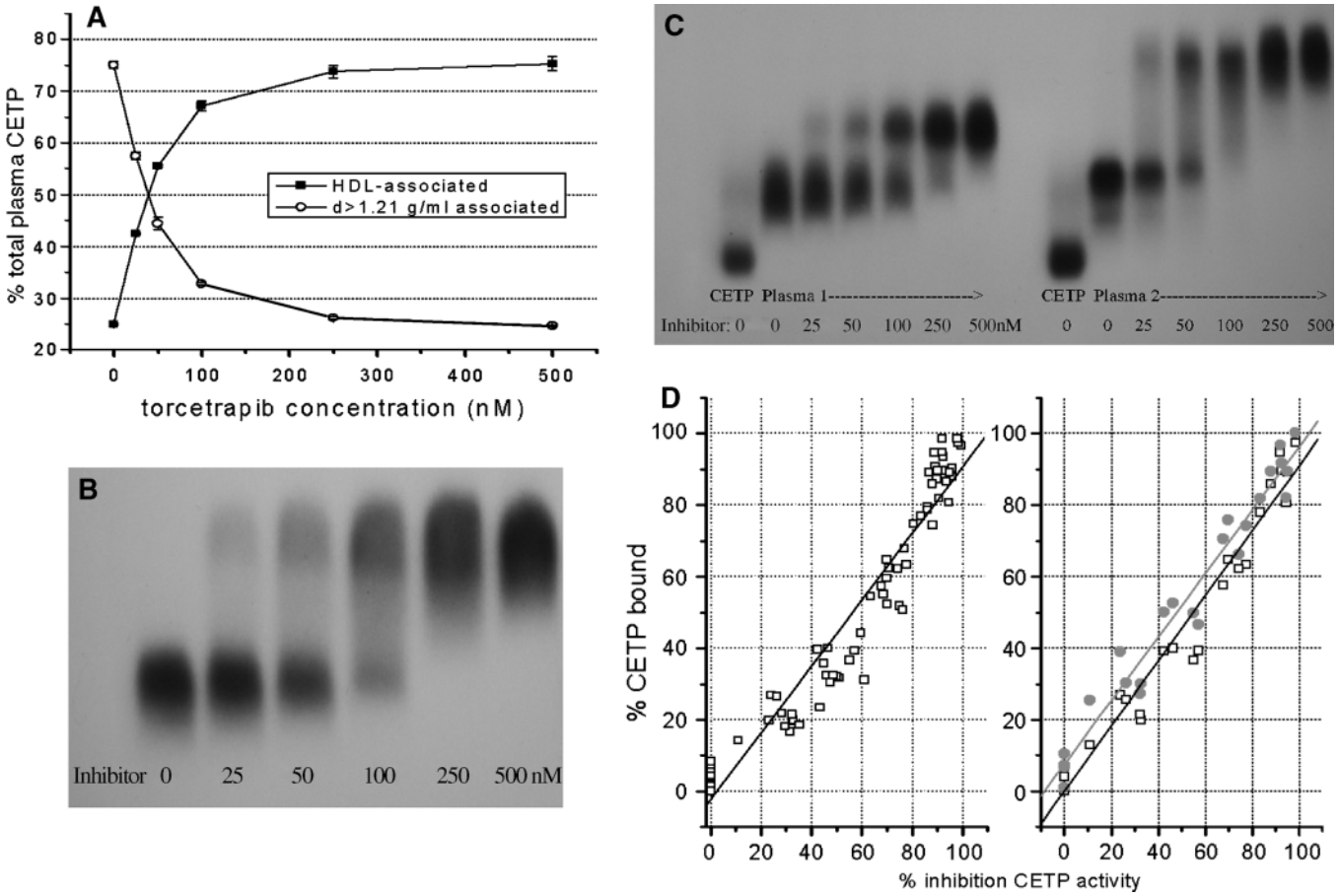


**Fig. 5.** A: Dose effect of torcetrapib added to plasma up to 500 nM on the distribution of plasma CETP between HDL (d = 1.063–1.21 g/ml) and the d > 1.21 g/ml fraction isolated by sequential centrifugation. Recovery of initial plasma CETP mass in the two fractions was 85 ± 3% (n = 6). CETP mass for all samples was assayed in triplicate. B: The same plasma series as for A but CETP distribution was determined by native PAGE/Western blot quantification using phosphor screen autoradiography. C: Comparison of torcetrapib-induced shift in Mr for CETP, determined as for B, for plasma from a male with low HDL-C (31 mg/dl) versus a female with high HDL-C (90 mg/dl). D: Twelve plasma samples, including the two analyzed in C, were analyzed by both native PAGE/CETP Western blotting and dual-label CE transfer assay (in triplicate). The left panel shows the correlation of percentage inhibition of CETP activity versus percentage HDL-bound CETP ($r$ = 0.965). The right panel shows the same correlation for four of the plasma series for both untreated samples (open squares) and samples treated with a combination of bis(sulfosuccinimidyl) suberate and disuccinimidyl suberate X-linking agents (solid circles). Free and HDL-bound forms of plasma CETP were detected equally by this Western protocol (see Materials and Methods). The ratio of total phosphorimager signal (free + bound) for the +500 nM inhibitor samples to the minus inhibitor samples was 1.008 ± 0.120 for n = 12. The mean $IC_{50}$ in the 12 individual plasma samples was 57 ± 21 nM. Error bars represent standard deviation.

