

# Inhibition of cholesteryl ester transfer protein by substituted dithiobisnicotinic acid dimethyl ester: involvement of a critical cysteine

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**Abstract** SC-71952, a substituted analog of dithiobisnicotinic acid dimethyl ester, was identified as a potent inhibitor of cholesteryl ester transfer protein (CETP). When tested in an in vitro assay, the concentration of SC-71952 required for half-maximal inhibition was 1  $\mu$ M. The potency of SC-71952 was enhanced 200-fold by preincubation of the inhibitor with CETP, and was decreased 50-fold by treatment with dithiothreitol. Analogs of SC-71952 that did not contain a disulfide linkage were less potent, did not display time dependency, and were not affected by dithiothreitol treatment. Kinetic and biochemical characterization of the inhibitory process of CETP by SC-71952 suggested that the inhibitor initially binds rapidly and reversibly to a hydrophobic site on CETP. With time, the bound inhibitor irreversibly inactivates CETP, presumably by reacting with one of the free cysteines of CETP. Liquid chromatography/mass spectroscopy (LC/MS) analyses of tryptic digests of untreated or SC-71952-inactivated CETP was used to identify which cysteine(s) were potentially involved in the time-dependent, irreversible component of inactivation by the inhibitor. One disulfide bond, Cys143–Cys184, was unaffected by treatment with the inhibitor. Inactivation of CETP by SC-71952 correlated with a progressive decrease in the abundance of free Cys-13 and Cys-333. Conversion of Cys-13 to alanine had no effect on the rapid reversible component of inactivation by SC-71952. However, it abolished the time-dependent enhancement in potency seen with the inhibitor when using wild-type CETP. These data indicate that Cys-13 is critical for the irreversible inactivation of CETP by SC-71952 and provides support for the structural model that places Cys-13 near the neutral lipid-binding site of CETP.—H. R. Hope, D. Heuvelman, K. Duffin, C. Smith, J. Zablocki, R. Schilling, S. Hegde, L. Lee, B. Witherbee, M. Baganoff, C. Bruce, A. R. Tall, E. Krul, K. Glenn, and D. T. Connolly. **Inhibition of cholesteryl ester transfer protein by substituted dithiobisnicotinic acid dimethyl ester: involvement of a critical cysteine.** *J. Lipid Res.* 2000. 41: 1604–1614.

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Cholesteryl ester transfer protein (CETP) transfers lipids between lipoprotein particles in the blood (1–3). It is thus an important regulator of lipoprotein composition. Previous studies have suggested that CETP transfers cholesteryl esters and triglycerides between lipoproteins by an exchange mechanism, possibly by binding of the neutral lipids to a high-affinity site on CETP (4–7). The exact details involved in the lipid transfer process are not known.

The overall amino acid composition of CETP is hydrophobic. The protein contains 476 amino acids, 7 of which are cysteine (8). Several studies have indicated that CETP can be inactivated by reagents that modify cysteine residues (4, 7, 9, 10). In a survey of more than 30 different compounds that could potentially modify cysteines, it was observed that there is substantial selectivity in the structural requirements for inactivation (10). Hydrophobic reagents were the most effective inhibitors whereas similar reagents that were polar or charged were much less inhibitory, implying that CETP contains a critical cysteine that resides in a hydrophobic environment. Because modification of this cysteine prevents lipid transfer, it was hypothesized that the critical cysteine could be in, or near, the lipid-binding site.

The design of CETP inhibitors presents a unique set of problems due to the amphipathic nature of CETP and the lipoprotein substrates. In addition, the neutral lipids trans-

Abbreviations: ACN, acetonitrile; BHK, baby hamster kidney; CAM-Cys, carboxyamidomethyl-cysteine; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); DTT, dithiothreitol; HDL, high density lipoprotein particles; LC/MS, liquid chromatography/mass spectroscopy; LDL, low density lipoprotein particles; PBS, phosphate buffered saline; PTH, phenylthiohydantoin; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TIC, total ion current; TFA, trifluoroacetic acid; TG, triglycerides; TNB<sup>2-</sup>, 2-nitro-5-thiobenzoate; VLDL, very low density lipoprotein particles.

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ferred by CETP, cholesteryl esters and triglycerides, are extremely nonpolar, and have high local concentrations within lipoprotein particles. It is likely that effective inhibitors of the CETP-mediated lipid transfer process must also share some combination of hydrophobicity or amphipathicity, and might also need to partition into specific regions of lipoprotein particles, without introducing substantial modifications in the lipoprotein (11). All these features represent formidable obstacles for the design of inhibitors that will be both potent and specific.

The present study describes a highly potent CETP inhibitor, SC-71952, which was discovered in a random screen of compounds from a chemical library. SC-71952 is a *bis* disulfide analog of nicotinic acid methyl ester. Other structurally related inhibitors were also identified, but these were less potent than SC-71952. The enhanced potency of SC-71952 is shown to be a function of its disulfide linkage and suggests that the mechanism by which it inactivates CETP involves a disulfide exchange reaction with susceptible cysteines on the protein. Structural analysis of CETP revealed heterogeneity with respect to disulfide pairings and oxidation state, and that specific activity was inversely related to the extent of cysteine oxidation. Three cysteines (Cys1, Cys13, and Cys333) were found both as free sulfhydryls and in disulfide linkages with each other. SC-71952 promoted the loss of Cys13 and Cys333. Taken together, these studies point to Cys1, Cys13, and Cys333 as residues whose oxidative state may have effects on CETP activity and provide support for the structural model of CETP (12) that places Cys13 near the neutral lipid-binding site.

## MATERIALS AND METHODS

### Purification of recombinant CETP

Recombinant human CETP was purified from stably transfected baby hamster kidney (BHK) cells by a modification of the immunoaffinity procedure previously described (10). Briefly, 1 liter of serum-free conditioned medium was neutralized by adding 100 ml of 10× concentrated Dulbecco's phosphate-buffered saline (PBS). Insoluble components were removed from the conditioned medium by centrifugation at 7,000 *g* for 30 min at 4°C followed by sequential filtration through 0.8-, 0.45-, and 0.2- $\mu$ m pore size filters (Millipore, Danvers, MA). Care was taken to maintain the pH at 7.5 to maintain disulfide bond integrity. The conditioned medium was then applied to an 80-ml immunoaffinity column consisting of M468 monoclonal IgG coupled to Sepharose 4B. The flow rate for loading, washing, and elution of the column was 1.5 ml/min. The column was washed with 1.5 liters of PBS containing 1 M NaCl followed by elution with 25 mM glycine, pH 3. Because of problems with precipitation of CETP, the fractions were not neutralized with concentrated Tris as was previously reported. The fractions containing CETP activity were pooled, dialyzed against deionized water (MilliQ; Millipore), and then concentrated in a Savant (Holbrook, NY) SpeedVac to a protein concentration of 0.5–1.0 mg/ml. Protein concentrations were determined by absorbance at 280 nm after correcting for light scattering as described by Connolly et al. (5). The CETP preparations were shown to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All samples were stored at –20°C. Numerous preparations were generated and labeled accordingly.

### CETP assay

CETP concentration was determined spectrophotometrically, using an extinction coefficient at 280 nm of 0.84 [(mg/ml)  $\text{cm}^{-1}$ ] (10). The [ $^3\text{H}$ ]CE transfer assay using high density lipoprotein (HDL) donor particles and low density lipoprotein (LDL) receptor particles, and characterization of CETP inhibition, have been described in detail (5, 10). Briefly, [ $^3\text{H}$ ]CE-labeled HDL (6  $\mu\text{g}/\text{ml}$  total cholesterol), LDL (50  $\mu\text{g}/\text{ml}$ ), and CETP (0.1–1  $\mu\text{g}/\text{ml}$ ) were incubated together for 2 h at 37°C in a volume of 0.2 ml in 96-well filter plates (Multiscreen DPB, 0.65  $\mu\text{m}$ ; Millipore). LDL was precipitated and separated from HDL with a dextran sulfate precipitation reagent, and the amount of radioactivity in the precipitate was quantitated by liquid scintillation counting. The assay was linear with respect to time and CETP concentration. Inhibitor solutions were prepared in dimethyl sulfoxide. Dilutions of inhibitor were made with 16% (v/v) dimethyl sulfoxide. Diluted inhibitor solutions were mixed with an equal volume of CETP solution in buffer [0.01 M Tris (pH 7.4), 0.14 M NaCl, 1 mM EDTA]. This solution was then diluted an additional 8-fold into the assay wells (1% final dimethyl sulfoxide concentration), or for preincubation experiments, incubated at 37°C. Dimethyl sulfoxide did not affect CETP at these concentrations. Nevertheless, control samples containing equal concentrations of dimethyl sulfoxide were included in all assays. All assays were carried out in triplicate and repeated to confirm the results.

