CP-346086: an MTP inhibitor that lowers plasma cholesterol and triglycerides in experimental animals and in humans

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Abstract **A microsomal triglyceride transfer protein (MTP) inhibitor, CP-346086, was identified that inhibited both human and rodent MTP activity [concentration giving half-maximal inhibition (IC_{50}) 2.0 nM]. In Hep-G2 cells, CP-346086 inhibited apolipoprotein B (apoB) and triglyceride secretion (IC_{50} 2.6 nM) without affecting apoA-I secretion or lipid synthesis. When administered orally to rats or mice, CP-346086 lowered plasma triglycerides [dose giving 30% triglyceride lowering (ED_{30}) 1.3 mg/kg] 2 h after a single dose. Coadministration with Tyloxapol demonstrated that triglyceride lowering was due to inhibition of hepatic and intestinal triglyceride secretion. A 2 week treatment with CP-346086 lowered total, VLDL, and LDL cholesterol and triglycerides dose dependently with 23%, 33%, 75%, and 62% reductions at 10 mg/kg/day. In these animals, MTP inhibition resulted in increased liver and intestinal triglycerides when CP-346086 was administered with food. When dosed away from meals, however, only hepatic triglycerides were increased. When administered as a single oral dose to healthy human volunteers, CP-346086 reduced plasma triglycerides and VLDL cholesterol dose dependently with ED_{50}s of 10 mg and 3 mg, and maximal inhibition (100 mg) of 66% and 87% when measured 4 h after treatment. After a 2 week treatment (30 mg/day), CP-346086 reduced total and LDL cholesterol and triglycerides by 47%, 72%, and 75%, relative to either individual baselines or placebo, with little change in HDL cholesterol. Together, these data support further evaluation of CP-346086 in hyperlipidemia.**—Chandler, C. E., D. E. Wilder, J. L. Pettini, Y. E. Savoy, S. F. Petras, G. Chang, J. Vincent, and H. J. Harwood, Jr. **CP-346086: an MTP inhibitor that lowers plasma cholesterol and triglycerides in experimental animals and in humans.** ***J. Lipid Res.*** **2003.** 44: **1887–1901.**

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Cardiovascular disease remains the leading cause of death in industrialized nations and accounted for 950,000, or 41%, of all deaths in the United States in 1998 (1). As a consequence of atherosclerosis, coronary heart disease (CHD) is the most common cause of cardiovascular morbidity and mortality, with an estimated 12 million people suffering from CHD in the United States alone (1). Elevated total and LDL cholesterol are both accepted primary risk factors for atherosclerosis (1–3). An estimated 101 million United States adults have elevated blood cholesterol (>200 mg/dl) and are candidates for LDL cholesterol lowering through dietary intervention (1, 4, 5). Of these, 41 million are considered high risk, having blood cholesterol greater than 240 mg/dl, and drug therapy is recommended (1, 4, 5).

Epidemiological studies have shown that elevated triglycerides and reduced HDL cholesterol are also contributing factors for the development of CHD (2, 3, 6–8). Among the adult United States population, 19% of people have low HDL cholesterol (<40 mg/dl) (3, 9, 10) and 21% have hypertriglyceridemia (>150 mg/dl) (3, 10). Thus, as important as elevated LDL cholesterol is as a risk factor for CHD, it is important to recognize that the most common spectrum of lipid abnormalities is atherogenic dyslipidemia, which is present in 45–50% of men with CHD (11, 12) and includes borderline high-risk LDL cholesterol (e.g., 130–159 mg/dl), elevated triglycerides, small dense LDL particles, and low HDL cholesterol.

The HMG-CoA reductase inhibitors (statins) are very effective in lowering LDL cholesterol and somewhat effective in reducing triglycerides, but they have only minimal effects on HDL cholesterol (2, 5, 13–15). Indeed, although numerous clinical trials have demonstrated that LDL cholesterol reduction can significantly reduce CHD risk, a great number of treated subjects who achieve substantial LDL cholesterol reduction still experience a clinical event (2, 3, 13–18). Therefore, with the goal of develop-

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Abbreviations: CETP, cholesterol ester transfer protein; CHD, coronary heart disease; ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein.

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ing a therapy for treating patients with dyslipidemias that extend beyond primary hypercholesterolemia, we targeted inhibition of microsomal triglyceride transfer protein (MTP) as a mechanism for preventing triglyceride-rich lipoprotein assembly in the liver and intestine.

MTP, which is located within the lumen of the endoplasmic reticulum (ER) in hepatocytes and absorptive enterocytes, is a heterodimeric protein consisting of a 97 kDa subunit, which confers all of the lipid transfer activity of the heterodimer, and the 58 kDa multifunctional protein disulfide isomerase (19). MTP plays a pivotal, if not obligatory role, in the assembly and secretion of triglyceride-rich, apolipoprotein B (apoB)-containing lipoproteins (VLDL and chylomicrons) from the liver and intestine and also catalyzes the transport of triglycerides, cholesteryl esters, and phospholipids between membranes (19–21). Although the exact role of MTP in the assembly of apoB-containing lipoproteins is still under investigation (21–23), MTP is proposed to transport lipids from the ER membrane to the growing apoB polypeptide chain in the ER lumen, thereby allowing proper translocation and folding of apoB to occur (19–24). MTP has also been proposed to mediate bulk triglyceride transfer into the ER lumen for incorporation into poorly lipidated apoB-containing lipoprotein particles during the process of VLDL and chylomicron assembly (25, 26). Recent studies have also suggested a role for MTP in the movement of cholesterol ester into the ER lumen for inclusion into nascent apoB-containing lipoprotein particles (27).

The initial suggestion that MTP inhibition could be a viable lipid-lowering therapy came with the discovery that functional MTP is absent in individuals with abetalipoproteinemia, a genetic disorder characterized by low plasma cholesterol and triglycerides due to a defect in the assembly and secretion of apoB-containing lipoproteins (28, 29). A similar phenotype is observed in MTP knockout mice (23, 30). Abetalipoproteinemia, however, represents an extreme example of MTP inhibition and is not without its clinical sequelae, all of which presumably are related directly or indirectly to fat malabsorption (steatorrhea), vitamin malabsorption, and hepatic and intestinal steatosis (29, 31). A less severe, and probably more relevant, example of the consequences of therapeutic MTP inhibition is a related genetic disease, hypobetalipoproteinemia, caused by mutations in apoB (32). Heterozygous individuals with this disease possess half of the normal levels of apoB-containing lipoproteins, lack the clinical signs and symptoms of abetalipoproteinemia, and have a normal lifespan (24).

We devised a two-stage empirical screening protocol for compound evaluation (33) to identify potent MTP inhibitors with the potential to inhibit hepatic and intestinal apoB-containing lipoprotein assembly and consequently lower plasma total, LDL, and VLDL cholesterol and triglycerides in experimental animals and in humans. In the first stage of the protocol, compounds were evaluated for their ability to inhibit apoB, but not apoA-I secretion, from Hep-G2 cells in a high-throughput, 96-well multiplexed format, essentially as described by Haghpassand, Wilder, and Moberly (34). In the second stage of the protocol, confirmed apoB secretion inhibitors were evaluated for their ability to inhibit the MTP-mediated transfer of radiolabeled triolein from synthetic phospholipid donor liposomes to acceptor liposomes (34). Using this two-stage screening protocol, we identified CP-94792, a potent inhibitor of apoB, but not apoA-I, secretion (33). Inhibition of apoB secretion was subsequently determined to be through inhibition of MTP activity (33, 35). However, although CP-94792 inhibited Hep-G2 cell apoB secretion with an half-maximal inhibition (IC_{50}) of 200 nM and inhibited MTP-mediated triglyceride transfer (rat MTP) with an IC_{50} of 250 nM, the compound displayed only weak triglyceride lowering activity when administered orally to rats (33).

The potent and orally efficacious MTP inhibitor, CP-346086 (4′-trifluoromethyl-biphenyl-2-carboxylic acid [2-(2H-[1,2,4]triazol-3-ylmethyl)-1,2,3,4-tetrahydro-isoquinolin-6-yl] amide); **Fig. 1**, inset), was ultimately identified (33, 35–37) by *1*) employing a robotics-assisted parallel synthesis strategy as a means of developing structure-activity relationships and improving in vitro potency, and *2*) using in vitro hepatic microsomal clearance and in vivo triglyceride lowering as guides for improving pharmacokinetic properties. In this report, we describe the biochemical

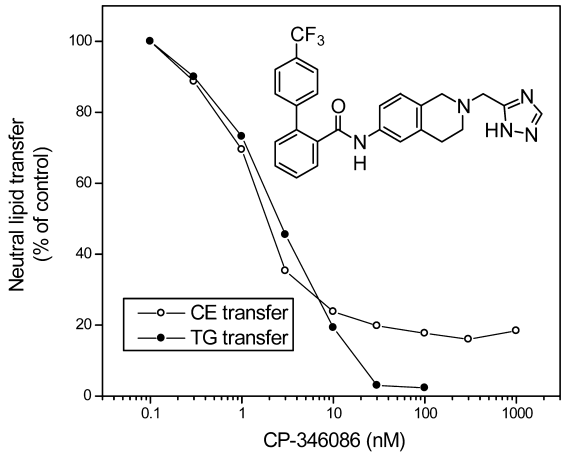

Fig. 1. Inhibition of human microsomal triglyceride transfer protein (MTP)-mediated neutral lipid transfer by CP-346086. Aliquots of solubilized human liver MTP, 150 μl, were incubated at 37°C for 45 min with 50 μl donor liposomes, 100 μl acceptor liposomes, and 200 μl assay buffer containing either 5% BSA (control) or 5% BSA plus sufficient CP-346086 to produce the indicated final concentrations of CP-346086, as described in Experimental Procedures. After incubation, triglyceride transfer was terminated by addition of 300 μl of a 50% (w/v) DEAE cellulose suspension in assay buffer. After a 4 min agitation, the donor liposomes, bound to DEAE cellulose, were selectively sedimented by low speed centrifugation (3,000 *g*, 5 min). An aliquot of the supernatant containing the acceptor liposomes was assessed for radioactivity as outlined in Experimental Procedures. Shown is the percentage of control [^{14}C]triolein or [^{14}C]cholesterol oleate transfer as a function of CP-346086 concentration. Control triolein and cholesterol oleate transfer during the 45 min assay averaged 42% and 13%, respectively. Inset: The structure of CP-346086 (4′-trifluoromethyl-biphenyl-2-carboxylic acid [2-(2H-[1,2,4]triazol-3-ylmethyl)-1,2,3,4-tetrahydro-isoquinolin-6-yl]amide).

mechanism of action of CP-346086 that leads to its LDL cholesterol-, VLDL cholesterol-, and triglyceride-lowering efficacy in experimental animals and in humans.