by both CE transfer assay to assess the inhibition of CETP activity and native PAGE/Western analysis to determine the distribution of CETP between free and HDL-bound states. Autoradiograms for two of these plasma series, the first for plasma from a male with low HDL-C (predominantly $HDL_3$) and the second for a female with high HDL-C (predominantly $HDL_2$), are shown in Fig. 5C. Purified CETP was included as a reference. A combination of Sudan black staining and apoA-I Western blotting of the two plasmas indicated the presence of several HDL size classes (data not shown). For plasma 1, the bulk of HDL fell within the particle size range of 7.6–8.4 nm diameter, and for plasma 2, most HDL was within the 8.5–11.7 nm diameter range. The addition of torcetrapib to plasma 1 and 2 resulted in mean apparent sizes for the CETP-containing complex of 11.5 and 15.0 nm, respectively. Comparison of the percentage inhibition of CETP activity with the percentage total CETP in an HDL-bound state demonstrated that the two end points were tightly correlated (Fig. 5D, left panel). The percentage total CETP shifted to the bound state, however, was in many cases slightly lower than the percentage inhibition. This may be attributable to some dissociation of the CETP-HDL complex during electrophoresis. For four of the plasma series, the samples were divided into two aliquots. One aliquot was treated with a mixture of water-soluble and water-insoluble X-linking agents after the addition of inhibitor and before electrophoresis. A comparison of the percentage shift in CETP observed for the treated and untreated samples is shown in Fig. 5D (right panel). In the absence of torcetrapib, X-linker treatment increased the percentage CETP bound only slightly, from 3.7% to 6.2%. The effect of X-linker was greater for the torcetrapib-containing samples, with a net increase of 10% observed for the 25–100 nM inhibitor series. This is consistent with the inhibitor inducing a tighter association between CETP and HDL.

### Immunoaffinity extraction of CETP from plasma confirms the involvement of apoA-I-containing lipoprotein in the inhibitor-induced complex

The complex induced by torcetrapib was examined using immunoaffinity to remove CETP from both untreated plasma and plasma containing torcetrapib. **Figure 6A** summarizes the mean results for three experiments. Although the amount of CETP recovered in the glycine elution was similar with and without inhibitor, the content of apoA-I was quite different. In the absence of inhibitor, only 0.38 ± 0.12 mol of apoA-I was recovered per mole of CETP, but with inhibitor, 1.7 ± 0.6 mol of apoA-I per CETP was collected ($P < 0.05$). The two plasmas, with predominantly $HDL_2$ (female) or $HDL_3$ (male), shown in Fig. 5C were included in this experiment. The fact that the moles of apoA-I recovered per mole of CETP was less for the former (1.5) than the latter (2.3) suggests that either the inhibitor-induced complex formation with HDL, or alternatively, the binding of the monoclonal antibody to the CETP-HDL complex, caused a loss of apoA-I from the larger HDL particles.

The CETP distribution in the glycine-eluted fraction was also examined by native PAGE and Western blotting. The results for one experiment, using the low-HDL plasma described above, is shown in Fig. 6B. Purified CETP was included for reference as well as the initial plasma samples

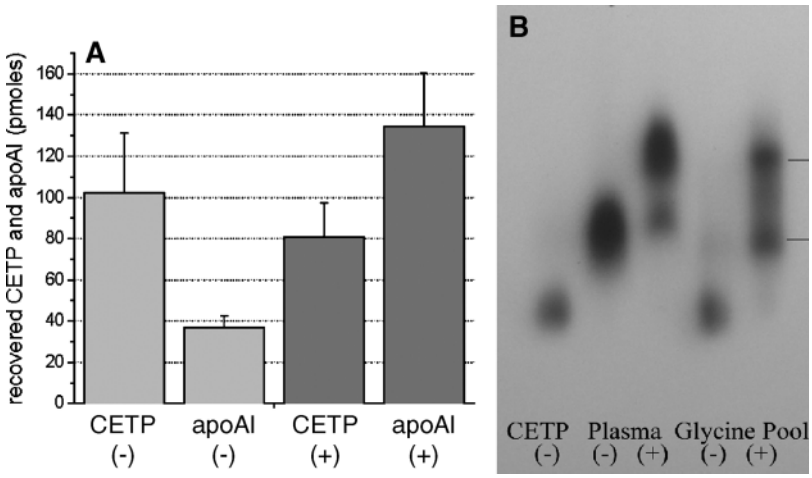


**Fig. 6.** CETP was extracted from plasma with or without 250 nM torcetrapib using anti-CETP immunoaffinity. Ten milliliters of plasma was mixed with monoclonal antibody 28B6 linked to agarose for 2.5 h and then transferred to columns as described in Materials and Methods. The affinity column was washed with both isotonic and low ionic strength buffer, followed by the elution of CETP with low-pH glycine and an additional PBS wash. CETP and apolipoprotein A-I (apoA-I) in all eluted fractions were assayed in duplicate by RIA and ELISA, respectively. A: The mean content (± SD) of CETP and apoA-I in the combined glycine and final PBS wash is shown for three separate plasma samples. B: Autoradiogram after native PAGE/CETP Western blotting of both starting plasma and the glycine/PBS-eluted fraction, with or without inhibitor, for one plasma (a low-HDL subject). Purified CETP was included for reference. Error bars represent standard deviation.

with or without inhibitor. For plasma, as expected, the addition of inhibitor induced most of the CETP to shift to an HDL-bound state. In the absence of inhibitor, CETP in the glycine-eluted fraction migrated at the same position as purified CETP, indicating that it was free and unaffected by the small amount of HDL present. In contrast, for the plus-inhibitor condition, CETP in the glycine-eluted sample migrated as two bands. The lower band was observed at a position more or less matching that of plasma CETP in the absence of inhibitor, suggesting a transient association of the eluted CETP with HDL, whereas the upper band was at a position similar to plasma CETP in the presence of inhibitor, indicating that it was tightly bound to HDL.