### DTNB studies

Aliquots (1 ml) of CETP (0.5–1.0 mg/ml in distilled  $\text{H}_2\text{O}$ ) were adjusted to 0.1 M sodium phosphate, pH 8.0, by the addition of 10-fold concentrated buffer. Blank samples were prepared with buffer alone. All samples were oxygen depleted by bubbling nitrogen through the solution. 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB, 5 mM in 0.1 M sodium phosphate, pH 8.0) was prepared immediately before use. The reactions were started by the addition of 100  $\mu\text{l}$  of DTNB to 1.1 ml of the buffered CETP samples, which were then transferred immediately to a cuvette, and sealed with Parafilm. The absorbance at 412 nm was monitored for 30 min, after which SDS was added to a final concentration of 0.8% (w/v). The absorbance was then monitored for an additional 15 min. The free cysteine content was calculated using the molar extinction coefficients ( $\epsilon_{412}$ ) for  $\text{TNB}^{2-}$  of 14,150  $\text{M}^{-1} \text{cm}^{-1}$  in 0.1 M sodium phosphate, pH 8.0 (13), and 12,300  $\text{M}^{-1} \text{cm}^{-1}$  in 0.1 M sodium phosphate, pH 8.0, plus 0.8% (w/v) SDS (determined as described below). To calculate the free sulfhydryl content of CETP, it is assumed that 1 mol of  $\text{TNB}^{2-}$  is formed per mole of cysteine.

Riddles, Blakeley, and Zerner (13) have reported that the molar extinction coefficient ( $\epsilon_{412}$ ) of  $\text{TNB}^{2-}$  varies under different solvent conditions. In particular, SDS was found to decrease the value of  $\epsilon_{412}$ . To determine the  $\epsilon_{412}$  of  $\text{TNB}^{2-}$  in 0.1 M sodium phosphate, pH 8.0, containing SDS (0–2%, w/v), standard curves were prepared by reacting excess DTNB with cysteine-HCl (Pierce, Rockford, IL). Comparison of the slopes of these curves with the slope in the absence of SDS allowed for the calculation of the molar extinction coefficient of  $\text{TNB}^{2-}$  at each concentration of SDS.

### Alkylation of recombinant human CETP

CETP [10 mM in PBS plus 0.04% (w/v) SDS] was either alkylated directly, or reduced with 10 mM dithiothreitol (DTT) at 37°C for 2 h prior to alkylation. Alkylations were carried out at 4°C for 1 h with 20 mM iodoacetamide for the nonreduced samples, or 40 mM iodoacetamide for the reduced samples. The samples were dialyzed against PBS overnight at 4°C. Samples were then trypsinized (see below) for liquid chromatography/mass spectroscopy (LC/MS) analysis.

## Tryptic digests

Trypsin [sequencing-grade tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-modified trypsin; Promega, Madison, WI] was dissolved at a concentration of 1 mg/ml in 40% (v/v) acetonitrile–0.1% (w/v) TFA. The alkylated, or reduced and alkylated, CETP samples were trypsinized overnight at 37°C, using a ratio of trypsin to CETP of 1:30 (w/w). After trypsinization, the samples were frozen at –20°C. The samples were thawed and acidified with trifluoroacetic acid [TFA; final concentration, 0.01% (w/v)] immediately before LC/MS analysis.

## NH<sub>2</sub>-terminal sequencing

Automated Edman degradation chemistry was used to determine the NH<sub>2</sub>-terminal protein sequence. A Perkin-Elmer/Applied Biosystems Division (Foster City, CA) Precise model 494A was employed for the degradations using gas-phase sequencing cycles. The respective phenylthiohydantoin (PTH-amino acid) derivatives were identified by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis with a Brownlee 2.1-mm i.d. PTH-C<sub>18</sub> column. Quantitation of PTH-amino acid derivatives in each degradation cycle was determined by measurement of peak area and comparison with a 10-pmol PTH-aa standard mixture.

## LC/MS of tryptic peptides

Twenty microliters of the digested protein solution was injected onto a Waters (Milford, MA) prototype capillary liquid chromatograph that contained a 0.32 mm i.d. × 15 cm capillary column packed with Vydac (Hesperia, CA) C<sub>18</sub> (300-Å) stationary phase. Tryptic peptides were eluted from the column with a gradient of 0–70% B mobile phase [A, H<sub>2</sub>O–0.1% TFA; B, acetonitrile (ACN)–H<sub>2</sub>O–TFA, 7:3:0.001] in 90 min at a flow of 5 µl/min. Column eluate passed via a 50-µm i.d. fused silica transfer capillary through a UV detector (wavelength, 214 nm) to the electrospray interface of a Sciex API III triple quadrupole mass spectrometer (Sciex, Thornhill, ON, Canada). The quadrupole mass analyzer was scanned from 300 to 2,400 units in 0.2-unit steps in approximately 4 sec. The retention times of eluted peptides were determined by an increase in the total ion current (TIC) of the mass chromatogram. Molecular weights of eluted peptides were determined after averaging the mass spectral scans over which the peptide eluted. Average molecular weights of high-mass peptides were determined by deconvoluting the multiply charged ion signals in the mass spectra, using software based on published equations (14).

## Synthesis of inhibitor compounds

Methyl 2-(difluoromethyl)-4-isobutyl-5-mercapto-6-(trifluoromethyl)-3-pyridinecarboxylate, SC-66960, was prepared by reaction of methyl 5-bromo-2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate with lithium sulfide. Dimethyl 5,5'-dithiobis[2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate], SC-71952, was prepared by reaction of SC-66960 with methanesulfonyl chloride, fluoroethanol, and triethylamine. Methyl 5-[(4-*t*-butylphenyl)thiomethyl]-2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate, SC-67201, was prepared by reaction of methyl 5-(chloromethyl)-2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate (14, 15) with 4-*t*-butylbenzenethiol.

Methyl 2-(difluoromethyl)-4-isobutyl-5-mercapto-6-(trifluoromethyl)-3-pyridinecarboxylate, SC-66960. To a stirred solution of 10.11 g (0.026 mol) of methyl 5-bromo-2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate in 75 ml of dry dimethylformamide was added 1.42 g (0.031 mol) of lithium sulfide in one portion. The reaction mixture was stirred overnight at room temperature, diluted with 150 ml of 10% HCl solution, and ex-

tracted with ether (three times, 100 ml each time). The combined ether extracts were washed with water, dried (MgSO<sub>4</sub>), and evaporated. The residue was purified by kugelrohr distillation at 1.5 torr (110°C) to give 7.35 g (83%) of SC-66960 as a yellow-green oil:  $n_D^{25} = 1.5833$ . Anal. Calcd. for C<sub>13</sub>H<sub>14</sub>F<sub>3</sub>NO<sub>2</sub>S: C, 45.48; H, 4.11; N, 4.08. Found: C, 45.58; H, 4.14; N, 4.08.

