Dual-action hypoglycemic and hypocholesterolemic agents that inhibit glycolgen phosphorylase and lanosterol demethylase


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Abstract Diabetic dyslipidemia requires simultaneous treatment with hypoglycemic agents and lipid-modulating drugs. We recently described glycolgen phosphorylase inhibitors that reduce glycogenolysis in cells and lower plasma glucose in ob/ob mice (J. Med. Chem., 41: 2934, 1998). In evaluating the series prototype, CP-320626, in dogs, up to 90% reduction in plasma cholesterol was noted after 2 week treatment. Cholesterol reductions were also noted in ob/ob mice and in rats. In HepG2 cells, CP-320626 acutely and dose-dependently inhibited cholesterolgenesis without affecting fatty acid synthesis. Inhibition occurred together with a dose-dependent increase in the cholesterol precursor, lanosterol, suggesting that cholesterolgenesis inhibition was due to lanosterol 14α-demethylase (CYP51) inhibition. In ob/ob mice, acute treatment with CP-320626 resulted in a decrease in hepatic cholesterolgenesis with concomitant lanosterol accumulation, further implicating CYP51 inhibition as the mechanism of cholesterol lowering in these animals. CP-320626 and analogs directly inhibited rhCYP51, and this inhibition was highly correlated with HepG2 cell cholesterolgenesis inhibition (R² = 0.77). These observations indicate that CP-320626 inhibits cholesterolgenesis via direct inhibition of CYP51, and that this is the mechanism whereby CP-320626 lowers plasma cholesterol in experimental animals. Dual-action glycolgenolysis and cholesterolgenesis inhibitors therefore have the potential to favorably affect both the hyperglycemia and the dyslipidemia of type 2 diabetes.—Harwood, Jr., H. James, S. F. Petras, D. J. Hoover, D. C. Mankowski, V. F. Soliman, E. D. Sugarman, B. Hulin, Y. Kwon, E. M. Gibbs, J. T. Mayne, and J. L. Treadway. Dual-action hypoglycemic and hypocholesterolemic agents that inhibit glycolgen phosphorylase and lanosterol demethylase. J. Lipid Res. 2005. 46: 547–563.

Supplementary key words cholesterol synthesis • glycolgenolysis • glycolgen phosphorylase • lanosterol 14α-demethylase • CYP51 • enzyme inhibition • type 2 diabetes • HepG2 cells • ob/ob mice

Type 2 diabetes is a severe and prevalent disease in the Western world and affects roughly 16 million persons in the US, and another 14 million people have impaired glucose tolerance (1). Projections indicate that the incidence of type 2 diabetes will increase to over 25 million by 2010 in the US, and to over 300 million worldwide by 2025 (1–3). The annual direct medical costs associated with type 2 diabetes, which in the United States was in excess of 44 billion dollars in 1997 (4), result primarily from secondary hyperglycemia-related complications, such as retinopathy, nephropathy, peripheral neuropathy, and cardiovascular, peripheral vascular, and cerebrovascular disease.

A high correlation exists between tighter glycemic control and reduction of these long-term complications in type 2 diabetes (5). Type 2 diabetics are currently treated with interventions to improve glycemia through a progressive regimen of diet, exercise, oral antidiabetic drugs (as monotherapy or in combination), and insulin (6). However, there is an ongoing need for additional oral antidiabetic agents that will achieve better glycemic control as monotherapy and/or work more safely or effectively in combination.

In addition to their hyperglycemia, patients with type 2 diabetes often present with a concomitant atherogenic dyslipidemia (elevated triglycerides, low HDL cholesterol, and small, dense LDL) that increases their risk of cardiovascular disease (7, 8). Because there is a high incidence of mortality for type 2 diabetics with their first myocardial infarction (7), aggressive therapy for treating diabetic dyslipidemia is recommended (7). It is suggested that initial lipid-modulating therapy be directed toward reducing LDL cholesterol levels to below 100 mg/dl through administration of a cholesterol synthesis inhibitor, such as a statin (HMG-CoA reductase inhibitor), and that this treatment
be combined with a fibrinolytic agent, such as fenofibrate, for patients with HDL cholesterol levels below 40 mg/dl and for patients with triglycerides that remain elevated (>150 mg/dl) after both improvement of glycemic control and initiation of statin therapy (7).

In pursuing new treatments for type 2 diabetes, we have targeted inhibition of glycerokinase phosphorylation (E.C. 2.4.1.1), the enzyme that catalyzes the hydrolytic release of glucose-1-phosphate from glycogen, as an approach to reducing hepatic glycolysis and thereby controlling plasma glucose levels (2). Through these efforts, we have identified a series of indole-2-carboxamide glycogen phosphorylase inhibitors (9, 10) that inhibit the human liver isoform of glycogen phosphorylase by binding at a unique allosteric regulatory site on the enzyme (11), reduce forskolin-induced glycolysis in SK-HEP-1 cells (10, 12), and exhibit glucose-lowering activity when given orally to diabetic ob/ob mice (10, 12).

Because of the potential pharmacological utility of this series of glycogen phosphorylase inhibitors, we have evaluated a representative analog, CP-320626 (Fig. 1); [IC$_{50}$ vs. human liver glycogen phosphorylase, 205 nM (10)], for its subchronic effects in diabetic ob/ob mice (10, 12), in rats, and in dogs. During the course of these studies, we discovered that CP-320626 reduced plasma cholesterol levels in a variety of normoglycemic, nondiabetic animals in a manner and magnitude inconsistent with its expected action as a glycogen phosphorylase inhibitor. Herein, we report identification of the mechanism responsible for the cholesterol-lowering action of CP-320626 as inhibition of the cholesterolgenic enzyme lanosterol 14α-demethylase (CYP51), and characterize structure–activity relationships for this activity within the series.