### Treatment of purified CETP with inhibitor increases its affinity for HDL

SPR was used to further characterize the effects of torcetrapib on the binding of CETP to HDL. In **Fig. 7**, the inhibitor is shown to increase total binding to immobilized HDL by >3-fold. In addition, the rate of CETP dissociation is clearly decreased. Attempts to fit the data to simple kinetic models indicated that binding is complex. Fitting dissociation data to two separate exponentials indicates that untreated CETP dissociates at two different rates, a fast rate of 0.2 $s^{-1}$ and a slow rate of 0.02 $s^{-1}$. Compound-treated CETP also dissociates at two rates, a fast rate of 0.03 $s^{-1}$ and a slow rate of 0.003 $s^{-1}$. Thus, compound treatment decreases the rates of dissociation from HDL by 6- to 7-fold. Attempts were made to estimate apparent affinities for torcetrapib-treated and untreated CETP by globally fitting kinetic data obtained over a range of CETP concentrations from 14 to 380 nM (data not shown). For binding of untreated CETP, a reasonable global fit was obtained using a two-sites model (the Biaeval heterogeneous ligand model). The results suggested that in the absence of compound, there are two kinetically different sites each having a dissociation constant of ~50 nM. Data obtained for compound-treated CETP over the same range of CETP concentrations could not be globally fit using the two-sites model. However, to obtain an estimate for the affinity of compound-treated CETP, the data in Fig. 7 were fit to the two-sites model. The results indicated two kinetically different sites each of ~10 nM. Thus, compound increases the apparent affinity of CETP for HDL by ~5-fold from 50 nM to ~10 nM.

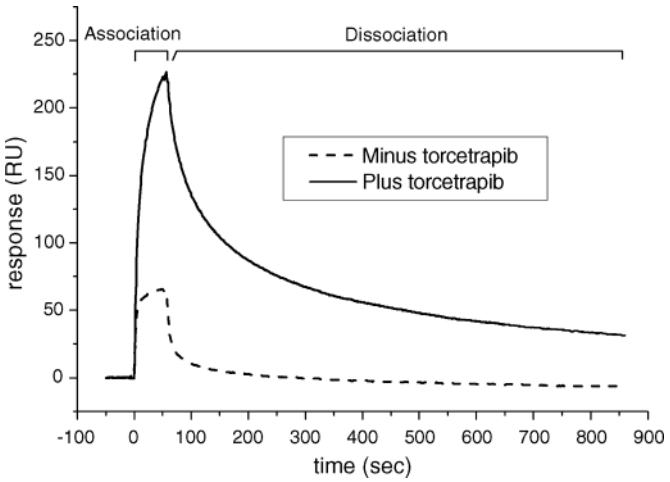


**Fig. 7.** Effect of torcetrapib on the kinetics of CETP binding to HDL as determined by surface plasmon resonance. Purified CETP (190 nM) was preincubated with or without torcetrapib (2 μM), then injected for 60 s over HDL immobilized on the surface of the Biacore chip. Dissociation was observed over 800 s. Inhibitor was present only in the injection volume. RU, resonance units.

### Inhibitor effects on lipid binding to CETP

The data described above indicate that the underlying mechanism of inhibition for torcetrapib is the formation of a nonproductive complex between CETP, inhibitor, and HDL. The normal association of CETP with lipoproteins involves both electrostatic and hydrophobic interactions (30). Altered charge does not appear to be involved in torcetrapib's effect on CETP binding to HDL, because the isoelectric point for native CETP is unchanged by the inhibitor (see supplementary Fig. I). On the other hand, formation of the CETP-HDL complex by inhibitor is reduced under conditions of low ionic strength, suggesting that increased hydrophobic interactions are involved in the mechanism (data not shown).

Given the lipophilic nature of the inhibitor and the fact that CETP possesses binding sites for both neutral lipid and PL, the question was asked whether the binding of torcetrapib to CETP would alter the subsequent binding of lipid. **Fig. 8A–C** summarizes three sets of experiments in which CETP was preincubated either alone or with $^{3}$H-labeled torcetrapib under conditions demonstrated previously to cause 1:1 binding of inhibitor to CETP. The CETP was then incubated with PL unilamellar vesicles containing [$^{14}$C]-labeled CE, PC, or TG, followed by the separation of free CETP from the liposomes. For the CE, PC, and TG binding experiments, pretreatment of CETP with inhibitor resulted in means of 1.24, 1.03 ± 0.06, and 1.09 ± 0.09 mol inhibitor bound per mole of CETP (for n = 2, 3, and 3 experiments each). No significant difference was found for the binding of CE (mean, 1.42 vs. 1.24 CE/CETP; n = 2) or PC (1.69 ± 0.06 vs. 1.74 ± 0.31 PC/CETP; n = 3) to torcetrapib-treated versus control CETP, respectively. For TG binding, initial experiments indicated reduced binding of TG to CETP pretreated with inhibitor. Using delipidated CETP in the absence of inhibitor, 0.082 ± 0.006 mol TG/CETP was bound (n = 6) versus 0.046 mol TG/CETP (n = 2) after inhibitor treatment, and with nondelipidated CETP, the results were similar, 0.107 mol TG/CETP minus inhibitor (n = 2), versus 0.039 mol TG/CETP (n = 1). In a second set of experiments, chromatography using Sephadex (Fig. 8C) instead of the Toyopearl resin used in Fig. 8A, B resulted in 0.303 ± 0.013 mol TG/CETP bound for control CETP (n = 3) versus 0.200 ± 0.063 for treated CETP (n = 3) based on TG content in the combined fractions containing free CETP. However, the main peak of TG observed