Dimethyl 5,5'-dithiobis[2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate], SC-71952. To a solution of 1.14 g (0.018 mol) of 2-fluoroethanol and 0.95 g (0.0094 mol) of triethylamine in 20 ml of dry tetrahydrofuran at –78°C was added 1.07 g (0.0094 mol) of methanesulfonyl chloride in 10 ml of dry tetrahydrofuran. After stirring the mixture for 30 min, 2.5 g (0.0073 mol) of SC-66960 and 0.95 g (0.0094 mol) of triethylamine were added. The reaction mixture was slowly warmed to room temperature and stirred for an additional 2 h. The reaction mixture was evaporated, and the residue was diluted with 100 ml of water and extracted with 125 ml of ether. The ether extract was washed with water, dried (MgSO<sub>4</sub>), and evaporated. The residue was purified by HPLC (8% ethyl acetate–hexane) to give 1.83 g (73%) of SC-71952 as a yellow oil. Anal. Calcd. for C<sub>26</sub>H<sub>26</sub>F<sub>10</sub>N<sub>2</sub>O<sub>4</sub>S<sub>2</sub>: C, 45.61; H, 3.83; N, 4.02. Found: C, 45.80; H, 3.87; N, 4.02.

Methyl 5-[(4-*t*-butylphenyl)thiomethyl]-2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate, SC-67201. The reaction of methyl 5-(chloromethyl)-2-(difluoromethyl)-4-isobutyl-6-(trifluoromethyl)-3-pyridinecarboxylate with 4-*t*-butylbenzenethiol was similar to the procedure previously described (15, 16), and gave SC-67201 as an oil. Anal. Calcd. for C<sub>24</sub>H<sub>28</sub>F<sub>5</sub>NO<sub>2</sub>S: C, 58.88; H, 5.77; N, 2.86. Found, C, 58.78; H, 6.04; N, 2.75.

## Expression of the Cys13Ala mutant CETP

The Cys13Ala mutant of CETP cDNA was cloned into the *Bam*HI site of the pCMV7 expression plasmid. This plasmid is a derivative of pCMV4 (17), having a generic intron (18) between its promoter and the multiple cloning site. Cos7 cells were transiently transfected with this construct, as well as with its wild-type equivalent and the empty vector, using the LipofectAMINE reagent (Life Technologies, Gaithersburg, MD) and then grown in serum-free Opti-MEM medium (Life Technologies) for 3 days. The cell media were concentrated 100× with Centricon-30 concentrators (Amicon, Beverly, MA) before assay.

## RESULTS

### Inhibition of CETP by SC-71952 and SC-67201 and the effect of DTT

SC-71952, a substituted analog of nicotinic acid methyl ester, was identified as a potent CETP inhibitor in a random screen of a chemical library. **Figure 1** shows the structures of the disulfide SC-71952, the reduced thiol SC-66960, and a related thioether analog, SC-67201. It should be noted that these compounds are extremely hydrophobic. A second notable feature of SC-71952 is the disulfide linkage, a structure that has the potential to undergo disulfide exchange with free cysteines on CETP. Several experiments were performed to examine whether cysteine modification was involved in inhibition by SC-71952. First, the reducing agent DTT was included in the CETP-mediated [<sup>3</sup>H]CE transfer assay along with SC-71952 (**Fig. 2**). DTT quantitatively converted the disulfide SC-71952 into the thiol SC-66960, as verified by HPLC analysis (not shown). DTT would also be expected to block or reverse mixed disulfide formation

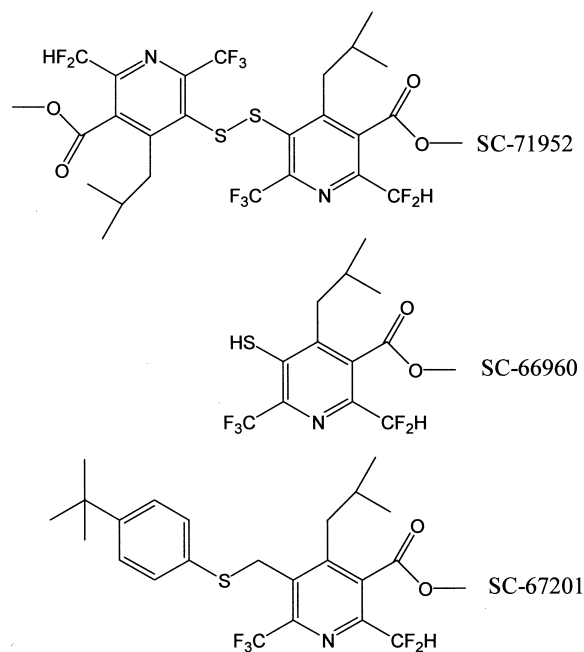


Fig. 1. Structures of substituted nicotinic acid CETP inhibitors.

between either of these compounds and cysteines on CETP. Figure 2 shows that in the absence of DTT, the concentration required for 50% inhibition ( $\text{IC}_{50}$ ) for SC-71952 was 1  $\mu\text{M}$ . In the presence of DTT, the  $\text{IC}_{50}$  was shifted to 50  $\mu\text{M}$ .

One explanation for the shift in  $\text{IC}_{50}$  from 1  $\mu\text{M}$  in the absence of DTT to 50  $\mu\text{M}$  in the presence of DTT is that SC-71952 is converted by reducing agents into the intrinsically less potent SC-66960. Alternatively, the effect could be due to modification of CETP by DTT. To distinguish between these two possibilities, the related analog SC-67201 was tested in the presence or absence of DTT. SC-67201 is structurally related to SC-71952 in that it contains the same substituted nicotinic acid methyl ester connected to a second hydrophobic ring by a two-atom bridge. However, instead of a disulfide linkage at the 5-position on the pyridyl ring, SC-67201 contains a thioether linkage connecting a methylene group (Fig. 1). The thioether linkage of SC-67201 cannot be reduced by reaction with DTT, and therefore cannot undergo disulfide exchange with cysteine. Figure 2 shows that although SC-67201 was still inhibitory ( $\text{IC}_{50} = 10 \mu\text{M}$ ), DTT had no effect on the potency of this compound. Taken together, these results suggest that the enhanced potency of SC-71952 relative to the other analogs is, at least in part, due to the presence of the disulfide.

#### Time dependence of inhibition by the disulfide SC-71952, but not by the thioether SC-67201

The time dependence of inhibition by SC-71952 or SC-67201 was examined by preincubating various concentrations of the respective inhibitors with CETP prior to assay. The inhibitors were delivered to solutions of CETP from concentrated stocks of dimethyl sulfoxide (DMSO). As noted above, both inhibitors are extremely nonpolar, and it was noted that significant cloudiness appeared on dilu-