EXPERIMENTAL PROCEDURES

Materials

Sodium [2-^{14}C]acetate (56 mCi/mmol), [^{14}C]triolein (110 mCi/mmol), cholesteryl [1-^{14}C]oleate (55 mCi/mmol), [^{3}H]triolein (25 Ci/mmol), [^{3}H]egg phosphatidylcholine (50 mCi/mmol), and Aquasol-2 were from New England Nuclear (Boston, MA). [^{3}H]glycerol (20 Ci/mmol) was from American Radiochemicals (St. Louis, MO). Ready Safe™ liquid scintillation cocktail was from Beckman Instruments (Fullerton, CA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, and gentamicin were from GIBCO Laboratories (Grand Island, NY). Heat-inactivated fetal bovine serum was from HyClone Laboratories (Logan, UT). DEAE cellulose was from Whatman International (Maidstone, England). Silica gel 60C TLC plates were from Eastman Kodak (Rochester, NY). BCA protein assay reagent was from Pierce (Rockford, IL). Cholesterol/HP reagent (Cat. 1127578), TG/GPO reagent (Cat. 1128027), Preciset Cholesterol Calibrator Set (Cat. 125512), and Precitrol-N serum (Cat. 620212) were from Boehringer Mannheim (Indianapolis, IN). Cholesterol CII reagent kit (Cat. 276-64909), Triglyceride E reagent kit (Cat. 432-40201), Standard Cholesterol CII Solution (Cat. 277-64939), and Standard Triglyceride G Solution (Cat. 998-69831) were from Waco Chemicals USA (Richmond, VA). Hep-G2 cells were from the American Type Culture Collection (Rockville, MD). Mouse anti-human apoB monoclonal antibodies (MoAB-012), goat anti-human apoB polyclonal antibodies (AB-742), mouse anti-human apoA-I monoclonal antibodies (MAB-011), goat anti-human apoA-I polyclonal antibodies (AB-740), and human apoA-I purified standard (ALP10) were from Chemicon (Temecula, CA). B6CBAF1J mice were from Jackson Laboratory (Bar Harbor, ME). Transgenic huA1/CIII/cholesteryl ester transfer protein (CETP) mice, originally obtained from Charles River (Boston, MA), were bred on site. Sprague Dawley rats were from Charles River. RMH 3200 laboratory meal was from Agway, Inc. (Syracuse, NY). AIN76A semipurified diet was from US Biochemicals (Cleveland, OH). F0739 liquid diet powder was from Bio-Serve, Inc. (Frenchtown, NJ). Fast protein liquid chromatography (FPLC) instrumentation was from Gilson, Inc. (Middletown, WI). Superose-6 gel filtration columns were from Pharmacia (Piscataway, NJ). Postcolumn reaction (PCX) instrumentation was from Pickering Laboratories (Mountain View, CA). All other chemicals and reagents were from previously listed sources (38, 39).

Preparation of human MTP

The procedure for isolating and solubilizing human MTP from frozen hepatic tissue is based on the method of Wetterau and Zilversmit (40) and was conducted essentially as described by Haghpassand, Wilder, and Moberly (34). Solubilized human liver MTP was stored at 4°C and was diluted 1:5 with assay buffer just before use. MTP preparations showed no notable loss of transfer activity with storage up to 30 days. Rat and mouse MTP were prepared similarly, except that freshly isolated liver tissue was used.

Preparation of phospholipid vesicles for use in lipid transfer assays

Donor and acceptor liposomes were prepared essentially as described by Wetterau et al. (28). Donor liposomes were prepared under nitrogen by room temperature bath sonication of a dispersion containing 447 μM egg phosphatidylcholine, 83 μM bovine heart cardiolipin, and 0.91 μM [^{14}C]triolein (110 Ci/mol). Acceptor liposomes were prepared under nitrogen by room temperature bath sonication of a dispersion containing 1.3 mM egg phosphatidylcholine, 2.6 μM triolein, and 0.5 nM [^{3}H]egg phosphatidylcholine in assay buffer. The donor and acceptor liposomes were centrifuged at 160,000 *g* for 2 h at 7°C. The upper 80% of the supernatant, containing small unilamellar liposomes, was carefully removed and stored at 4°C until use.

Measurement of MTP inhibitory activity

MTP inhibitory activity as outlined by Haghpassand, Wilder, and Moberly (34) was determined by adding 200 μl of a buffer containing either 5% BSA (control) or 5% BSA plus CP-346086 to a mixture containing 50 μl donor liposomes, 100 μl acceptor liposomes, and 150 μl of solubilized, dialyzed MTP protein diluted in assay buffer as outlined above. After incubation at 37°C for 45 min, triglyceride transfer was terminated by addition of 300 μl of a 50% (w/v) DEAE cellulose suspension in assay buffer. After 4 min of agitation, the donor liposomes, bound to DEAE cellulose, were selectively sedimented by low speed centrifugation (3,000 *g*; 5 min). An aliquot of the supernatant containing the acceptor liposomes was assessed for radioactivity, and the ^{14}C and ^{3}H counts obtained were used to calculate the percent recovery of acceptor liposomes and the percent triglyceride transfer using first order kinetics.

Measurement of Hep-G2 cell apoB and apoA-I secretion inhibition

HepG2 cells were grown in DMEM containing 10% fetal bovine serum in 96-well plates in a humidified, 5% CO_2 atmosphere at 37°C until ~70% confluent. CP-346086 was dissolved in DMSO, diluted to 1 μM in growth medium, serially diluted in growth medium to the desired concentration range, and added in 100 μl aliquots to separate wells of 96-well culture plates containing HepG2 cells. Twenty-four hours later, growth medium was collected and assessed for apoB and apoA-I concentrations as described by Haghpassand and Moberly (41).

Measurement of Hep-G2 cell cholesterol and fatty acid synthesis

Cholesterol and fatty acid synthesis were evaluated in Hep-G2 cells by measuring incorporation of [2-^{14}C]acetate into cellular lipids as previously described (38, 39), with the following modifications to allow simultaneous assessment of both cholesterol and fatty acid synthesis. After collection and assessment of the hexane fraction containing cholesterol and nonsaponifiable lipids as previously described (38, 39), the remaining aqueous phase (containing fatty acid sodium salts) was acidified to pH <2 by addition of 0.5 ml of 12 M HCl. The resulting mixtures were then transferred to glass conical tubes and extracted three times with 4.5 ml hexane. The pooled organic fractions were dried under nitrogen, resuspended in 50 μl of chloroform-methanol (1:1; v/v), and applied to 1 × 20 cm channels of Silica Gel 60C TLC plates. Channels containing nonradioactive fatty acids were included on selected TLC plates as separation markers. TLC plates were developed in hexane-diethyl ether-acetic acid (70:30:2; v/v/v) and air dried, and the region of chromatograms corresponding to fatty acid mobility was removed and assessed for radioactivity in Aquasol-2 using a Beckmann LS6500 liquid scintillation counter. Cholesterol and fatty acid synthesis are expressed as dpm [2-^{14}C]acetate incorporated into either cholesterol or saponifiable lipids during the 6 h incubation at 37°C per mg cellular protein.

Measurement of HepG2 cell triglyceride synthesis and secretion

HepG2 cells, grown in T-75 flasks as previously described (38, 39), were seeded into 24-well plates at 4–6 ×10^{5} cells/well and

maintained in a humidified, 5% CO_2 atmosphere at 37°C for 48 h prior to use. At the beginning of each experiment, media were removed and replaced with fresh media containing 0.2% DMSO ± CP-346086. One hour after compound addition, 25 μl of media containing 50 μCi of [^{3}H]glycerol was added to each incubation well. Plates were then sealed with Parafilm to avoid evaporation, and cells were incubated at 37°C for 6 h with gentle shaking. After incubation, the media were removed, and the cells were washed three times with PBS and scraped from wells into PBS. Lipids were extracted from the media and the cell lysate with chloroform-methanol (2:1; v/v) and applied to 1 × 20 cm channels of Silica Gel 60C TLC plates. Channels containing nonradioactive triglycerides were included on selected TLC plates as separation markers. TLC plates were developed in petroleum ether-diethyl ether-acetic acid (75:25:1; v/v/v) and air dried. The region of chromatograms corresponding to triglyceride mobility was removed and assessed for radioactivity in Aquasol-2 using a Beckman LS6500 liquid scintillation counter. Triglyceride synthesis and triglyceride secretion are expressed as dpm [^{3}H]glycerol incorporated into cellular triglycerides or secreted into the culture medium during the 6 h incubation at 37°C per mg cellular protein.

Measurement of plasma cholesterol and triglyceride levels

Plasma triglyceride and total cholesterol concentrations were determined using Cholesterol CII and Triglyceride E reagent kits and employing Standard Cholesterol CII Solution and Standard Triglyceride G Solution as calibration standards.

Measurement of hepatic and intestinal cholesterol and triglyceride levels

Hepatic and intestinal cholesterol and triglyceride levels were measured as previously described (42) with the following modifications. Sections, 0.5 g, of hepatic or intestinal tissue were homogenized at 4°C in 3.0 ml PBS using a Polytron tissue homogenizer. Aliquots, 200 μl, of the homogenates were transferred to 15 ml screw-capped glass tubes containing 7.5 ml of a mixture of $CHCl_3$-MeOH (2:1; v/v) and mixed vigorously for 20 s. One milliliter of ddH_2O was then added, and the resulting suspension was mixed vigorously for 15 s then centrifuged at 3,000 rpm for 5 min at room temperature in a Sorvall RT-6000 bench-top centrifuge. The chloroform-methanol layer was removed, placed in a 13 × 100 mm test tube, and evaporated to dryness under nitrogen at 60°C. The lipid residue was resuspended in 200 μl 1% Triton X-100 in absolute EtOH, and the cholesterol and triglyceride concentrations were determined using Cholesterol CII and Triglyceride E reagent kits, adapted for colorimetric analysis in 96-well plate format.