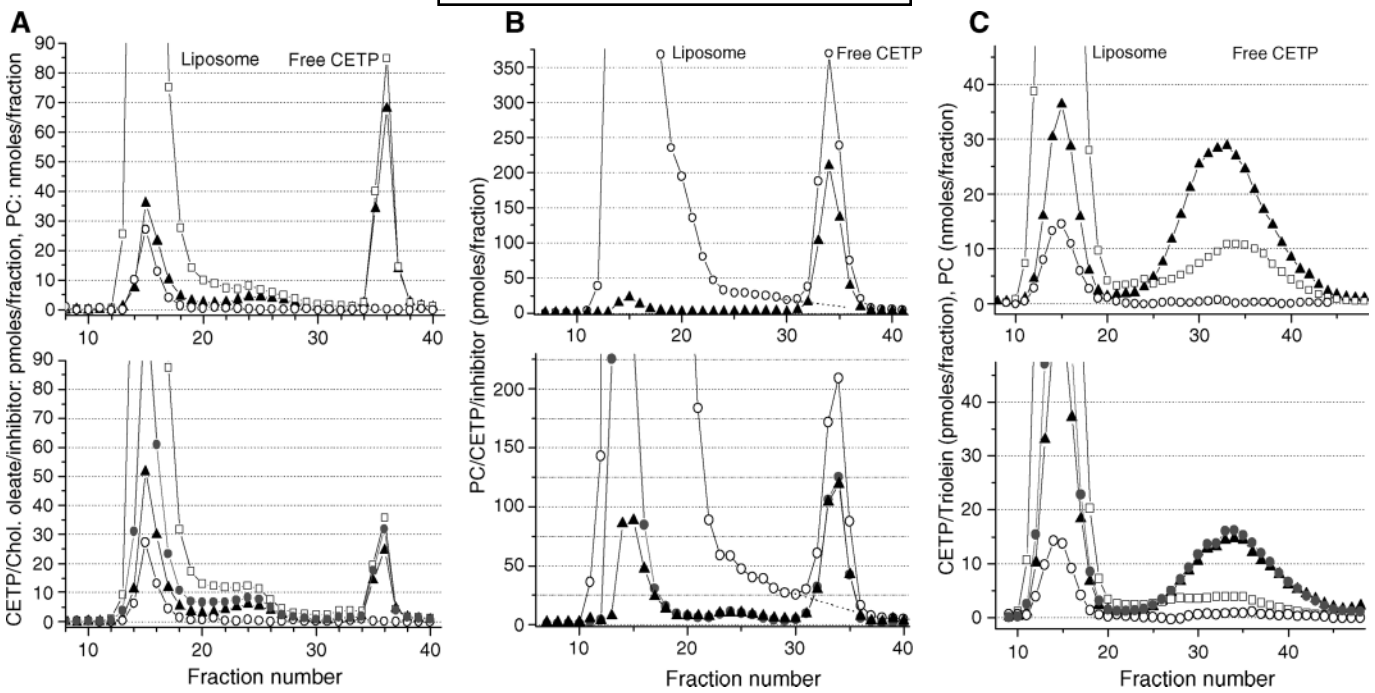


**Fig. 8.** Effect of Pfizer A on binding of lipids to CETP. For A–C, upper panels show the absence of inhibitor, and lower panels show the presence of inhibitor. A: Delipidated, purified CETP was preincubated with $^{3}$H-inhibitor for 10 min, followed by the addition of [$^{14}$C]cholesteryl oleate-labeled liposomes. The sample was incubated for 1.5 h, followed by FPLC fractionation and liquid scintillation counting of labels and assay of CETP mass and PL. Plotted are mean values for two separate experiments both with and without inhibitor. Open circles, PL; open squares, cholesteryl oleate; closed circles, inhibitor; triangles, CETP. See Materials and Methods for additional details. B: PL binding to purified, nondelipidated CETP examined as for A, except that labeled phosphatidylcholine (PC; 1-palmitoyl-2-[1-[$^{14}$C]]oleoyl; open circles) was used. Values shown are means of three separate experiments. C: TG binding as for A using [$^{14}$C]triolein (open squares), except that sample was incubated for 4 h instead of 1.5 h. Values shown are means of three experiments. Mean recoveries of CETP for the three sets of experiments were 76 ± 9%, 76 ± 6%, and 73 ± 10% for A–C, respectively. For FPLC, low ionic strength buffer (10 mM Tris) was used to ensure that a significant portion of CETP would elute free of the liposome peak.

coeluting with CETP in the absence of torcetrapib was in large part abolished by inhibitor. For all of these experiments, in spite of the low ionic strength conditions used for FPLC fractionation, the addition of torcetrapib increased the portion of total CETP coeluting with the liposomes versus that eluting free. Thus, the presence of apolipoprotein is not required for the inhibitor-induced complex formation between CETP and its substrates.