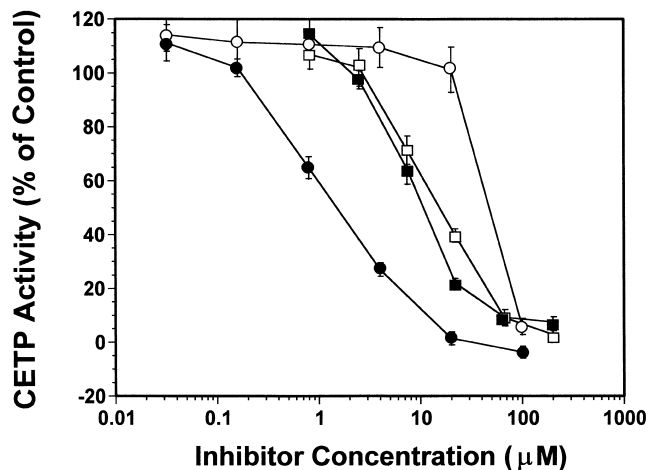
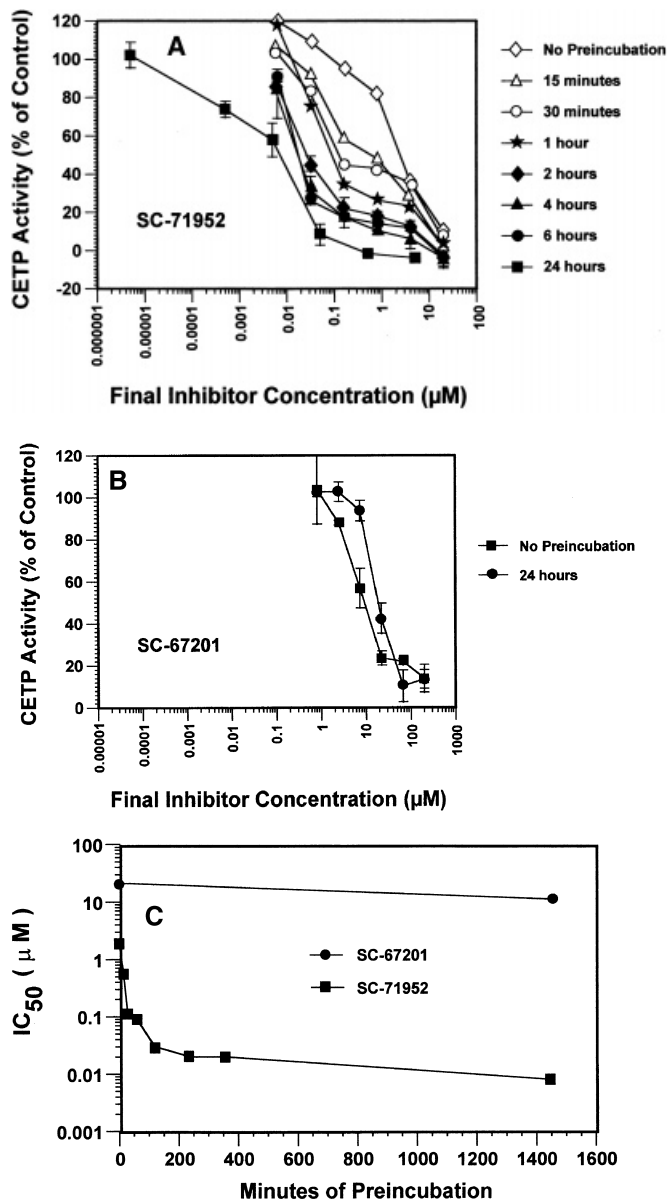


Fig. 2. Effect of DTT on inhibition of CETP by SC-71952 or SC-67201. Inhibitor compounds in 16% dimethyl sulfoxide were treated with 16 mM DTT for 15 min at room temperature. The inhibitors were then mixed (1:1) with CETP immediately prior to assay, and then diluted an additional 8-fold into the assay. The amount of CETP-mediated transfer of [ $^3\text{H}$ ]CE from HDL to LDL was determined. SC-71952, untreated (solid circles); SC-71952 plus DTT (open circles); SC-67201, untreated (solid squares); SC-67201 plus DTT (open squares). Data points represent triplicate assays.

tion into the aqueous buffer of the solution containing CETP. This cloudiness could be due to precipitation of the inhibitor, possibly lowering its concentration. Nevertheless, SC-71952 was evidently able to interact with CETP under these conditions. Figure 3A shows that the  $\text{IC}_{50}$  for inhibition by SC-71952 was shifted from 2  $\mu\text{M}$  in this experiment to 0.01  $\mu\text{M}$  after 24 h of incubation, representing a 200-fold increase in potency. In contrast, the  $\text{IC}_{50}$  for SC-67201 changed only slightly on preincubation, from 20 to 10  $\mu\text{M}$  (Fig. 3B). The change in  $\text{IC}_{50}$  values on preincubation with CETP is plotted (log scale) as a function of time for both inhibitors in Fig. 3C. Preincubation times of 1 h or less produced substantial, but incomplete, shifts in the  $\text{IC}_{50}$  for SC-71952. The shift in  $\text{IC}_{50}$  was essentially complete by 4 h of preincubation. It should be noted that, in addition to the preincubation period, the inhibitor was present with CETP during the 2-h assay period. Thus the time needed for enhanced inhibition cannot be precisely determined from these data. Nevertheless, it can be concluded that SC-71952, but not SC-67201, interacted with CETP during the first 1 to 4 h of incubation in such a way as to drastically enhance the potency of inhibition.

Interestingly, while preincubation of CETP with SC-71952 enhanced the potency of the inhibitor with respect to CE transfer, no significant effects were seen with respect to triglyceride transfer. Using HDL labeled with both [ $^3\text{H}$ ]CE and [ $^{14}\text{C}$ ]triglyceride (TG), preincubation of CETP with SC-71952 resulted in the typical 200-fold increase in potency for CE transfer while only a 2-fold shift (10 to 5  $\mu\text{M}$ ) was seen for TG transfer (data not shown). Therefore, while SC-71952 did inhibit TG transfer, no time-dependent increase in potency was seen. This difference in the inhibition profiles of SC-71952 for the transfer of these lipids is consistent with the work of others, in which both small molecules (9)

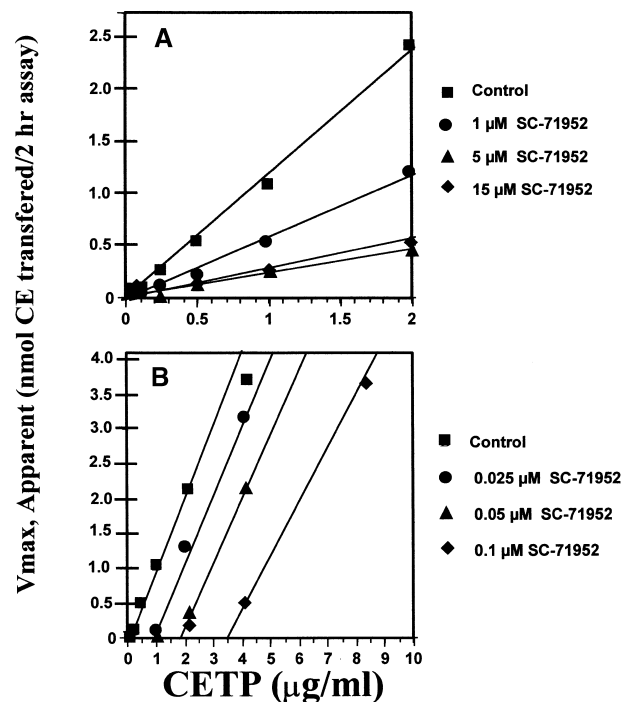


**Fig. 3.** Effect of preincubation with CETP on the inhibitory potency of SC-71952 or SC-67201. Various concentrations of SC-71952 or SC-67201 were mixed with CETP and incubated for different periods of time prior to addition to the [<sup>3</sup>H]CE transfer assay. (A) IC<sub>50</sub> curves for SC-71952 preincubated for different periods of time with CETP prior to assay, or added without preincubation. (B) IC<sub>50</sub> curves for SC-67201 incubated with CETP for 18 h prior to assay or added without preincubation. (C) Change in IC<sub>50</sub> values obtained for SC-71952 or SC-67201 after different times of preincubation with CETP. All data points represent triplicate assays.

and neutralizing monoclonal antibodies (19) have been shown to effect CE and TG transfer quite differently.

#### Determination of $V_{max}$ in the presence of different concentrations of SC-71952, with or without preincubation with CETP

The time-dependent enhancement of IC<sub>50</sub> displayed by SC-71952 suggests that the inhibition might have an irreversible component. To test this, assay conditions were adjusted to measure the apparent  $V_{max}$  of transfer at different



**Fig. 4.** Determination of  $V_{max}$  in the presence of various concentrations of SC-71952, with or without preincubation with CETP. The lipoprotein concentrations in the lipid transfer assay were increased so that the rates of transfer approached  $V_{max}$ . The concentrations were as follows: [<sup>3</sup>H]CE-HDL (60 μg/ml total cholesterol) and LDL (100 μg/ml total cholesterol). CETP was mixed with different concentrations of SC-71952, and either added directly to the [<sup>3</sup>H]CE transfer assay (A), or preincubated for 18 h prior to assay (B). The indicated concentrations of SC-71952 represent final concentrations in the assay after 8-fold dilution. All data points represent triplicate assays.