Plasma lipoprotein subfractionation and visualization by FPLC and PCX

Plasma lipoproteins were separated on a single Superose-6 column by FPLC, and the effluent was split into two equal streams. One stream was reacted postcolumn with cholesterol enzymatic assay reagents for the determination of lipoprotein cholesterol. The second stream was reacted postcolumn with triglyceride enzymatic assay reagents for the determination of lipoprotein triglyceride. Lipoprotein FPLC utilized a single Gilson autoinjector and dual PCX setups, each consisting of a Pickering nitrogen pressurized reagent delivery system, a Gilson reagent pump, a Pickering CRX 400 PCX with a custom 2.8 ml knitted reaction coil, and a Gilson spectrophotometer set at 500 nm. The stream splitting method used one Gilson mobile phase pump, one Pharmacia Superose-6 10 × 300 mm column, and multiple switching valves for controlling effluent stream splitting and PCX reagent flow. The entire system was computer controlled by Gilson model 715-system controller software running under Microsoft Windows. The mobile phase was 154 mM NaCl, 1 mM EDTA, 0.02% sodium azide (pH 8.1) at a flow rate of 0.36 ml/min. The cholesterol-PCX reagent, Cholesterol/HP, was prepared at 2× concentration by adding deionized water. The triglyceride-PCX reagent, TG/GPO, was prepared at 2× concentration by adding two bottles of enzyme to one bottle of buffer. The sterile filtered PCX reagents were stored and used under nitrogen in the dark at 4°C at a flow rate of 0.06 ml/min. Preciset Cholesterol Calibrators, Standard Cholesterol CII, Standard Triglyceride G, Precitrol-N serum, fresh human plasma (EDTA), and unknown samples were serially diluted in mobile phase and placed in a 2°C refrigerated rack for FPLC sampling. Aliquots of 50–500 μl were injected automatically. Plasma standards were included to compare peak areas obtained via direct PCX injection (e.g., Superose-6 column bypassed), with peak areas obtained after column fractionation via FPLC-PCX and as an intra/inter-assay control sample, which was used before, interdispersed with, and after the unknown samples. The split-column eluent was combined with the cholesterol or triglyceride reagent in the PCX module forming a reaction product that was measured spectrophotometrically at 500 nm after an 11 min pass through the 37°C reaction coil. The Gilson 715 software performed the analysis of the spectrophotometer output. The peak start, peak end, and baselines were checked visually and adjusted as necessary for each standard, control plasma, and sample prior to integrating the peak areas. In the case of unresolved peaks, a perpendicular was drawn from each peak valley to the horizontal baseline for determining the peak area. A plot of peak area versus quantity of cholesterol injected was made for the cholesterol and triglyceride standards, and a least squares regression analysis was performed and then used to convert chromatographic peaks from unknown samples into cholesterol and triglyceride concentrations in units of mg/dl.

Studies using experimental animals

The Institutional Animal Care and Use Procedures Review Board approved all procedures using experimental animals. B6CBAF1J mice, mice hemizygous for the human genes encoding apoA-I, apoC-III, and CETP (huA1/CIII/CETP mice), or Sprague Dawley rats were given food (RMH3200 laboratory meal or AIN76A semipurified diet) and water ad libitum and treated orally at a volume of 1.0 ml/200 g body weight (rats) or 0.25 ml/25 g body weight (mice) with either an aqueous solution containing 0.5% methyl cellulose (vehicle) or an aqueous solution containing 0.5% methyl cellulose plus CP-346086. In experiments in which CP-346086 was administered in the feed, animals were fed powdered RMH3200 laboratory meal for 1 week prior to commencing administration of CP-346086 as a dietary supplement to powdered chow. In studies in which rates of hepatic and intestinal triglyceride secretion were determined after Tyloxapol treatment, Tyloxapol (1.0 ml; 125 mg/ml) was administered intravenously 60 min after oral administration of CP-346086. In experiments in which rates of intestinal triglyceride absorption were determined in the absence of Tyloxapol, [^{14}C]triolein mixed in Bioserve F0739 liquid diet [17.7% (w/w) fat] at a concentration of 0.5 μCi/ml was administered orally to fasted mice as a 0.5 ml aliquot.

Human subjects and study design

Protocols involving human subjects were approved by the Institutional Protocol Review Board of Pfizer, Inc., and by the Protocol Review Board of the Food and Drug Administration. Forty-eight healthy male adults, aged 18–45 years, participated in the randomized, double-blind, placebo-controlled, escalating single-dose study, and 30 healthy male adults, aged 18–45 years, partici-

pated in the 2 week randomized, double-blind, placebo-controlled, parallel-group multidose study. Inclusion criteria for both studies were body weight within 10% of ideal based on 1983 Metropolitan Life Insurance Height and Weight Tables (43), fasting plasma cholesterol levels in the upper 50% of the normal range for the American population based on age, sex, and race (e.g., 180–250 mg/dl) (1), fasting plasma triglyceride levels >150 mg/dl and <400 mg/dl, no evidence of clinically significant disease based on complete medical history, full physical examination, 12-lead ECG and clinical laboratory testing, and no prescription, OTC, or other drug usage for at least 2 weeks prior to beginning the study. Subjects were confined to the Clinical Research Unit and fed standardized meals (30% of daily calories from fat).

In the single-dose study, after randomization (six subjects/group; eight groups) each group was assigned a CP-346086 dose ranging in half-log intervals from 0.1 mg to 300 mg. At 7 AM on the day of study, after fasting for at least 8 h, four subjects from each group received their respective doses of CP-346086, and two subjects received placebo. Plasma samples were obtained at various times over the next 72 h for use in determining total and lipoprotein cholesterol and triglyceride levels by FPLC (see above).

In the multidose study, subjects were randomized with eight receiving 30 mg CP-346086 and six receiving placebo at 10 PM (bedtime) for 14 days. Plasma samples were obtained daily predose, frozen at −20°C, and forwarded to Medical Research Labs (39 Excelsiorlaan Zaventem, Brussels, Belgium) for determination of total and lipoprotein cholesterol and triglyceride levels using standardized autoanalyzer technology.

RESULTS

Inhibition of MTP-mediated lipid transfer by CP-346086

When donor and acceptor liposomes, prepared as described in Experimental Procedures, were incubated with varying concentrations of inhibitor, CP-346086 dose dependently inhibited human MTP-mediated triglyceride transfer between vesicles with a concentration giving an IC_{50} equal to 2.0 nM (Fig. 1). Similarly, when the radiolabeled triolein of donor vesicles was replaced by radiolabeled cholesteryl oleate, CP-346086 inhibited transfer of cholesteryl oleate between vesicles with an IC_{50} of 1.9 nM (Fig. 1), demonstrating the compound's ability to equally inhibit transfer of both neutral lipids. Similar IC_{50} values were also noted for inhibition of rat (1.7 nM) and mouse (6.7 nM) MTP activity.

Inhibition of neutral lipid transfer by CP-346086 was specific to MTP-mediated lipid transfer, however, as in an identical assay in which MTP was replaced by CETP, no inhibition of CETP-mediated lipid transfer was observed at concentrations of CP-346086 up to 10 μM (data not shown), indicating the compound's specificity for inhibition of MTP-mediated lipid transfer and demonstrating the lack of an effect of CP-346086 on the physicochemical properties of the donor and acceptor vesicles.

Inhibition of apoB and triglyceride secretion from Hep-G2 cells by CP-346086

When Hep-G2 cells were incubated with various concentrations of CP-346086 for 24 h at 37°C, and the culture media was evaluated for human apoB, apoA-I, and apoC-III concentrations by ELISA, CP-346086 inhibited Hep-G2 cell apoB secretion with an IC_{50} of 2.0 nM (**Fig. 2A**). Under these conditions, neither apoA-I secretion (Fig. 2A) nor apoC-III secretion (not shown) were inhibited by CP-

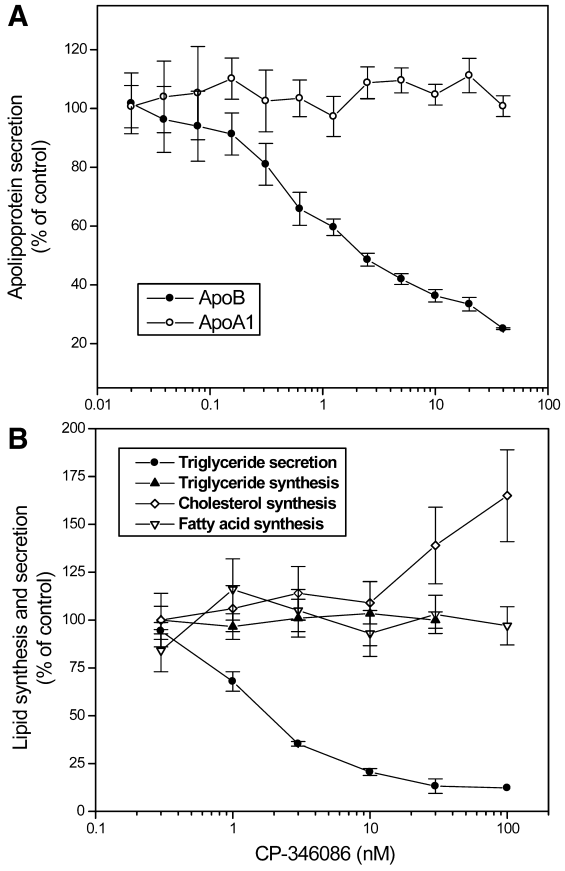

Fig. 2. Inhibition of Hep-G2 cell apolipoprotein B (apoB) and triglyceride secretion by CP-346086. A: Hep-G2 cells seeded and maintained in culture as described in Experimental Procedures were incubated for 24 h at 37°C in supplemented DMEM plus the indicated concentrations of CP-346086. After incubation, the medium was removed and assessed for apoB and apoA-I concentrations by apolipoprotein-specific ELISAs as outlined in Experimental Procedures. Data for apoB and apoA-I secretion are the mean of triplicate determinations ± SD and are expressed as a percentage of control apolipoprotein secretion as a function of CP-346086 concentration. B: Hep-G2 cells seeded and maintained in culture as described in Experimental Procedures were incubated for 6 h at 37°C in supplemented DMEM containing either 1% DMSO (control) or 1% DMSO containing CP-346086 sufficient to produce the indicated final inhibitor concentrations. For measurement of triglyceride synthesis and secretion, cells also received 50 μCi of [^{3}H]glycerol (closed symbols). After incubation, the media was removed, the cells were washed three times with PBS, and the secreted (media) and cellular triglycerides were quantitated as described in Experimental Procedures. For measurement of cholesterol and fatty acid synthesis, cells also received 4 μCi of [2-^{14}C]acetate (open symbols). After incubation, newly synthesized cholesterol and fatty acids were separated and quantitated as described in Experimental Procedures. Data for triglyceride secretion (closed circles), triglyceride synthesis (closed triangles), cholesterol synthesis (open diamonds), and fatty acid synthesis (open triangles) are the mean of triplicate determinations ± SD and are expressed as a percentage of control synthesis and secretion as a function of CP-346086 concentration.