### Inhibition of CETP by torcetrapib is reversible

Preincubation of torcetrapib with CETP for increasing time periods produced a slight increase in the potency of inhibition in the subsequent CE transfer assay (**Fig. 9A**). Conversely, titration of the assay mix with increasing concentrations of LDL or VLDL resulted in the progressive decrease of inhibition (Fig. 9B). This apparent reversal of inhibition could also be accomplished using intralipid, a PL- and TG-rich emulsion lacking CE (Fig. 9C). In this case, the degree to which inhibition was reduced by intralipid was the same whether CETP and inhibitor were preincubated for long (3 h) or short (10 min) periods before assay. These experiments demonstrate that inhibition by torcetrapib is reversible. They also suggest that the observation that longer preincubation times lead to a slight increase in potency is likely due to a solubility effect. Torcetrapib has low aqueous solubility, and when combined with CETP at similar concentrations the extended preincubations favor inhibitor-CETP complex formation. The reversal of inhibition could also be demonstrated using whole plasma. Figure 9D shows the effects of increasing plasma TG concentration in plasma from 68 mg/ml (nonsupplemented) up to 816 mg/dl (12-fold increase) using intralipid. In this case, CETP activity was determined by the transfer of [$^{14}$C]triolein from labeled LDL to the HDL fraction. The double reciprocal plot of the data (Fig. 9E) indicates a noncompetitive effect of intralipid on the ability of torcetrapib to inhibit triolein transfer.

### Within the torcetrapib series, potency for inhibition of CE transfer correlates with the ability to form a stable CETP-HDL complex

Added insight into the relationship between torcetrapib's ability to inhibit CETP activity and to induce a CETP-HDL complex is provided by a comparison of torcetrapib with two less potent analogs (**Fig. 10**). Both CETP distribution, by native PAGE/Western analysis, and CETP activity in whole plasma were determined. For torcetrapib and the Pfizer 1 and 2 analogs, the measured $IC_{50}$ values were 54, 380, and 705 nM, respectively. The shift in CETP distribution patterns was notably different. For torcetra-