CETP and inhibitor concentrations. Reversible inhibition should produce sets of nonparallel lines that extrapolate to the origin whereas an irreversible inhibitor should produce sets of parallel lines that intersect on the abscissa (20). **Figure 4A** shows that when SC-71952 was added directly to the assay without preincubation,  $V_{max}$  was reduced as the inhibitor concentration was increased, and that all of the nonparallel lines extrapolate to the origin as expected for reversible inhibition. However, when the inhibitor was preincubated with CETP for 18 h prior to the assay, sets of nearly parallel lines that intersected on the abscissa were produced, consistent with irreversible inhibition (Fig. 4B). The preincubation time of 18 h was chosen as a time sufficient for achieving maximal inhibition. Note that the concentrations necessary for inhibition were much higher without preincubation than with preincubation, consistent with the shift in IC<sub>50</sub> noted above. These results demonstrate that inhibition by SC-71952 initially occurred by a reversible mechanism, but that inhibition became irreversible on extended incubation with CETP.

In previous experiments, it was demonstrated that certain CETP inhibitors such as cholesteryl sulfate, endotoxin, or apolipoproteins exerted their inhibitory effect, at least in part, by modifying the structure of either HDL

or LDL (11). We used similar analytical methods to determine whether SC-71952 modified lipoprotein structure. Nondenaturing gradient gel electrophoresis and agarose gel electrophoresis were used to analyze lipoprotein samples treated with SC-71952. No alterations were detected by these electrophoretic methods at concentrations up to 100  $\mu\text{M}$  (data not shown).

### Quantitation of the free sulfhydryl content of CETP

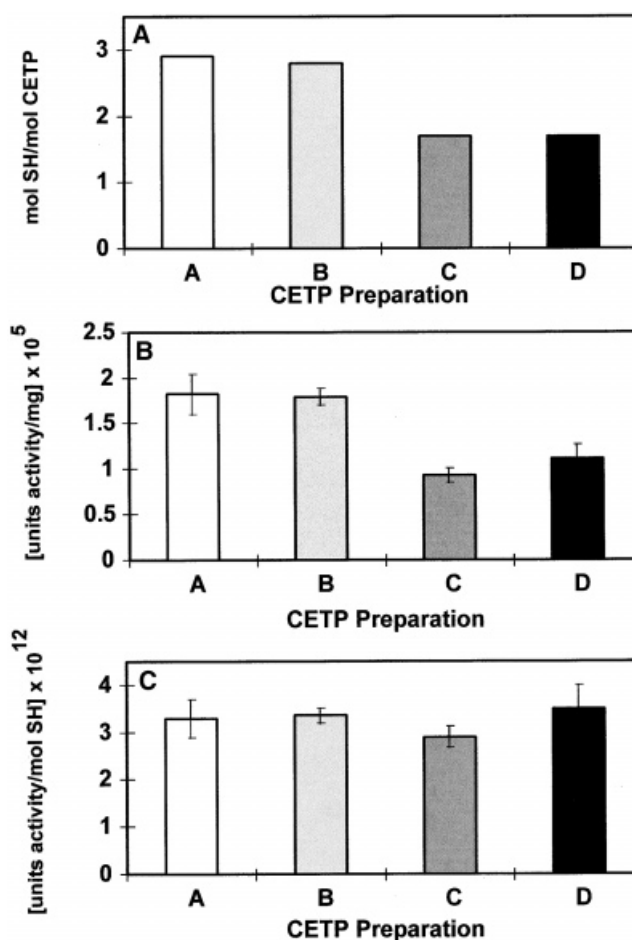
Assuming that the time-dependent inhibition of CETP by SC-71952 resulted from the reaction of the inhibitor with free cysteines, it was necessary to determine the free sulfhydryl content of the CETP. Free sulfhydryls were titrated with DTNB, and quantitated spectrophotometrically by monitoring the formation of TNB<sup>2-</sup>. The ability of DTNB to react with free sulfhydryls on CETP was shown to be enhanced by the addition of the denaturant SDS (data not shown). This suggests that, in the native conformation, some of the free sulfhydryls were not accessible for reaction with DTNB. Titration of the free sulfhydryls was therefore performed both in the absence and presence of SDS (0.8%) to distinguish between accessible and nonaccessible cysteines.

The free sulfhydryl content of CETP was determined for several different preparations of pure recombinant human CETP. In the absence of SDS, the number of free sulfhydryls per mole of CETP ranged from 0.2 to 1.1 in different CETP preparations (data not shown). The values obtained in the presence of SDS, conditions under which CETP is presumably unfolded, were substantially higher than those obtained in the absence of SDS, but they also showed variability. These values ranged from 1.55 to 2.8 mol/mol CETP in the four different CETP preparations that were examined (Fig. 5A). The sulfhydryl content of different preparations varied over about a 2-fold range, the highest value being nearly 3 mol of cysteine per mole of CETP. Conversely, because CETP is known to possess a total of seven cysteines, this would suggest that the disulfide content likewise varies between two and three disulfides per mole of CETP.

The cholesteryl ester transfer activities were also measured for the same CETP preparations, and specific activities were calculated on the basis of the amount of protein added in each assay (Fig. 5B). The specific activities of the CETP preparations varied over a 2-fold range, from 1 to  $2 \times 10^6$  units (% transfer of [<sup>3</sup>H]CE/2-h assay) per milligram of protein. Comparison of the data in Fig. 5A and B suggests a correlation between free sulfhydryl content and activity of CETP. The preparations with the highest free sulfhydryl contents also had the highest specific activities. The relationship of free sulfhydryl content to lipid transfer activity of CETP is shown clearly in Fig. 5C, in which the sulfhydryl content is normalized to activity rather than protein concentration. All four preparations showed nearly identical ratios of units of CETP activity per mole of free sulfhydryl.

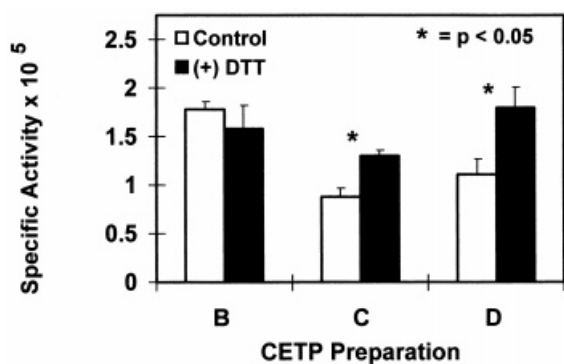
### Effect of DTT treatment on specific activity

One hypothesis explaining the relationship of free sulfhydryls to intrinsic activity of the CETP preparations with fewer titratable sulfhydryls is that disulfide forma-



**Fig. 5.** Correlation of the free sulfhydryl content with the specific activities of various recombinant human CETP preparations. Various preparations of CETP were analyzed for free sulfhydryl content (A) and specific activities (% transfer CE/2-h assay/mg protein) (B) as described in Materials and Methods. (C) The sulfhydryl titration data are normalized relative to the specific activity (moles free sulfhydryl/unit CETP activity, where 1 unit CETP activity = 1% CE transfer/2-h assay). In both (B) and (C), the error bars represent standard deviations of the mean of three determinations (CETP preparation, columns A and D) or six determinations (CETP preparations, columns B and C).

tion leads to the decrease in lipid transfer activity. If this is correct, then treatment with the disulfide reducing agent, DTT, might restore the specific activities of the preparations containing lower free cysteine content. To test this hypothesis, three preparations of CETP were incubated for 20 min at 4°C in the presence or absence of ~1,000-fold molar excess of DTT. The samples were then diluted and assayed. The specific activity of CETP preparation B, which had a free cysteine content of 2.8 mol/mol, was unaffected by treatment with DTT (Fig. 6). In contrast, the specific activities of preparations C and D, which had free sulfhydryl contents of only 1.5 and 1.6 mol/mol free cysteine, respectively, were increased to levels approaching preparation B. Thus, DTT treatment restored the activity of the preparations that had low free cysteine content to levels comparable to preparation B, which had a maximal free sulfhydryl content of 2.8 mol



**Fig. 6.** Effect of DTT treatment on specific activity. Three preparations of CETP were treated with DTT as described in Materials and Methods, and then assayed for their ability to transfer [<sup>3</sup>H]CE from HDL to LDL. The preparations varied in their free sulfhydryl content: CETP preparation B (2.6 mol SH/mol CETP), CETP preparation C (1.6 mol SH/mol CETP), CETP preparation D (1.55 mol SH/mol CETP). The specific activities of the DTT-treated samples were compared with untreated control samples. Increases in the specific activities of CETP preparations C and D on DTT treatment were determined to be statistically significant (\*) on the basis of standard Student's *t*-test analysis. The LDL preparation used was LDL149. One unit of CETP activity = 1% CE transfer/2-h assay. Specific activity = units of activity per milligram protein.

of SH per mole of CETP. This occurred presumably by reducing disulfide bonds.