346086 at concentrations up to 30 nM, indicating that the reduction in apoB secretion by CP-346086 was not a consequence of a generalized reduction in protein secretion.

CP-346086 also inhibited apoB secretion in Hep-G2 cells whose rate of apoB secretion had been stimulated by oleate administration. For example, when Hep-G2 cells were incubated in medium containing additional glucose (5.5 mM) and oleic acid (300 μM), apoB secretion was stimulated by 2.8-fold. Under these conditions, 3.0 nM CP-346086 inhibited oleate-stimulated apoB secretion by 88%.

Similarly, when Hep-G2 cells were incubated with various concentrations of CP-346086 and simultaneously treated for 6 h at 37°C with either [^{14}C]acetate, to assess synthesis of nascent fatty acids and cholesterol, or [^{3}H] glycerol, to assess synthesis and secretion of nascent triglycerides, CP-346086 inhibited nascent triglyceride secretion with an IC_{50} of 1.7 nM (Fig. 2B). Inhibition of triglyceride secretion occurred without concomitant inhibition of cholesterol, fatty acid, or triglyceride synthesis (Fig. 2B), or of cholesterol esterification at concentrations of up to 10 μM (data not shown). That the IC_{50} values for inhibition of apoB secretion and triglyceride secretion are essentially identical is consistent with the proposed role of MTP-mediated triglyceride transfer in both the formation and lipidation of apoB-containing lipoproteins within the ER lumen (19–26).

Inhibition of hepatic and intestinal triglyceride secretion by CP-346086

CP-346086 also inhibited hepatic and intestinal triglyceride secretion in experimental animals. This was demonstrated by measuring inhibition by CP-346086 of triglyceride accumulation in the plasma of animals treated with Tyloxapol (Triton WR1339), which prevents the catabolism of triglyceride-rich lipoproteins by lipoprotein lipase (44) and allows a linear increase in plasma triglyceride levels with respect to time to be observed. The rate of plasma triglyceride elevation after Tyloxapol treatment, therefore, reflects the triglyceride secretion rate from the liver and intestine. In the fasted state, where little lipoprotein production from the intestine occurs, hepatic VLDL production is the predominant source of nascent plasma triglycerides (24). In the postprandial state, however, substantial VLDL and chylomicron formation occurs in the intestine, and the pool of nascent plasma triglycerides arises from a combination of hepatic VLDL production and intestinal VLDL and chylomicron output.

When fasted rats were administered Tyloxapol intravenously and the rate of hepatic triglyceride accumulation in plasma was assessed, the rate of hepatic triglyceride secretion was estimated to be 7.5 mg/dl/min (180 mg/kg/h; **Fig. 3**, closed circles). Treatment of fasted rats with CP-346086 at a dose of 25 mg/kg, 60 min prior to Tyloxapol administration, resulted in an almost complete inhibition of triglyceride accumulation (Fig. 3, open circles), indicating that CP-346086 inhibits hepatic VLDL production. When Tyloxapol-treated rats were given an oral bolus of [^{3}H]triolein-labeled corn oil, radioactivity began appearing in the plasma within 25 min. Eighty minutes after bolus

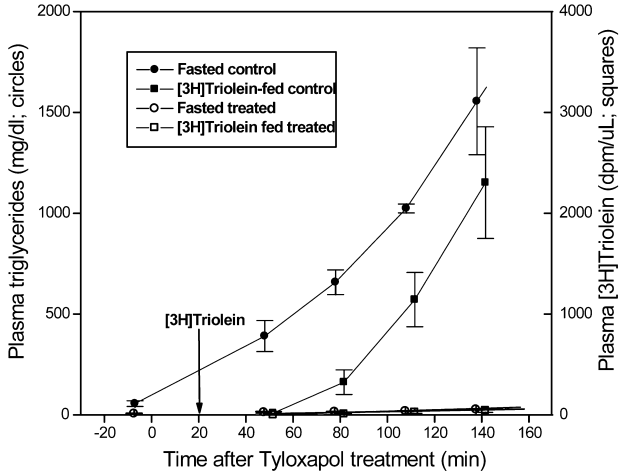

Fig. 3. Inhibition of hepatic and intestinal triglyceride secretion by CP-346086. Sprague Dawley rats given RMH3200 laboratory meal and water ad libitum were fasted overnight and then treated orally at a volume of 1.0 ml/200 g body weight with either 0.5% methyl cellulose (vehicle control) or an aqueous solution containing 0.5% methyl cellulose and 25 mg/kg CP-346086. Fifty-three minutes after treatment, plasma samples were obtained for the determination of baseline triglyceride levels. Sixty minutes after treatment (t = 0 min) all rats received an intravenous dose of Tyloxapol (1.0 ml; 125 mg/ml). Plasma was again sampled at 45, 80, 110, and 140 min. At t = 20 min, all rats received an oral bolus (0.25 ml) of corn oil containing [^{3}H]triolein (60 μCi/ml). Plasma samples were assessed for triglyceride concentration and for radioactivity comigrating with triglycerides after TLC separation as outlined in Experimental Procedures. Shown are plasma triglyceride levels for vehicle-treated control animals (filled circles) and CP-346086-treated animals (open circles), and plasma [^{3}H]triglycerides for vehicle-treated control animals (filled squares) and CP-346086-treated animals (open squares) as a function of time after Tyloxapol treatment. The data are the mean of triplicate determinations ± SD; n = 3.

administration, the rate of triglyceride accumulation in the plasma was estimated to be 15 mg/dl/min (360 mg/kg/h; Fig. 3, closed squares). The appearance of [^{3}H]triglycerides in the plasma after corn oil administration, indicative of intestinal triglyceride absorption, was also almost completely inhibited by treatment with 25 mg/kg CP-346086 (Fig. 3, open squares), indicating that CP-346086 also inhibits intestinal VLDL and chylomicron secretion. Similar effects of CP-346086 were also noted in mice, where the control triglyceride secretion rate was 5.4 mg/dl/min and where orally administered CP-346086 at doses of 10 mg/kg and 30 mg/kg decreased triglyceride secretion to 0.5 mg/dl/min and 0.1 mg/dl/min, respectively.

In fed animals, inhibition of intestinal triglyceride absorption by CP-346086 also resulted in an accumulation of radiolabeled triglycerides within the small intestine with a time course similar to that of reduced appearance in the plasma. For example, in untreated mice administered an oral bolus of [^{14}C]triolein, ~20% of the radiolabel remained associated with the small intestine 2 h after administration, implying that 80% of the administered radiolabel had been hydrolyzed within the intestinal lumen, taken up by enterocytes, reesterified with glycerol to form triglycerides, packaged into VLDL and chylomicron parti-

cles, and absorbed. After a single oral treatment with CP-346086, the radiolabel retained within the small intestine increased in a dose-responsive manner to ~70% (**Fig. 4A**), indicating that, at the highest doses evaluated, only 30% of the administered bolus had been absorbed within the 2 h period. The ED_{50} for intestinal triglyceride retention was 2.0 mg/kg (Fig. 4A), further indicating that CP-346086 inhibits intestinal triglyceride absorption.

Hepatic and intestinal triglyceride secretion was also inhibited when CP-346086 was administered intravenously, demonstrating that its actions in the intestine are not restricted to the intestinal lumen. For example, after treatment with Tyloxapol, fasted and corn oil-fed rats accumulated triglyceride in their plasma at rates of 4.5 mg/dl/min and 6.1 mg/dl/min, respectively. CP-346086, administered either orally at 25 mg/kg or intravenously at 20 mg/kg 60 min prior to Tyloxapol treatment, prevented both intestinal triglyceride secretion (corn oil-fed rats) and hepatic triglyceride secretion (fasted rats) by >95%. Taken together, these observations indicate that CP-346086 induces a decrease in triglyceride secretion rather than an increase in triglyceride clearance from plasma and that CP-346086 is pharmacologically active in both the liver and the small intestine.

Acute plasma triglyceride lowering by CP-346086

Inhibition of hepatic and intestinal triglyceride secretion by CP-346086 leads to a reduction in plasma triglyceride concentration that occurs within 90 min of treatment. When B6CBAF1J mice were treated orally with CP-346086 and plasma triglyceride levels were determined 90 min later, plasma triglycerides were reduced in a dose-dependent manner with a dose giving 30% triglyceride lowering (ED_{30}) of 1.6 mg/kg (Fig. 4B). Based on FPLC analysis of lipoprotein cholesterol and triglycerides, the acute reduction in plasma triglycerides was restricted to the VLDL and LDL fractions, with little reduction in HDL triglycerides. Plasma cholesterol levels were not acutely affected by CP-346086 in any species evaluated (data not shown).

Reduction in plasma total, VLDL, and LDL cholesterol and triglycerides after a 2 week treatment with CP-346086

When B6CBAF1J mice were administered CP-346086 by oral gavage at doses between 1 mg/kg and 100 mg/kg once daily for 2 weeks, and plasma was collected and analyzed for lipoprotein cholesterol and triglycerides by FPLC, LDL cholesterol, VLDL cholesterol, and triglycerides were all reduced in a dose-dependent manner, with ED_{50} values of 5.4, 2.1, and 2.9 mg/kg/day, respectively (**Fig. 5**). A markedly smaller percentage reduction in HDL cholesterol also occurred at the upper end of the dosing range (Fig. 5). For the LDL and HDL subfractions, there was a shift toward the smaller LDL and HDL size classes with a reduction in the proportion of larger particles.