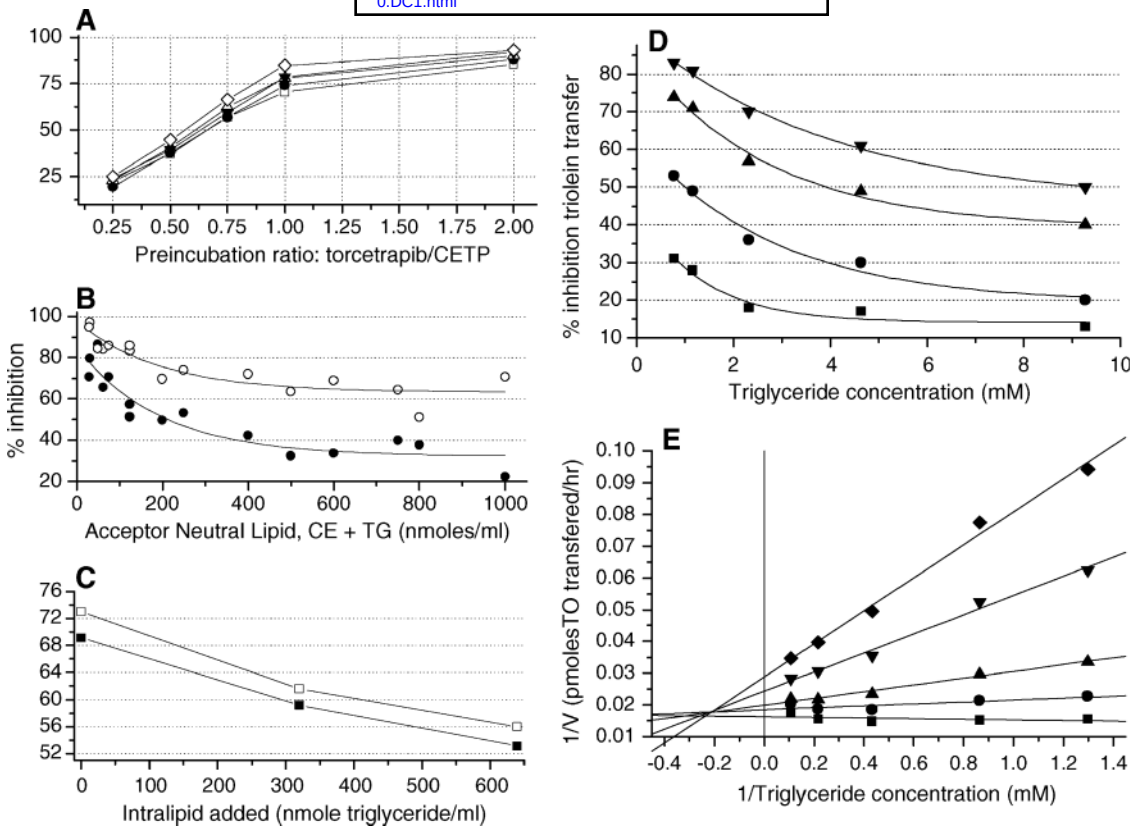


**Fig. 9.** Reversibility: effects of preincubation and lipid substrate titration on CETP inhibition by torcetrapib. A: Torcetrapib was added to purified CETP in PBS in the stoichiometries shown and preincubated at 37°C for periods of 5 min (open squares), 20 min (closed circles), 1.25 h (open triangles), 3 h (closed inverted triangles), and 6 h (open diamonds). The CE transfer assay was started by diluting the CETP/inhibitor solution 150-fold into the [$^3$H]LDL/HDL assay mix, then incubating for 30 min. Final assay concentrations were as follows: CETP (38 nM), [$^3$H]LDL (65 μM), and [$^3$H]HDL (130 μM). B: Purified CETP with or without torcetrapib was preincubated with donor [$^3$H]HDL for 10 min at 37°C, then added to aliquots of acceptor VLDL or LDL to start the CE transfer assay. The final concentrations of CETP and torcetrapib were 1.0 and 2.5 nM, respectively. Donor [$^3$H]HDL was held constant at 27 μM CE, whereas acceptor VLDL (closed squares) and LDL (open squares) were titrated to the levels shown. Total neutral lipid in VLDL consisted of 80% TG and 20% CE (mol%), and that in LDL consisted of 20% TG and 80% CE. C: Effect of preincubation period on reversibility. Purified CETP (300 μg/ml) with or without torcetrapib (1:1 molar ratio) was preincubated for 10 min (closed squares) versus 3 h (open squares), diluted 150-fold into an assay mix containing [$^3$H]LDL/HDL (1:2; 160 nmol CE/ml) and 0 nmol/ml, 320 nmol/ml (2×), or 640 nmol/ml (4×) intralipid TG, and incubated for 30 min at 37°C. D: Inhibition of CETP by 25 nM (closed squares), 50 nM (closed circles), 100 nM (closed triangles), and 150 nM (closed inverted triangles) torcetrapib was determined in plasma alone (0.77 mM TG) or plasma supplemented with intralipid to increase TG content by 0.5-, 3-, 6-, or 12-fold. Torcetrapib was added to prewarmed plasma and incubated for 3 min, PBS or intralipid was added, incubation was continued for 3 min, and then all samples were cooled on ice. [$^{14}$C]triolein-labeled LDL was added, and the samples were returned to 37°C to start the 2 h assay. For B–D, percentage inhibition is relative to the substrate-matched controls minus torcetrapib. E: Double reciprocal plot of the data from D. All CETP activity assays were performed in quadruplicate. TO, triolein.

pib, the titration with inhibitor produced an incremental shift in CETP from a lower to a higher molecular weight form, with little material in between. The lower band represents CETP that was only transiently associated with HDL during electrophoresis, and the upper band represents tightly bound CETP. The fact that a shift occurs at a concentration of torcetrapib (25 nM) lower than that for the plasma CETP (34 nM) indicates that once the inhibitor has bound CETP and an HDL complex has formed, the association is strong enough, for the most part, to be maintained during electrophoresis. For the less potent analogs, no shift is seen until multiple moles of inhibitor are present per CETP, then with increasing concentration all CETP displays a greater and greater delay in migration. This pattern could be explained by the lower affinity binding of the two analogs to CETP and therefore a higher concentration required to maintain the inhibitor saturation of CETP and thereby a CETP-HDL complex.

### A second inhibitor series with an apparently similar mode of action

A number of *N,N*-disubstituted trifluoro-3-amino-2-propanols, represented by SC-795, have previously been reported to bind specifically to CETP and block CE binding (31). Studies of CETP binding to immobilized synthetic HDL disks by SPR detected no difference in either the presence or the absence of SC-795. Therefore, it was concluded that the mechanism for the inhibition of CE and TG transfer was the competitive binding of these inhibitors to the neutral lipid binding site. Subsequently, in

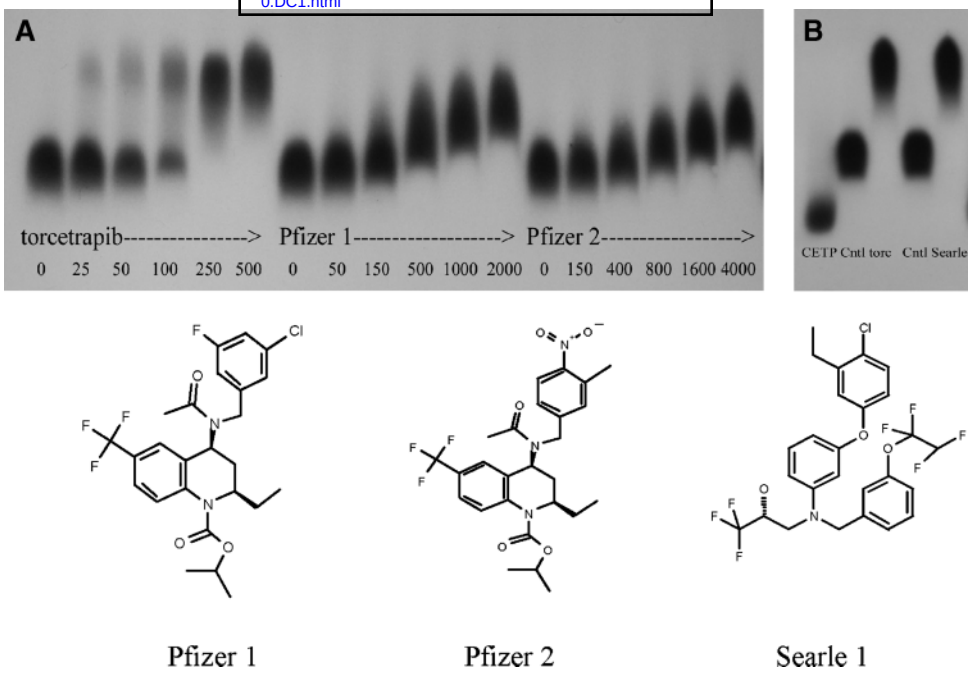


**Fig. 10.** A: The shift in plasma CETP to an HDL-bound state with the addition of torcetrapib was compared with that seen with two less potent analogs, Pfizer 1 and Pfizer 2. A dual-label activity assay was conducted in parallel for the three inhibitors. The inhibitors were added to plasma at the concentrations indicated (nM), followed by native PAGE and CETP Western blotting. The autoradiograms show the distribution in CETP. B: A member of another series of potent CETP inhibitors, Searle 1, also induces a shift in CETP to an HDL-bound state. Torcetrapib and Searle 1 were both tested at 500 nM.