#### Liquid chromatography/mass spectroscopy analysis of tryptic peptides

To isolate and map the disulfide-containing peptides of recombinant CETP, LC/MS was performed on tryptic digests of alkylated samples. Controls consisted of samples that were completely reduced with DTT prior to alkylation and trypsinization. These experiments were performed with three different CETP preparations, two of which had high free sulfhydryl content and high specific activities (CETP preparations B and E) while the third had a lower free sulfhydryl content and lower specific activity (CETP preparation D).

The cDNA sequence of CETP predicts that 37 peptides should be obtained on trypsin treatment. We identified 31 peptides (84% of the total sequence), including 3 of the 4 peptides that contain potential sites of asparagine-linked glycosylation. The potential structures of the carbohydrates were deduced from the masses of the respective peptides, and were complex tri- and tetraantennary structures capped by sialic acids, typical of glycoproteins produced in BHK cells. The fourth site, Asn341, was found as a nonglycosylated peptide. These data were in agreement with those of Stevenson et al. (21), who found the same three sites to be glycosylated in recombinant human CETP produced in Chinese hamster ovary cells. Two of these sites, Asn396 and Asn88, were previously shown by using site-directed mutagenesis to be necessary for the secretion of CETP. Asn341 was found to be variably glycosylated, and it was suggested that this variability may affect the specific activity of CETP.

The disulfide maps of the two CETP preparations with

high free sulfhydryl content (preparations B and E) were similar, but not identical (Table 1 and Fig. 7). One disulfide, Cys143–Cys184, was found consistently in both preparations. The identity of this peptide was verified by amino acid sequencing (data not shown). Neither Cys143 nor Cys184 was found in the free sulfhydryl form.

Two cysteines (Cys13 and Cys333) were found both as free sulfhydryls and in the disulfide combinations Cys1–Cys13 and Cys13–Cys333. Cys1 was not found in a disulfide linkage with Cys333, nor was it isolated as a free sulfhydryl. The latter result was possibly due to the small size of the Cys1 peptide (three amino acids), which might have rendered it difficult to resolve from the salt front on the column. The Cys13–Cys333 peptide was obtained in low yield, and in only one of the two high specific activity CETP preparations. The Cys1–Cys13 disulfide was found in both preparations and was confirmed by tandem mass spectroscopy (MS/MS) (data not shown).

LC/MS analysis of CETP preparation D (lower free sulfhydryl content and lower specific activity) showed a relative increase in the Cys13–Cys333 disulfide when compared with preparations with higher free sulfhydryl content/higher specific activities (Table 1). No other differences were observed.

We were unable to identify peptides containing Cys131 or Cys325 either as free sulfhydryls, or in disulfide linkages from untreated samples, or in reduced and alkylated control samples. On the basis of the DTNB and LC/MS data obtained for the other cysteines, it would appear that Cys131 and Cys325 are either disulfide linked with each other or are inaccessible to modification. Because both peptides are large and hydrophobic in nature, they may not be soluble in aqueous solution after trypsinization or may not elute off of the C<sub>18</sub> HPLC column.

#### Liquid chromatography-mass spectroscopy analysis of CETP treated with SC-71952

To determine whether the irreversible inactivation of CETP by SC-71952 was associated with changes in CETP structure, LC/MS analysis was performed on CETP samples that were incubated for various times with the inhibitor. Samples of CETP preparation E were incubated for 5 min or 4 h with SC-71952, and then alkylated with iodoacetamide. After alkylation, the samples were dialyzed and then digested with trypsin and analyzed by LC/MS. Controls consisted of a sample that was not treated with the inhibitor and a sample that was treated with the inhibitor for 4 h, and then denatured with 2% SDS prior to alkylation, dialysis, and trypsin digestion. This latter sample served as a control for the potential complications resulting from incomplete alkylation.

The Cys143–Cys184 disulfide was detected in all of the samples with or without SC-71952 treatment. Control CETP (alkylated, no SC-71952 treatment) possessed the Cys1–Cys13 disulfide and low levels of the Cys13–Cys333 disulfide. Cys13 and Cys333 were also detected as free sulfhydryls. This was consistent with the previous LC/MS analysis of this preparation of CETP. As shown in Fig. 8, the levels of the Cys1–Cys13 disulfide, free Cys13, and free

TABLE 1. Disulfides and free sulfhydryls detected by LC/MS analysis of tryptic digests of various preparations of recombinant CETP

Tryptic Peptide	[M + H] <sup>+</sup>		
	Calculated <sup>a</sup>	Observed <sup>a</sup>	
<i>m/z</i>			
CETP preparation B (2.8 mol SH/mol CETP)			
Disulfides			
Cys1–Cys13	T1–T2	1,464.67	1,464.0
Cys143–Cys184	T12/13–T15/16/17/18	6,729.98	6,729.0
Cys143–Cys184	T12/13/14–T15/16/17/18 (pyroE)	7,440.84 (7,423.84)	7,440.0 (7,423.0)
Free SH			
Cys13	T2	1,187.32	1,186.8
Cys333	T28	749.86	749.6
CETP preparation E (3.1 mol SH/mol CETP)			
Disulfides			
Cys1–Cys13 <sup>b</sup>	T1–T2	1,464.67	1,463.4
Cys13–Cys333 <sup>b</sup>	T2–T28	1,820.06	1,819.4
Cys143–Cys184	T12/13–T15/16/17/18	6,729.98	6,729.0
Cys143–Cys184	T12/13/14–T15/16/17/18 (pyroE)	7,440.84 (7,423.84)	7,439.0 (7,423.0)
Free SH			
Cys13	T2	1,187.32	1,186.6
Cys333	T28	749.86	749.2
CETP preparation D (1.7 mol SH/mol CETP)			
Disulfides			
Cys1–Cys13 <sup>c</sup>	T1–T2	1,464.67	1,464.2
Cys13–Cys333 <sup>c</sup>	T2–T28	1,820.06	1,820.0
Cys143–Cys184	T12–T15/16/17/18 (pyroE)	5,669.69 (5,652.69)	5,669.0 (5,652.0)
Cys143–Cys184	T12/13–T15/16/17/18 (pyroE)	6,729.98 (6,712.98)	6,729.0 (6,711.0)
Cys143–Cys184	T12/13/14–T15/16/17/18 (pyroE)	7,440.84 (7,423.84)	7,440.0 (7,423.0)
Free SH			
None found			

Samples from various preparations of recombinant CEPT were alkylated, trypsinized, and analyzed, and analyzed by LC/MS as described in Materials and Methods. Controls consisted of samples that were completely reduced with DTT prior to alkylation and trypsinization.

<sup>a</sup> Isotopically averaged *m/z*.

<sup>b</sup> Yield of Cys1–Cys13 greater than yield of Cys13–Cys333.

<sup>c</sup> Equal yields.