Triglyceride accumulation in the liver and intestine of animals treated with CP-346086

Inhibition of hepatic and intestinal triglyceride secretion by CP-346086 results in an accumulation of triglycer-

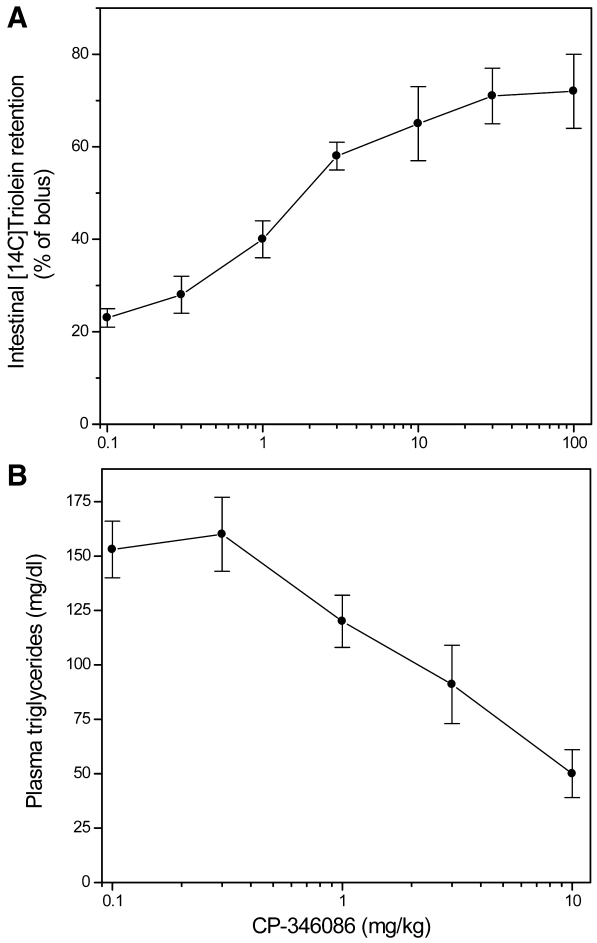

Fig. 4. Acute triglyceride absorption inhibition and plasma triglyceride lowering by CP-346086. A: Forty male B6CBAF1J mice given RMH3200 laboratory meal and water ad libitum were separated into eight groups of five animals each and fasted overnight. The following morning, animals were treated orally at a volume of 0.25 ml/25 g body weight with either an aqueous solution containing 0.5% methyl cellulose (vehicle control) or an aqueous solution containing 0.5% methyl cellulose and the indicated amounts of CP-346086, and were then immediately administered a 0.5 ml oral bolus of Bioserve F0739 liquid diet containing 0.25 μCi [^{14}C]triolein. Two hours after treatment, animals were killed and their small intestines were removed, placed in 15 ml glass conical tubes without removal of the intestinal contents, and saponified for 3 h at 75°C in 3.0 ml of 2.5 M KOH. After saponification, 200 μl aliquots of the resulting mixtures were transferred to 20 ml liquid scintillation vials, decolorized for 30 min by addition of 200 μl of 30% H_2O_2, neutralized by addition of 200 μl of 3 M HCl, mixed with 10 ml of ReadySafe liquid scintillation fluid, and assessed for radioactivity in a Beckman LS6500 liquid scintillation counter. B: Thirty male B6CBAF1J mice given RMH3200 laboratory meal and water ad libitum were separated into six groups of five animals each and treated orally at a volume of 0.25 ml/25 g body weight with either an aqueous solution containing 0.5% methyl cellulose (vehicle control) or an aqueous solution containing 0.5% methyl cellulose and the indicated amounts of CP-346086. Plasma samples were obtained 90 min later and assessed for triglyceride concentration as outlined in Experimental Procedures. Shown are the amount of [^{14}C]triolein remaining within the small intestinal tissue 2 h after bolus administration ± SD (A) and mean plasma triglyceride concentrations ± SD (B) as a function of dose of CP-346086.

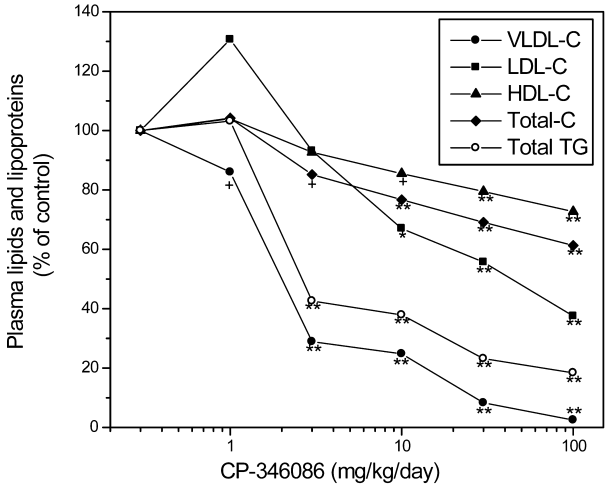

Fig. 5. Lipid and lipoprotein cholesterol lowering in mice treated for 14 days with CP-346086. Thirty male B6CBAF1J mice given RMH3200 laboratory meal and water ad libitum were separated into six groups of five animals each and treated orally at a volume of 0.25 ml/25 g body weight once daily for 14 days with either an aqueous solution containing 0.5% methyl cellulose (vehicle control) or an aqueous solution containing 0.5% methyl cellulose and the indicated amounts of CP-346086. Plasma samples were obtained on the final day of the study, 90 min after the last dose, fractionated into the major lipoprotein subfractions by fast protein liquid chromatography (FPLC), and analyzed for cholesterol and triglyceride content using a flow-through postcolumn reactor as outlined in Experimental Procedures. Shown are the percentage of control plasma cholesterol, triglycerides, VLDL cholesterol, LDL cholesterol, and HDL cholesterol as a function of CP-346086 dose. Control lipids and lipoproteins were as follows: total cholesterol, 103 mg/dl; triglycerides, 190 mg/dl; VLDL cholesterol, 12.1 mg/dl; LDL cholesterol, 8.8 mg/dl; HDL cholesterol, 82 mg/dl. Statistical significance assessed relative to placebo-treated animals was as follows: + $P < 0.05$; * $P < 0.01$; ** $P < 0.001$.

TABLE 1. Triglyceride accumulation in liver and intestine after multidose treatment with CP-346086

Treatment Regimen	Intestinal Triglyceride Content			Hepatic Triglyceride Content		
	mg/g	*% of control*	*P*[a]	*mg/g*	*% of control*	*P*[a]
2 Week in-feed admin. dose (% of diet)						
0	0.80	—	—	9.3	—	—
0.0015	0.97	121	ns	7.7	83	ns
0.005	1.14	142	ns	6.7	72	ns
0.015	0.62	78	ns	8.7	94	ns
0.05	1.61	201	0.0434	16.6	178	0.0021
0.15	2.87	358	<0.0001	26.5	285	<0.0001
2 Week oral admin. dose (mg/kg/day)						
0	1.11	—	—	6.1	—	—
1	0.83	75	ns	8.4	138	ns
3	1.62	146	ns	10.2	166	0.0252
10	0.42	38	0.0366	12.7	208	0.0007
30	0.41	37	ns	13.6	222	0.0002
100	0.59	53	0.0346	14.8	242	<0.0001

ns, not significant.

Sixty male B6CBAF1J mice given water ad libitum were separated into 12 groups of five animals each. Six groups were fed RMH3200 laboratory meal ad libitum and treated orally at a volume of 0.25 ml/25 g body weight once daily at 9 AM, 2 h after the onset of lighting (at the nadir of their feeding cycle), for 14 days with either an aqueous solution containing 0.5% methyl cellulose (vehicle control) or an aqueous solution containing 0.5% methyl cellulose and the indicated amounts of CP-346086. The remaining six groups were fed powdered RMH3200 laboratory meal for seven days and then were fed powdered meal containing the indicated amounts of CP-346086 for 14 days. Based on average body weights and food consumption, 0.15% (w/w), for example, is equivalent to a daily dose of 30 mg/kg. On the final day of the study, 90 min after the last dose, hepatic and small intestinal tissue samples were homogenized, extracted, and assessed for tissue triglycerides as outlined in Experimental Procedures.

[a] *P* values versus control were determined by Fisher's PLSD after one-way ANOVA.

ides within both tissues. As shown in **Table 1**, when CP-346086 was given to B6CBAF1J mice periprandially through administration as a dietary admix for 2 weeks at doses ranging between 0.0015% and 0.15% of the diet (equivalent to 0.3 mg/kg/day to 30 mg/kg/day based on body weights and daily food consumption rates), both hepatic and intestinal triglycerides increased in a dose-dependent manner, with hepatic triglycerides increased by up to 2.9-fold and intestinal triglycerides increased by up to 3.6-fold. Tissue triglyceride accumulation occurred concomitant with reductions in plasma lipoprotein cholesterol and triglyceride levels such that, for example, at a dose of 0.15% CP-346086 in the diet, total cholesterol, VLDL cholesterol, and LDL cholesterol were reduced by 47%, 35%, and 69%, respectively, and hepatic and intestinal triglycerides were increased by 185% and 256%.

When B6CBAF1J mice were given CP-346086 orally at the nadir of their feeding cycle (2 h after the onset of room lighting) for 2 weeks at daily doses between 1 mg/kg and 100 mg/kg, hepatic triglycerides were likewise dose-dependently increased by up to 2.4-fold (Table 1) in a manner that again paralleled lipoprotein cholesterol and triglyceride reductions (Table 1, Fig. 5). For example, at a dose of 30 mg/kg/day, total cholesterol, VLDL cholesterol, LDL cholesterol, and total triglycerides were reduced by 31%, 92%, 44%, and 82%, respectively, where hepatic triglyceride concentrations were increased by 142%. Hepatic triglyceride accumulation was apparent even at doses for which plasma lipid and lipoprotein levels were only modestly reduced (Table 1, Fig. 5) and increased in parallel with these reductions at increasing doses of CP-346086 (Table 1, Fig. 5). However, in contrast to administration of CP-346086 in the fed state, in the absence of food in the intestine, intestinal triglycerides showed no systematic elevations with increased dose administration, but rather were lower than control animals at all doses evaluated (Table 1). This difference in tissue accumulation between the intestine and liver in the fasting state is not unexpected, because the liver is continually forming and secreting triglyceride-rich lipoproteins in both the fed and fasted state, whereas the intestine primarily secretes triglyceride-rich lipoproteins that are prepared using triglycerides formed from free fatty acids obtained from dietary sources.