2003, Reinhard et al. (32) described additional members of this series. A 4-chloro-3-ethyl analog was particularly potent, exhibiting an $IC_{50}$ in human serum of ~50 nM versus 640 nM for SC-795. We have synthesized this analog, designated Searle 1, and in experiments performed in parallel to those shown in Fig. 2A found that it not only inhibited neutral lipid transfer but CETP-mediated PL transfer as well (data not shown). This raised the question of how a low molecular weight inhibitor, competitive with CE for binding to a common site on CETP, was able to block PL transfer as well. When tested side-by-side in a gel-shift experiment, Searle 1 produced identical shifts in plasma CETP distribution, as did torcetrapib (Fig. 10B), indicating that it too increased binding to HDL. Searle 1 also duplicated torcetrapib's enhancement of CETP binding to native HDL determined by SPR (data not shown). These results suggest that although the torcetrapib and SC-795 series may bind to different sites on CETP, they share a common mechanism of inhibition: formation of a nonproductive complex between CETP and HDL.

## DISCUSSION

CETP catalyzes the exchange and transfer of both neutral lipid and PL between lipoproteins and has binding sites for both lipid classes (19, 20). For lipid transfer to occur, CETP must be able not only to bind lipid but also to form transient associations with donor and acceptor particles. Therefore, the means of inhibition include blocking lipid binding or preventing its release once bound and causing the excessive or insufficient binding of CETP to its lipoprotein substrates. Although a variety of small molecule inhibitors have been reported, few of these have been characterized with regard to their mechanism of action. Numerous hydrophobic cysteine-modifying reagents have been shown to inhibit CETP in a time-dependent, irreversible manner (10, 22, 33, 34). Evidence for two of these, SC-71952 (34), a bis-disulfide analog of nicotinic acid methyl ester, and JTT-705 (10), a thioester, indicate that inhibition involves the formation of a disulfide bond with cysteine 13 of CETP. Consequently, cysteine 13 has been proposed to be near the neutral lipid binding site for CETP. U-95,594 (22), an amino steroid, demonstrated kinetic aspects of a competitive inhibitor, consistent with its structural similarities to CE. Similarly, a nonsteroid inhibitor, SC-795 (31), was shown to block CE binding to CETP. Both reversible agents were proposed as competitive inhibitors of CETP, although they also inhibited TG transfer. Interestingly, U-617, an organomercurial derivative of cholesterol, was shown to inhibit CE transfer but not TG transfer (22). Additional reports that a number of synthetic compounds, as well as antibodies and their fragments, inhibit CE and TG transfer selectively are consistent with the idea that different lipids may rely on different sites on CETP for binding and transfer (35).

In this report, torcetrapib was shown to be a potent and selective inhibitor that appears to block all of the major lipid transfer functions of plasma CETP by causing a nonproductive complex between the transfer protein and

HDL. Although CETP has been shown to associate with a variety of lipoproteins and synthetic substrates in isolation (36, 37), analysis of its distribution in plasma has suggested a preferential association with HDL (38–40). Groener, Rozen, and Erkelens (38) found that whereas sequential, multiple centrifugations caused CETP to dissociate from lipoprotein, leaving it in the d > 1.21 g/ml fraction, a single step gradient centrifugation isolated more than half of CETP with $HDL_3$ at d = 1.125–1.21 g/ml. Using a similar gradient centrifugation protocol, Marcel et al. (39) also isolated approximately half of plasma CETP within the density for $HDL_3$ and most of the remainder within the bulk protein fraction at d > 1.21 g/ml. By native PAGE/CETP Western analysis of plasma, the latter study found that CETP exhibited a mass of 129–154 kDa. Another study using FPLC fractionation found that plasma CETP eluted with a mass of 140–180 kDa (41). These results are consistent with the data presented in this report.

In the absence of torcetrapib, the mass for plasma CETP was 150–180 kDa, which is ~25–55 kDa greater than that of purified CETP. This difference appears too small to be caused by a stable complex between CETP and HDL. As suggested by the incremental shift in Mr for purified CETP in the presence of increasing concentrations of HDL, these small shifts in apparent size likely result from the transient association of CETP with HDL during the initial steps of either FPLC fractionation or native PAGE, followed by the release of CETP and its subsequent migration as free protein. On the other hand, the shift in size resulting from the addition of torcetrapib is much greater, as would be expected for the tight association of an HDL particle and CETP. Biophysical measurements have indicated that CETP has an elongated shape as well as secondary structure percentages similar to another member of this family of lipid binding proteins, bactericidal/permeability-increasing protein (BPI) (20). The crystal structure of BPI has shown it to be a boomerang-shaped protein formed by two domains with similar structures (42). Each domain possesses a hydrophobic pocket on the concave side of the protein occupied by a PL molecule. The dimensions of the protein are 13.5 × 3.5 × 3.5 nm. By analogy, if one imagines CETP bound to a spherical HDL particle such that its concave surface conforms to the curvature of the lipoprotein, then the effective diameter of the complex with regard to mobility by native PAGE would be approximately that of the HDL plus 3.5 nm. An increment of 3.5 nm is close to the difference between the $HDL_{3b}$ (7.9 nm) and $HDL_{2b}$ (10.9 nm) particles included in the experiment described in Fig. 4E and the diameter of the apparent CETP-HDL complexes, 11.4 and 15.25 nm, respectively, formed with the addition of torcetrapib. The magnitude of the inhibitor-induced shift in apparent size for CETP in plasma indicates that recruitment of HDL into the inhibitor-CETP complex may favor the mid-sized or larger HDL particles rather than the smaller HDL. This was also suggested by the more complete shift of CETP to an HDL-bound state at lower HDL concentrations, relative to physiological concentrations, in the presence of large HDL versus smaller HDL. Furthermore, torcetrapib added to mixtures of large and small HDL produced shifts in CETP Mr much closer to those seen with large HDL alone than with small HDL (see supplementary Fig. II). As noted above, previous suggestions that plasma CETP associated with small HDL failed to take into account the large apparent size of CETP itself. Likewise, the recovery of CETP in the d = 1.125–1.21 g/ml density range ( 38, 39) does not necessarily mean an association with $HDL_3$ but could just as likely result from the combined densities of CETP and $HDL_2$.