Cys333 dramatically decreased on increased incubation time with SC-71952. Surprisingly, no significant increases were seen in any disulfides, nor was there the appearance of any new disulfide correlating with irreversible inactivation. Likewise, no mixed disulfides with the inhibitor were detected. The profiles of the 4-h SC-71952 treatment samples were the same whether or not SDS was included prior

to alkylation (data not shown). These data show that the alkylation was complete in the samples that were not denatured with SDS, thereby eliminating the possibility of complications due to the presence of reactive cysteines. A possible explanation for the loss of cysteine-containing peptides is that the modification of these peptide, via formation of a disulfide pair or a mixed disulfide with the inhibitor,

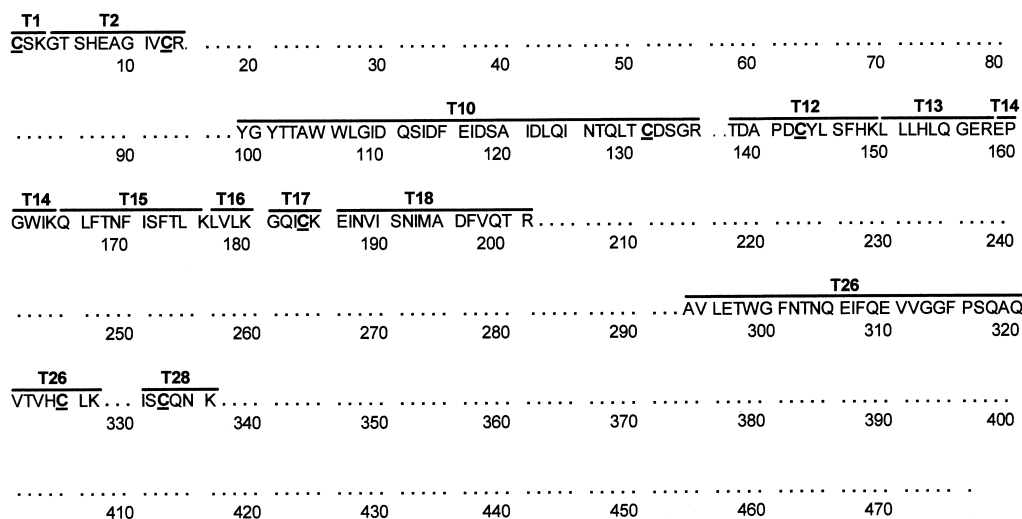
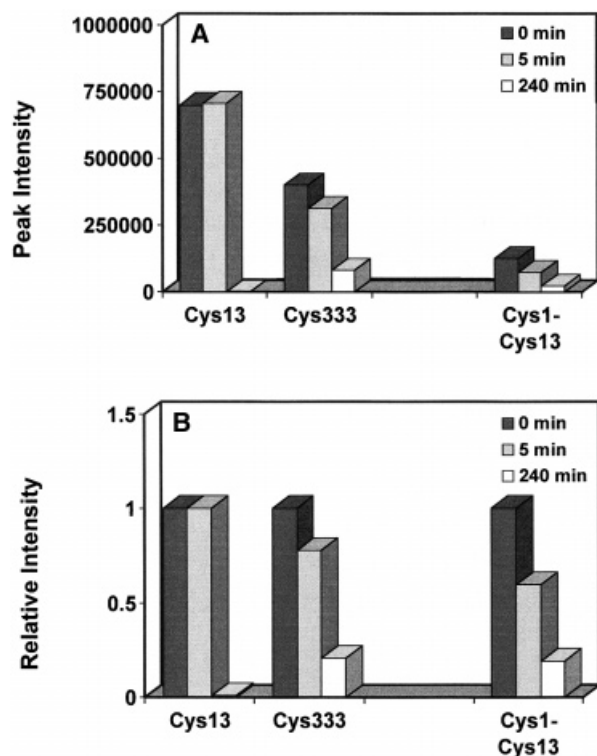


Fig. 7. Location and amino acid sequence of the various cysteine-containing tryptic peptides.



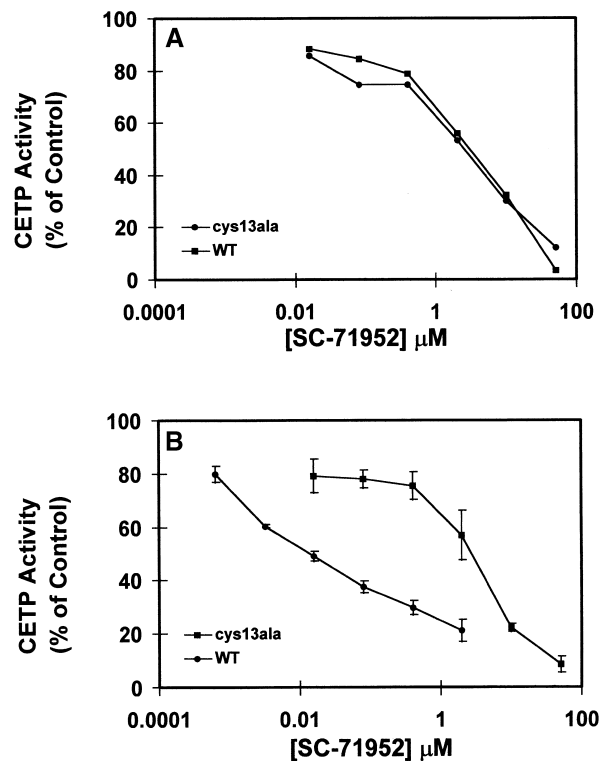


**Fig. 8.** Changes in relative amounts of Cys1-Cys13, free Cys13, and free Cys333-containing peptides on treatment with SC-71952. Samples of CETP preparation E were treated with SC-71952 for 5 min or 4 h. The samples were then alkylated and trypsin digested as described in Materials and Methods. The control consisted of an equal sample that was alkylated only prior to trypsin digestion. Samples were analyzed by LC/MS and normalized for total protein. (A) Data are presented as relative peak intensity with respect to total protein. (B) Data are presented as changes in peak intensity with respect to the alkylated only control.

renders the peptide insoluble or otherwise difficult to elute from the column. These results, however, implicate the involvement of at least one of the three partially oxidized cysteines (Cys1, Cys13, and Cys333) in the irreversible modification of CETP by SC-71952.

#### Loss of time dependence of inhibition by SC-71952 in Cys13Ala mutant CETP

Cys13 was chosen as the most likely of the three cysteines to be involved in the irreversible inactivation of the protein by the inhibitor, based on a model of CETP constructed by Bruce, Beamer, and Tall (12). In this model, Cys13 sits at the bottom of the neutral lipid binding pocket in the N-terminal domain of the protein. This residue was converted to alanine and the resulting mutant CETP was tested for its susceptibility to both the rapid reversible and the time-dependent irreversible inactivation by the inhibitor. Both the wild-type and the mutant CETP showed similar specific activities, suggesting no obvious impact of the mutation on normal CE transfer (data not shown). As shown in **Fig. 9**, the inhibition of [ $^3$ H]CE transfer activity by SC-71952 was identical for both the wild-type CETP and the Cys13Ala mutant CETP in assays



**Fig. 9.** Effects of preincubation with wild-type CETP and the Cys13Ala mutant CETP on the inhibitory potency of SC-71952. Various concentrations of SC-71952 were mixed with wild-type CETP or Cys13Ala mutant CETP either immediately prior to or 18 h prior to the [ $^3$ H]CE transfer assay. (A) IC<sub>50</sub> curves for SC-71952 with wild-type CETP or Cys13Ala mutant CETP with no preincubation with the inhibitor. (B) IC<sub>50</sub> curves for SC-71952 with wild-type CETP or Cys13Ala mutant CETP incubated with the inhibitor for 18 h prior to assay. Assays were carried out with equal concentrations of wild-type and mutant protein, with comparable values of total transfer observed.

where there was no preincubation (**Fig. 9A**). However, when the inhibitor was mixed with either wild-type CETP or the Cys13Ala mutant CETP for 18 h prior to assay, a dramatic difference was noted between mutant and wild-type enzymes. The IC<sub>50</sub> of the inhibitor for the wild-type CETP showed its characteristic 200-fold increase in potency while no shift was seen using the Cys13Ala mutant CETP. These data confirm that Cys13 is required for the time-dependent enhancement in potency seen with SC-71952.