The accumulation of triglycerides within the liver and intestines of animals treated with CP-346086 was further exacerbated through supplementation of the diet with ad-

ditional fat. For example, when B6CBAF1J mice were fed semipurified diets containing either 13% of calories as fat (AIN76A; standard diet) or 43% of calories as fat (modified AIN76A in which milk fat was substituted for the sucrose; high-fat diet) and were treated orally for 4 days with CP-346086 (25 mg/kg/day), equal reductions in plasma VLDL cholesterol (87% vs. 90%), LDL cholesterol (81% vs. 74%), and total triglycerides (69% vs. 68%) were noted. Increases in hepatic and intestinal triglyceride concentrations after treatment with CP-346086, however, were markedly greater in animals fed the high-fat diet. Indeed, whereas hepatic and intestinal triglyceride levels were increased by 2.0-fold and 2.3-fold in animals fed the standard diet, hepatic and intestinal triglyceride increases of 3.5-fold and 4.7-fold were observed in animals receiving the high-fat diet.

After treatment of rats with efficacious doses of CP-346086, accumulation of cholesterol in the liver and intestine also occurred, but to a lesser degree than the accumulation of triglycerides. For example, treatment of male (female) Sprague Dawley rats with CP-346086 for 90 days by oral gavage at a dose of 20 mg/kg resulted in a 66% (62%) reduction in plasma triglycerides, a 32% (29%) reduction in plasma cholesterol, a 5.9-fold (7.0-fold) increase in hepatic triglycerides, and a 2.5-fold (2.3-fold) increase in hepatic cholesterol. Similarly, male (female) beagle dogs, treated with CP-346086 at the time of feeding for 90 days at a dose of 12.5 mg/kg, exhibited a 54% (71%) reduction in plasma triglycerides, a 79% (60%) reduction in plasma cholesterol, a 3.4-fold (4.6-fold) increase in hepatic triglycerides, and a 17.7-fold (13.7-fold) increase in intestinal triglyceride levels, with no change in either hepatic or intestinal cholesterol levels.

Plasma lipoprotein reduction in hypertriglyceridemic mice after a 2 week treatment with CP-346086

Mice that are hemizygous for the human genes encoding apoA-I, apoC-III, and CETP (huA1/CIII/CETP mice) present with a dyslipidemia characterized by dramatically elevated plasma triglycerides, elevated VLDL and chylomicron levels, and markedly reduced HDL cholesterol levels (45). The lipid and lipoprotein profiles of this mouse are unique among rodents in that the primary carrier of plasma cholesterol is an apoB-containing lipoprotein. Indeed, huA1/CIII/CETP mice carry >90% of their cholesterol in VLDL (**Table 2**). When huA1/CIII/CETP mice were treated with CP-346086 orally for 2 weeks at daily doses between 1 mg/kg and 100 mg/kg, plasma total cholesterol, triglycerides, VLDL cholesterol, and LDL cholesterol were all reduced in a dose-dependent manner with maximal reductions of 64%, 63%, 66%, and 61%, respectively (Table 2). The ratio of plasma triglyceride to VLDL cholesterol remained constant at 17:1, consistent with a decrease in particle number rather than particle size. This ratio is the same as that seen in nontransgenic mice, consistent with the apoC-III-mediated defect in triglyceride clearance in these animals (45). HDL cholesterol was not altered in these animals by treatment with CP-346086.

TABLE 2. Lipid and lipoprotein cholesterol lowering in huAI/CIII/CETP mice treated for 14 days with CP-346086

CP-346086 Dose	Plasma Lipid and Lipoprotein Parameters									
	VLDL-Cholesterol		LDL-Cholesterol		HDL-Cholesterol		Total Cholesterol		Total Triglycerides	
(mg/kg/day)	*mg/dl*	*% cont.*	*mg/dl*	*% cont.*	*mg/dl*	*% cont.*	*mg/dl*	*% cont.*	*mg/dl*	*% cont.*
0	401	—	15.7	—	13.5	—	430	—	6,750	—
1	275[b]	69	12.6[a]	80	13.7	101	301[b]	70	4,777[a]	71
3	199[c]	50	10.8[b]	69	14.0	104	224[c]	52	2,833[c]	42
10	193[c]	48	9.3[c]	59	14.7	109	217[c]	50	3,373[c]	50
30	136[c]	34	6.6[c]	42	14.1	104	156[c]	36	3,188[c]	47
100	146[c]	36	6.2[c]	39	12.0	89	164[c]	38	2,475[c]	37

CETP, cholesteryl ester transfer protein.

Thirty hypertriglyceridemic male huA1/CIII/CETP transgenic mice given RMH3200 laboratory meal and water ad libitum were separated into six groups of five animals each and treated orally at a volume of 0.25 ml/25 g body weight once daily for 14 days with either an aqueous solution containing 0.5% methyl cellulose (vehicle control) or an aqueous solution containing 0.5% methyl cellulose and the indicated amounts of CP-346086. Plasma samples were obtained on the final day of study, 90 min after the last dose, fractionated into the major lipoprotein subfractions by fast protein liquid chromatography, and analyzed for cholesterol and triglyceride content using a flow-through postcolumn reactor as outlined in Experimental Procedures. *P* values versus control were determined by Fisher's PLSD after one-way ANOVA.

[a] $P < 0.01$.
[b] $P < 0.001$.
[c] $P < 0.0001$.

Time- and dose-dependent lipid and lipoprotein cholesterol lowering after single oral dose administration in humans

To demonstrate the acute efficacy of CP-346086-mediated MTP inhibition in lowering plasma lipids and lipoproteins in humans, healthy male adults were treated orally with CP-346086 at doses ranging from 0 mg (placebo) to 300 mg, as outlined in Experimental Procedures. As shown in **Fig. 6**, when plasma triglyceride levels were measured at varying intervals of up to 24 h after dose administration, plasma triglycerides were reduced in a time-dependent and dose-dependent manner, with an ED_{50} of 10 mg and a maximal reduction of ~80% noted at the highest dose administered. At the lowest doses administered, triglyceride levels rebounded to greater than predose levels (Fig. 6), presumably as a consequence of the release into the plasma of triglycerides accumulated in the liver in response to MTP inhibition, as the plasma concentrations of CP-346086 were reduced to below efficacious levels. At higher doses, where plasma drug levels did not fall below efficacious concentrations over the entire 24 h duration, a similar rebound was not observed (Fig. 6).

When plasma samples obtained at varying times after single-dose administration across the entire concentration range were subjected to analysis for lipoprotein cholesterol and triglycerides by FPLC, the analysis revealed that plasma VLDL cholesterol levels were also reduced in both a time-dependent and dose-related manner that closely paralleled plasma triglyceride reductions, exhibiting an ED_{50} for VLDL cholesterol reduction of 3 mg and a maximal reduction of 87% at the 100 mg dose. For example,

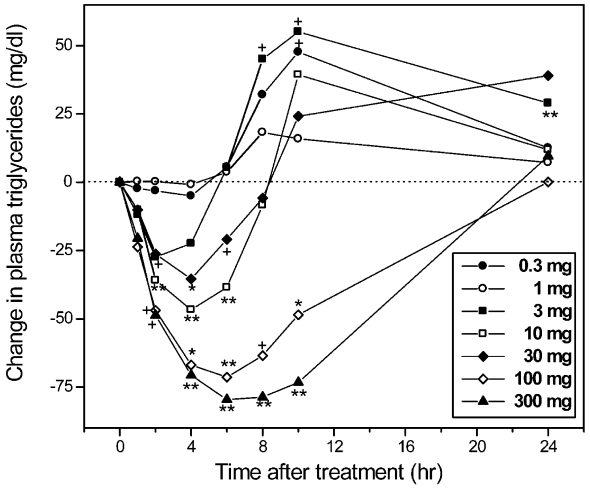

Fig. 6. Time- and dose-dependent triglyceride lowering after single-dose administration in humans. Forty-eight healthy male adults, aged 18–45 years and meeting the selection criteria outlined in Experimental Procedures, were randomized to eight groups of six subjects each and assigned in a double-blind manner to CP-346086 dose groups ranging from 0.1 mg to 300 mg. At 7 AM on the day of the study, after a meal-free interval of at least 8 h, plasma samples were obtained for assessment of baseline lipid and lipoprotein concentration, then four subjects from each group received their respective oral doses of CP-346086 and two subjects received a placebo. Meals were administered at 4.5 h and 10.5 h after dose administration. Plasma samples were obtained at the indicated times after treatment and assessed for triglyceride concentration as outlined in Experimental Procedures. Shown are the average changes in plasma triglycerides relative to individual predose baseline concentrations as a function of time after treatment for each dose of CP-346086. Predose baseline triglycerides averaged 142.7 mg/dl. No significant changes in plasma triglyceride levels were noted in placebo-treated subjects for the entire duration of the study. Statistical significance assessed relative to baseline triglyceride levels was as follows: + $P < 0.05$; * $P < 0.02$; ** $P < 0.01$.

Fig. 7A shows a dose-responsive reduction of both VLDL cholesterol and triglycerides 4 h after treatment, whereas Fig. 7B shows the time-dependent reduction and rebound of plasma VLDL cholesterol and triglycerides after a 30 mg dose. Consistent with the data obtained from studies in experimental animals (see above), total cholesterol, LDL cholesterol, and HDL cholesterol levels were not reduced after a single treatment with CP-346086 (Fig. 7). However, for the LDL and HDL subfractions, there was a shift toward the smaller LDL and HDL size classes with a reduction in the proportion of larger particles.