In whole plasma, the mechanism for inhibition appears restricted, for the most part, to complex formation with HDL lipoprotein. However, in the absence of HDL, torcetrapib also enhances the association of CETP with isolated LDL (see supplementary Fig. III) and to apolipoprotein-free liposomes (Fig. 8). The reason the effect is restricted mainly to HDL particles in plasma is likely the result of both a higher affinity binding of CETP inhibitor to HDL compared with LDL or VLDL lipoprotein and the ~20-fold greater concentration of plasma HDL versus non-HDL lipoprotein.

The effects of torcetrapib on CETP binding to HDL are reminiscent of the previously described anti-CETP monoclonal antibody TP2 (43, 44). The TP2 monoclonal antibody was found to inhibit CE, TG, and PL transfer mediated by CETP but enhanced the binding of CETP to plasma lipoproteins and synthetic liposomes. Unlike torcetrapib, however, TP2 binding to CETP depends, at least in part, on amino acids at the C terminus of the transfer protein and does not bind to the 470–475 residue deletion mutant. In contrast, torcetrapib binds with 1:1 stoichiometry to both WT and the Δ470–475 CETP mutant. Also, whereas the TP2 antibody blocks both CE and TG binding to CETP, the torcetrapib series has an apparent effect on TG binding but no effect on CE binding. The C-terminal region of CETP forms an amphipathic helix, and point mutagenesis within this area revealed that TP2 binding involved polar residues on one face of this helix, whereas the binding of CE and TG required the presence of hydrophobic amino acids on the opposite face (45). Based on the BPI structure, a model was constructed for CETP in which two lipid binding pockets were also present (24). The C terminus of CETP, relative to that of BPI, has a 12 residue extension, and in the model for CETP this helical region was in close proximity to the entrance of the N-terminal domain lipid binding pocket. Therefore, it was proposed that this amphipathic helix might form a lid over the N-terminal pocket and so convert what was a PL binding site in BPI into the neutral lipid binding site of CETP. The C-terminal pocket was presumed to remain specialized for PL binding. However, whether CETP possesses two or more lipid binding sites, and the extent to which these exist separately or overlap, have not yet been defined.

Although decreased lipid uptake by CETP had been proposed as the major mechanism for the inhibition of lipid transfer by TP2, the antibody-enhanced association with lipoprotein was acknowledged as a possible additional mechanism (44). The antibody was reported to block TG

and CE binding to CETP, but its effect on PL binding was not described. Preventing the dissociation of CETP from lipoprotein, as appears to be the case for torcetrapib, would explain this monoclonal antibody's ability to inhibit PL as well as neutral lipid transfer (43).

By our measurements, normal human plasma CETP concentrations range between 20 and 65 nM. Therefore, the finding with Biacore that the dissociation constant for CETP binding to HDL was decreased by torcetrapib from ~50 nM to 10 nM could explain a difference between the relatively loose association of CETP to HDL that is permissive for lipid transfer and a tighter binding that is inhibitory. In humans, we have found that CETP mass levels increase between 12 and 24 h after a single low dose of torcetrapib (13). Also, this increase in mass is accompanied by a shift in CETP to an HDL-bound state, as indicated by ex vivo plasma analysis using the gel-shift assay. The fact that this occurs before significant changes in plasma lipid levels take place indicates a delayed clearance for CETP, as a result of its HDL-bound state rather than increased expression. Thus, the increase in the affinity of CETP for HDL caused by torcetrapib is physiologically relevant. Whether effects on the binding or release of lipid may also contribute to the inhibition achieved by torcetrapib has not been ruled out, however, and remains a subject of active study. We found no apparent effect of prebound torcetrapib on the subsequent binding of CE or PC to CETP but a reduction in binding of TG. Previous studies, using similar methods to assess lipid binding (19, 23), also reported a fraction of a mole of TG bound per CETP. A nonspecific loss of TG during chromatography was suggested as a possible explanation. This may also occur in these experiments, and if so, it is likewise possible that the inhibitor, which is not totally recovered from the columns, may enhance such loss and so reduce TG binding to CETP nonspecifically rather than directly. In any case, the fact that increasing TG concentration reduces inhibition in a noncompetitive manner (Fig. 9D, E) indicates that TG and inhibitor do not compete for a shared binding site. That TG-rich VLDL is better able than CE-rich LDL to reverse inhibition (Fig. 9B) is consistent with the plasma distribution (Fig. 3B), in which the inhibitor is disproportionately associated with VLDL compared with LDL or HDL. This, in turn, may reflect both a higher solubility of the inhibitor in TG-rich versus CE-rich cores and the ability of the former particles to better sequester drug as a result of their larger diameters.

We have found that another series of potent CETP inhibitors, represented by SC-795, apparently blocks CETP-mediated lipid transfer through a means similar to torcetrapib. Why a previous study had found no effect of SC-795 on CETP binding to HDL is not known (31). However, in the latter case, synthetic HDL was used for SPR experiments, whereas our experiments used native HDL. As is the case for the TP2 antibody, the fact that the Searle inhibitor is similar to torcetrapib at inducing complex formation between CETP and HDL would likewise explain its ability to block the multiple lipid transfer functions of the protein. It also suggests that as more CETP inhibitors are characterized, this mechanism for inhibition may become a common theme.

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