## DISCUSSION

CETP plays a role in lipoprotein metabolism by mediating both the transfer of CE from HDL to VLDL and the reciprocal transfer of TG. In doing so, it can affect the balance of the various lipoprotein particles. Several studies, both clinical and epidemiological, have clearly shown that alterations in lipoprotein profiles can have profound effects on an individual's risk for coronary heart disease (CHD) (22, 23). Although there are some conflicting data with respect to the role of CETP in CHD, an emerging hypothesis is that a potentially complex dose relationship exists

between CETP levels, HDL-cholesterol levels, and CHD (24, 25). CETP inhibition may be therapeutically beneficial when treating patients with excessive CETP levels. The hypothesis suggests that potent CETP inhibitors (>50% inhibition *in vivo*) could reduce CHD risk if they can raise HDL cholesterol levels above 60 mg/dl (25). In addition, CETP inhibitors may be used as tools to delineate the biochemical mechanisms of CETP-mediated lipid transport.

Our strategy to identify CETP inhibitors involved screening chemical libraries for compounds that prevent CE transfer from HDL to LDL. SC-71952 was identified as a potent, time-dependent inhibitor of CETP. When added directly into the [<sup>3</sup>H]CE transfer assay containing HDL and LDL, an IC<sub>50</sub> of about 1 μM was obtained. At 1 μM, the SC-71952 concentration is about 0.3 mol% relative to total cholesterol in the assay, indicating that the inhibitor is potent even in the presence of excess lipid. On preincubation with CETP, the IC<sub>50</sub> was lowered about 200-fold, from 2 μM to 10 nM. The CETP concentration used in the assay was 0.5 nM, and thus the ratio of SC-71952 to CETP under preincubation conditions was only about 20:1.

The potency of SC71952 was reduced about 50-fold, to 50 μM, by inclusion of the disulfide-reducing agent DTT. This suggests that disulfide exchange with a cysteine on CETP may be key to the potency of SC-71952.

SC-67201 is structurally related to SC-71952. One important difference between the two inhibitors is the substitution of a thioether for the disulfide. SC-67201 is therefore unable to undergo disulfide exchange with cysteine. Even so, SC-67201 was still a relatively potent inhibitor of CETP (IC<sub>50</sub> = 10 μM), demonstrating that although disulfide exchange enhances potency, it is not required for inhibition. This has been verified with other structural analogs of SC-67201 that do not contain a disulfide but still inhibit CETP (data not shown). This observation suggests that CETP contains a specific hydrophobic binding site that can accommodate SC-67201 or related compounds, and that binding to this site is sufficient to inhibit CETP. The enhanced potency of SC-71952 relative to other molecules in this class arises from its ability to undergo disulfide exchange with a cysteine that is in or near this site, thus irreversibly inhibiting CETP. It is not known whether this cysteine is also near the neutral lipid-binding site. Results from kinetic analysis of inhibition do not provide insight into the question of competition of inhibitor with substrate because both V<sub>max</sub> and K<sub>m</sub> effects were observed with the reversible analog SC-67201, even though SC-71952 was observed to prevent CE and TG binding to CETP in direct binding experiments (data not shown).

Disulfide modification by certain hydrophobic reagents has previously been shown to inactivate CETP (4, 7, 9, 10). However, most of the disulfide modification reagents tested were not effective CETP inhibitors (10). In particular, polar or charged reagents were ineffective. It is of interest that 4,4'-dithiodipyridine, a compound structurally similar to SC-71952, was one of the most potent inhibitors identified. However, 4,4'-dithiodipyridine was found to require preincubation with CETP and was noninhibitory when added directly into the transfer assay. In contrast,

SC-71952 was potent even when added immediately prior to the assay.

Structural analysis of CETP was undertaken to identify the cysteine(s) with which SC-71952 reacts to irreversibly inactivate CETP. The degree of sulfhydryl oxidation varied between CETP preparations and was inversely correlated with the respective specific activities of the preparations. The cysteines contributing to the heterogeneity were Cys1, Cys13, and Cys333. These cysteines were found as free sulfhydryls and in disulfide pairs. Of the remaining cysteines, Cys143 and Cys184 were found exclusively in a disulfide linkage with each other while, unfortunately, peptides containing Cys131 and Cys325 were not detected. However, direct measurement of the free sulfhydryl content indicated that there was less than 3 mol of free cysteine per mole of CETP. There are two possible explanations to account for these data. First, Cys131 and Cys325 could be disulfide linked. Alternatively, they could be inaccessible to modification by DTNB, even in the presence of SDS. Beamer, Carroll, and Eisenberg (26) published the crystal structure of bactericidal permeability increasing protein (BPI), a protein that is structurally related to CETP. Bruce, Beamer, and Tall (12) constructed a model of CETP based on the structure of BPI. In this model, Cys131 and Cys325 are not in close enough proximity to allow for a disulfide linkage (C. Bruce and A. R. Tall, unpublished observation). The combination of inaccessibility to modification and recovery difficulties might explain the "missing in action" status of these two cysteines.

The free sulfhydryl content of the various preparations of CETP ranged from 1.55 to 2.8 mol of free sulfhydryl per mole of CETP. The noninteger nature of these values suggests that, in any given preparation of CETP, there is a mixed population. Our data indicate that the variable disulfide pairs are Cys1–Cys13 and Cys13–Cys333. Although attempts were made to separate these "thioforms" of CETP by ion-exchange and size-exclusion chromatography (data not shown), our attempts thus far have been unsuccessful. The requirement for SDS for complete titration of the free sulfhydryls indicates that approximately two of the three free sulfhydryl-containing cysteines are located in a hydrophobic environment that is inaccessible to DTNB. Importantly, the specific activities of recombinant CETP preparations that possessed free sulfhydryl contents close to 3 mol of sulfhydryl per mole of CETP were equal to or better than the specific activity of human serum CETP (data not shown).

Treatment of CETP with SC-71952 resulted in a time-dependent reduction in the relative amounts of the Cys1–Cys13 disulfide, free Cys13, and free Cys333, correlating with loss of CETP activity. Although these experiments, along with the previously mentioned biochemical data, did not allow for the unequivocal identification of the cysteine or cysteines involved in the time-dependent inactivation of CETP by SC-71952, they did allow attentions to be focused on Cys1, Cys13, and Cys333. They also provided evidence of inhibitor-dependent changes in the oxidative states of these cysteines, thereby supporting the hypothesis that mixed disulfide formation with SC-71952, or mixed

disulfide formation that is followed by disulfide rearrangement, leads to inactivation of CETP.

Critical information was obtained by using a Cys13-to-alanine mutant of CETP. This mutant was indistinguishable from wild-type CETP with respect to both specific activity and the rapid, reversible component of inactivation by SC-71952. However, conversion of Cys13 to alanine resulted in a protein that was not susceptible to the time-dependent component of inactivation by SC-71952. This suggests that the presence of Cys13 is required for the time-dependent inactivation of CETP by SC-71952, and strengthens the evidence placing this residue in or near the neutral lipid binding site of CETP. We have no reason to believe that Cys13 is involved in the mechanism of CE transfer by CETP, especially because the CE transfer activities of the mutant and wild-type CETP appeared to be indistinguishable in the absence of the inhibitor. Differences were seen only with respect to the time-dependent inactivation of CETP by SC-71952. Cys13 may therefore participate in disulfide exchange with the inhibitor or serve as a site for inhibitor-catalyzed disulfide formation with one of the other cysteines of CETP. Both of these possibilities could result in a lipid-binding site that is no longer able to bind CE. Further study of this mutant, as well as the study of other mutations at Cys1, Cys13, and Cys333, will provide insight into both the structure of CETP and the mechanism by which it transfers CE and TG. [Fig](#)

#### NOTED ADDED IN PROOF

While this article was in press, Okamoto et al. published the following article: A cholesteryl ester transfer protein inhibitor attenuates atherosclerosis in rabbits. *Nature*. **406**: 203–205. Using an inhibitor structurally similar to SC-71952, they also demonstrated potent inhibition of CETP by a disulfide-containing compound and likewise identified Cys13 as a residue critical to the inhibition. These data are consistent with our data.

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