Lipid and lipoprotein cholesterol lowering in subjects treated for 14 days with 30 mg/day CP-346086 versus placebo

The most common adverse events reported in a dose-dependent manner in the single-dose study (see above) were gastrointestinal disturbances such as flatulence, abdominal pain, diarrhea, nausea, and an impulse to defecate. Indeed, in the presence of a high-fat meal, all subjects receiving the single 100 mg dose experienced mild to moderate diarrhea and abdominal discomfort, flatulence, and nausea.

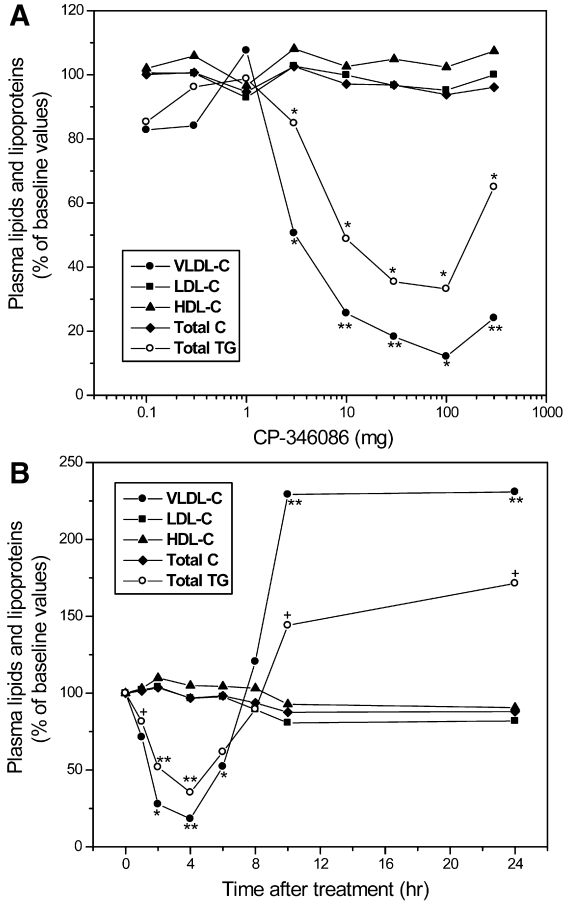

Fig. 7. Time- and dose-dependent lipid and lipoprotein cholesterol lowering after single-dose administration in humans. Plasma samples obtained as outlined in the legend to Fig. 6 were fractionated into the major lipoprotein subfractions by FPLC and analyzed for cholesterol and triglyceride content using a flow-through post-column reactor as outlined in Experimental Procedures. Shown as examples are the mean percentage of individual baseline plasma cholesterol, triglycerides, VLDL cholesterol, LDL cholesterol, and HDL cholesterol as a function of CP-346086 dose 4 h after treatment (A) and time after treatment with 30 mg of CP-346086 (B). Mean baseline lipids and lipoproteins were as follows: total cholesterol, 192.2 mg/dl; triglycerides, 142.7 mg/dl; VLDL cholesterol, 11.9 mg/dl; LDL cholesterol, 131.4 mg/dl; HDL cholesterol, 48.8 mg/dl. Average standard deviations were as follows: total cholesterol, ±4.6%; triglycerides, ±11.7%; LDL cholesterol, ±4.3%; HDL cholesterol, ±5.1%; VLDL cholesterol, ±19.5%. No significant changes in plasma lipid and lipoprotein levels were noted in placebo-treated subjects for the entire duration of the study. Statistical significance assessed relative to baseline lipid and lipoprotein levels was as follows: + $P < 0.05$; * $P < 0.02$; ** $P < 0.01$.

As studies in experimental animals (see previous) demonstrated that intestinal triglyceride accumulation and also, presumably, reported gastrointestinal disturbances could be avoided by administration of CP-346086 away from meals, a 2 week multi-dose study was designed with treatment at 10 PM (bedtime), a minimum of 4 h after the final meal of the day. Therefore, to demonstrate the chronic effectiveness of CP-346086-mediated MTP inhibition in lowering plasma lipids and lipoproteins in humans, healthy adult males were treated orally with 30 mg

CP-346086 or placebo once daily at 10 PM for 14 days as outlined in Experimental Procedures. Under this dosing regimen, the only gastrointestinal symptoms occasionally reported were mild diarrhea and flatulence, and these were self limiting.

As shown in **Fig. 8A**, when daily plasma samples were subjected to analysis for lipoprotein cholesterol and triglycerides and for apoB concentration, the analysis revealed that total cholesterol, LDL cholesterol, HDL cholesterol, total triglycerides, and apoB levels were unaffected by treatment with placebo. By contrast, in the treated subjects total cholesterol, LDL cholesterol, and apoB levels were reduced in a dose-dependent manner with maximal reductions of 47%, 68%, and 52% relative to either individual baseline values or placebo controls noted by the end of the study (Fig. 8B). HDL cholesterol levels, however, remained within 10% of prestudy values (Fig. 8).

Plasma triglycerides were also reduced by up to 75% in a time-dependent manner (**Fig. 9**) reflective of the time-course demonstrated in the single-dose study (see above), such that maximal plasma triglyceride lowering occurred between 4 and 8 h after dose administration, with plasma triglycerides returning to baseline just prior to the next treatment (Fig. 9). As a consequence, daily fasting plasma triglyceride levels, measured just prior to the next dose administration, remained relatively unchanged throughout the course of the study (Fig. 8B).

Serum vitamin A levels were unchanged by treatment with CP-346086, consistent with a minimal effect of CP-346086-mediated MTP inhibition on whole-body vitamin A status when administered away from meals. Serum vitamin E levels were decreased from predose levels by 43% ± 10% (SD; n = 8) by the end of the study. The reduction in vitamin E levels closely paralleled the reductions in total cholesterol (47%) and LDL cholesterol (68%) levels, with no significant change in either vitamin E-to-total cholesterol or vitamin E-to-LDL ratios, consistent with a minimal effect of CP-346086-mediated MTP inhibition on whole-body vitamin E status during the course of study.

An isolated elevation in alanine aminotransferase (ALT) was reported after 2 weeks of dosing in one subject administered placebo and in three subjects receiving the 30 mg evening dose of CP-346086. In two of these subjects, the

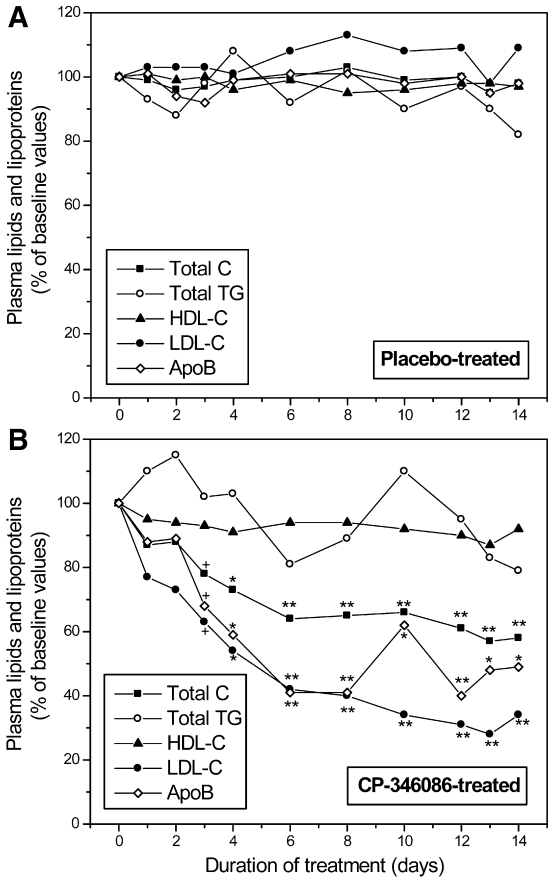

Fig. 8. Lipid and lipoprotein cholesterol lowering in subjects treated for 14 days with 30 mg/day CP-346086 versus placebo. Fourteen healthy male adults, aged 18–45 years and meeting the selection criteria outlined in Experimental Procedures, were randomized in a double-blind manner, with eight receiving a 30 mg daily oral dose of CP-346086 and six receiving placebo at 10 PM (bedtime) for 14 days. Plasma samples were obtained daily just prior to dose administration, with the sample obtained prior to the first dose used for determining baseline plasma lipid and lipoprotein levels. Plasma samples were analyzed for lipoprotein cholesterol and triglycerides and for apoB concentration as outlined in Experimental Procedures. Shown are the mean percentage (relative to baseline values) of individual plasma cholesterol, triglycerides, VLDL cholesterol, LDL cholesterol, and HDL cholesterol as a function of duration of treatment with 30 mg of CP-346086 for the placebo-treated (A) and CP-346086-treated (B) groups. Mean baseline lipids and lipoproteins were as follows: total cholesterol, 196 mg/dl; triglycerides, 172 mg/dl; LDL cholesterol, 109 mg/dl; HDL cholesterol, 52.4 mg/dl. Average standard deviations were as follows: total cholesterol, ±6.8%; triglycerides, ±21.4%; LDL cholesterol, ±15.0%; HDL cholesterol, ±10.1%; apoB, ±9.3%. Statistical significance assessed relative to baseline lipid and lipoprotein levels was as follows: + $P < 0.05$; * $P < 0.01$; ** $P < 0.001$.

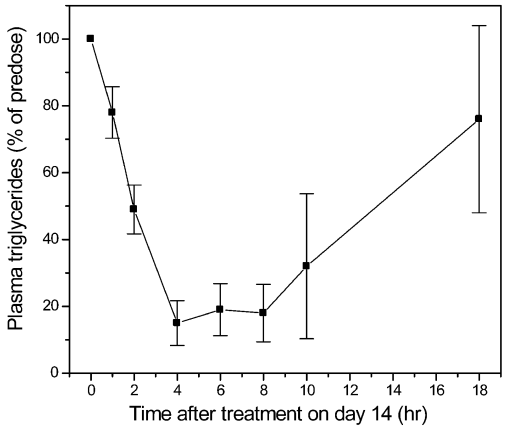

Fig. 9. Intraday plasma triglyceride levels after 2 week administration of CP-346086 to humans. On day 14, the final day of the study outlined in the legend to Fig. 8, plasma samples were collected at various times up to 18 h after administration of CP-346086 and assessed for triglyceride concentration as outlined in Experimental Procedures. Shown is the mean percentage of predose plasma triglyceride concentrations ±SD as a function of time after administration of the day 14, 30 mg dose of CP-346086. Predose plasma triglyceride concentration averaged 203 mg/dl.

ALT elevation was approximately two times the upper limit of normal, and in the third subject, the ALT elevation was six times the upper limit of normal. In almost all of these subjects, ALT levels returned to within normal values within 7 days of discontinued dosing. The other five subjects treated with CP-346086 did not show elevations in ALT during the course of the study. No other liver enzymes were elevated in any of the placebo- or CP-346086-treated subjects, and none of the treated subjects complained of any symptoms.

MRI of the liver indicated no significant reduction in signal intensity after 14 days of evening treatment with 30 mg CP-346086 compared with predose, indicating that fatty infiltration into the liver was minimal, or not quantifiable at the doses evaluated.

DISCUSSION

In this report, we describe a potent inhibitor of MTP-mediated neutral lipid transfer that reduces hepatic and intestinal triglyceride-rich lipoprotein secretion and thereby markedly lowers plasma levels of the apoB-containing lipoproteins VLDL and LDL in experimental animals and in humans without significantly affecting plasma HDL levels. The actions of CP-346086 are specific for MTP-mediated neutral lipid transfer, because the compound neither affects the activity of other neutral lipid transfer proteins (e.g., CETP) nor alters the intracellular production of cholesterol, fatty acids, or triglycerides. Inhibition of triglyceride secretion by CP-346086 occurs as a consequence of a reduction in the rate of apoB-containing particle secretion and not through a change in particle size or composition.

As anticipated from the mechanism of action, inhibition of triglyceride-rich lipoprotein secretion and subsequent plasma LDL and VLDL cholesterol reductions occur concomitant with an increase in intrahepatic and intraintestinal triglyceride concentrations. The latter, which presumably is the cause of the gastrointestinal disturbances observed when CP-346086 is administered together with a fatty meal, can be attenuated by administering the compound away from food.

The mechanism of lipid-lowering action of CP-346086 in experimental animals and in humans, that of inhibition of VLDL particle secretion from the liver and VLDL and chylomicron secretion from the intestine after a meal, is distinctly different from the primary mechanisms of action of the currently marketed lipid-lowering agents such as the statins, which inhibit cholesterol production in the liver (46), the fibrates, which stimulate both lipoprotein lipase-mediated triglyceride clearance from the plasma and intracellular fatty acid oxidation (46), and the bile acid sequestrants, which interfere with the enterohepatic recirculation of bile acids (46). Thus, CP-346086 and related MTP inhibitors should be effective in combination with these agents. A combination with fibrates, and indeed with the more potent peroxisome proliferator-activated receptor α (PPARα) agonists in development (46), might be especially attractive, as the PPAR agonist-mediated increases in intrahepatocellular fatty acid oxidation could potentially help in the removal of the triglycerides retained in the liver after MTP inhibition. The increased removal of plasma triglycerides by the fibrates, in combination with the reduced secretion of nascent triglyceride-rich lipoproteins by an MTP inhibitor, could potentially lead to an even greater magnitude of VLDL- and LDL-lowering efficacy.

While the ability of CP-346086 and other MTP inhibitors to reduce atherosclerosis and favorably affect the morbidity and mortality of CHD has not yet been demonstrated in clinical trials, a variety of studies in experimental animals have recently been reported that suggest that MTP inhibition may favorably affect these disease manifestations. For example, in apoE knockout mice fed a Western-type diet, treatment for 14 weeks with efficacious concentrations of the MTP inhibitor, BAY 13-9952, significantly reduced atherosclerotic lesions and lesion lipid content (47–49). In this study, the average cross-sectional plaque areas of the aortic root, determined by computer-aided morphometric analysis, were reduced by up to 93% and the lipid content reduced by up to 99% (47–49). This reduction in lesion development and lesion lipid content translated to a dose-related increase in survival time such that, while only 1 out of 25 untreated mice was still alive after 18 months, up to 24 out of 25 mice were still alive after 18 months of treatment (47, 49). In addition, BAY 13-9952 also reportedly reduced control levels of fatty streak formation in New Zealand White rabbits fed a 0.5% cholesterol-enriched diet for 3 months (50).

Furthermore, because there is increasing evidence that delayed clearance of postprandial lipemia is an important contributing factor to the development of atherosclerosis (51), the potential exists for MTP inhibitor-mediated attenuation of postprandial lipemia to further contribute to reducing the development and progression of atherosclerosis. Indeed, in studies in rats and dogs, a Novartis MTP inhibitor (compound 8aR) (52) administered just prior to an oral fat load effectively prevented the elevation of plasma triglycerides in response to the fat bolus (52), providing further evidence that MTP inhibition in the intestinal mucosa can effectively attenuate postprandial lipemia. In a clinical study in which BAY 13-9952 was administered to healthy volunteers for 4 weeks at 160 mg/day, a dose that decreased total cholesterol, LDL cholesterol, and triglycerides by 45%, 55%, and 29%, respectively (53), the postprandial hyperlipemia following a high-fat meal was almost totally prevented (47, 54).

While the potential benefits of MTP inhibition-mediated plasma LDL and VLDL lowering and postprandial lipemia reduction to the attenuation of atherosclerosis progression and of CHD incidences are appealing, several major developmental issues confront the advancement of an MTP inhibitor. These issues include those related to the potential for adverse effects associated with mechanism-based fat malabsorption (steatorrhea), fat-soluble vitamin (e.g., vitamin A, vitamin E, vitamin K) malabsorption, and fat accumulation in the liver and intestine (31), all of which have been observed preclinically with various MTP

inhibitors (see below). Indeed, such adverse findings are also observed in patients with abetalipoproteinemia (29, 31) and thus have the potential to occur in clinical trials with MTP inhibition, especially if the degree of inhibition is marked or the exposure to inhibitor is prolonged. In general, however, the preclinical adverse effect profiles of MTP inhibitors have been promising and related mainly to gastrointestinal disturbances, presumably associated with steatorrhea and/or intestinal mucosal lipid accumulation and liver function abnormalities, estimated by elevations of plasma aspartate aminotransferase (AST) and ALT levels, which are presumed to be related to hepatic lipid accumulation.

For example, in clinical studies with a variety of MTP inhibitors, gastrointestinal toleration issues associated with steatorrhea were observed in phase I multidose studies (47, 50, 53), particularly when the inhibitors were administered together with meals. Indeed, in clinical trials, efficacious doses of BAY 13-9952 resulted in a high incidence of digestive adverse effects (mainly diarrhea) (47, 53). By using a regimen of dosing away from meals, however, it is possible to circumvent these toleration issues, as was noted with CP-346086 (this report).

Also, although vitamin deficiencies are a hallmark of abetalipoproteinemia (29, 31), to date no evidence for vitamin deficiencies has been noted in subjects treated with MTP inhibitors. For example, 4 week clinical studies with efficacious concentrations of BAY 13-9952 have demonstrated only minor changes in circulating vitamin A levels (55). Likewise, no changes in serum vitamin A levels were noted after 2 weeks of treatment with efficacious doses of CP-346086 (this report). Changes in vitamin E levels after treatment with either BAY 13-9952 (55) or CP-346086 (this report) paralleled LDL cholesterol lowering with no significant changes in vitamin E/LDL or vitamin E/HDL ratios, consistent with a minimal effect of MTP inhibition on whole-body vitamin E status in these studies.

Increases in liver and intestinal triglyceride content that are correlated with plasma lipid lowering appear to be a consistent finding among MTP inhibitors. For example, in addition to the increases in both liver and intestinal triglycerides observed when efficacious doses of CP-346086 were administered to mice, rats, and dogs in close temporal proximity to eating (this report), a Novartis MTP inhibitor (compound 8aR) (52) increased rat hepatic cholesterol and triglyceride content in a dose-dependent manner that was correlated with plasma lipid lowering and was reversed after treatment withdrawal (52), efficacious doses of BMS 201038-induced fat accumulation in the enterocytes and liver of hamsters that returned to control levels after the termination of treatment (24), and efficacious concentrations of BAY 13-9952-induced accumulation of both triglycerides and cholesterol in the livers of Watanabe heritable hyperlipidemic (WHHL) rabbits after 4 weeks of treatment (56) and in the small intestines of apoE knockout mice (47, 49).

It is important to note, however, that although hepatic lipid levels were markedly increased with treatment, hamsters administered efficacious doses of BMS 201038 for 3 weeks showed minimal change in liver weight, and plasma ALT and AST levels did not rise significantly during the treatment period (24). Similarly, WHHL rabbits treated with efficacious doses of BMS 201038 for 2 weeks showed no alteration in plasma AST or ALT levels, despite normalization of plasma lipids (24). Therefore, a causal relationship between hepatic lipid accumulation and serum transaminase elevations still remains to be demonstrated.

While the impact of MTP inhibition-mediated fat accumulation in the liver and intestine remains to be evaluated in the clinical setting, particularly in patients with impaired liver function and in patients suffering from diabetes or gastrointestinal abnormalities, it is important to note that plasma ALT and AST levels were increased three times above normal in 12–27% of the patients receiving 80 mg/day and 160 mg/day doses of BAY 13-9952 (53). Similar AST and ALT elevations, of a magnitude sufficient to halt development of BMS 201038, were also reported (57). Whether these transaminase elevations are a consequence of hepatic lipid accumulation or are structure specific still remains to be determined experimentally. It is interesting to note, however, that the isolated ALT, but not AST, elevations noted in three of eight subjects treated with CP-346086 (this report) were not associated with measurable increases in hepatic lipid levels.

Thus, although significant and encouraging lipid-lowering efficacy of CP-346086 has been demonstrated in clinical studies (this report), the potential for mechanism-related adverse effects, as outlined in this article, needs to be thoroughly evaluated. While those related to steatorrhea or fat-soluble vitamin malabsorption could be readily corrected by either drug administration away from meals (e.g., PM dosing) or by coadministration of a vitamin admix, the potential for adverse effects relating to hepatic lipid accumulation in a clinical setting needs to be further defined in order to assess its true potential for chronic use.

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