**EXPERIMENTAL PROCEDURES**

**Materials**

Lanosterol, NADP+, glucose-1-phosphate, glycogen, isocitrate, isocitrate dehydrogenase, dioleoyl phosphatidylcholine, ergosterol, tyloxapol, ketocazoxole, quinidine, sulfaphenazole, cytochrome-P450 (CYP) reductase, PEG400, and diagnostic kits for measuring plasma lactate and β-hydroxybutyrate were from Sigma Chemical Co. (St. Louis, MO). TMSI + Pyridine, 1:4 (Sylon TP) in 1 ml aliquots were from Supelco (Belleville, PA). Sodium [2,3-3H]acetate (36 mCi/mmol), R,S-[2,3-3C]mevalonolactone (38 mCi/mmol), and Aquasol-2 were from New England Nuclear (Boston, MA). Ready-Safe was from Beckman Instruments (Fullerton, CA). [2,6,11,12,15,23-3H]lanosterol was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). 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). Silica gel 60C TLC plates were from Eastman Kodak (Rochester, NY). BCA protein assay reagent was from Pierce (Rockford, IL). A-Gent™ Glucose-U, A-Gent™ Triglyceride and A-Gent™ Cholesterol Test reagent systems were from Abbott Laboratories (Irving, TX). Pluronic P105 Block Copolymer Surfactant was from BASF (Parsippany, NY). Sprague Dawley rats and Beagle dogs were from Charles Rivers (Boston, MA). C57BL/6j-ob/ob mice were from Jackson Laboratory (Bar Harbor, ME). RMH 3290 laboratory meal and Agway Respond 2000 laboratory dog chow were from Agway, Inc. (Syracuse, NY). HepG2 cells were from the American Type Culture Collection (Rockville, MD). All other chemicals and reagents were from previously listed sources (13–16).

**Studies using experimental animals**

All procedures using experimental animals were approved by the Institutional Animal Care and Use Procedures Review Board. Sprague Dawley rats, C57BL/6j-ob/ob mice, and Beagle dogs were given food and water ad libitum and treated orally at a volume of 1.0 ml/200 g body weight (rats), 0.25 ml/25 g body weight (mice), or 1.0 ml/kg body weight (dogs) with either an aqueous solution of 0.1% pluronic P-105 in 10% DMSO (vehicle) or an aqueous solution of 0.1% pluronic P-105 in 10% DMSO plus CP-320626.

**Measurement of plasma, cholesterol, triglyceride, glucose, and related metabolite levels**

Serum glucose triglyceride and total cholesterol concentrations were determined by the Abbott VP™ and VP Super System™ Auto-analyzer using the A-Gent™ Glucose-U, A-Gent™ Triglyceride and A-Gent™ Cholesterol Test reagent systems. Serum insulin and glucagon concentrations were determined by radioimmunooassay (RIA) using kits from Binax (Portland, ME) and Amersham Corp. (Arlington Heights, IL), respectively. β-Hydroxybutyrate concentration was determined spectrophotometrically using kits from Sigma. Free fatty acid concentration was determined using a kit from Wako (Richmond, VA). The serum glucose, insulin, glucagon, triglyceride, total cholesterol, β-hydroxybutyrate, and free fatty acid-lowering activity of test compounds were determined by statistical analysis (unpaired $t$-test) with the vehicle-treated control group.

**Measurement of plasma CP-320626 levels**

To 100 μl aliquots of plasma were added 50 μl of an internal standard (2 μg/ml CP-89816 in methanol), 5 ml methyl tert-butyl ether, and 1 ml of 0.5 M sodium carbonate (pH 9). After vigorous mixing and centrifugation, the ether layers were removed and evaporated to dryness, and the resulting solid was reconstituted with 75 μl mobile phase [45% acetonitrile, 55% 50 mM sodium phosphate monobasic, and 30 mM triethylamine (pH 3)]. Aliquots (30 μl) of reconstituted samples were injected onto a 4 μm Waters Nova-Pak C-18 (Waters Corp, Bedford, MA) reverse phase column (3.9 × 150 mm), with a mobile phase flow rate of 1 ml/min. CP-320626 and internal standard were detected by fluorescence (excitation at 290 nm and emission at 348 nm). The linear dynamic range was between 0.1 μg/ml (lower limit of quantification) and 1 μg/ml (upper limit of quantification). $C_{max}$ was the concentration in the blood sample in which the highest plasma concentration was measured. The area under the plasma concentration time curve (AUC) from 0 to last (AUC$_{last}$) was calculated using a linear trapezoidal approximation, where last is the time point of the last quantifiable plasma concentration.

**Measurement of liver and plasma precursor sterols by gas chromatography-mass spectrometry**

Nonsaponifiable lipids were isolated from liver and plasma and quantitated by gas chromatography-mass spectrometry (GC/
Samples of plasma (0.75 ml) and liver (0.75 g) were saponified at 70°C for 120 min in 2.5 ml of 2.5 M NaOH, then 5 ml of absolute EtOH was added to each sample and the solutions were mixed. Ten milliliters of petroleum ether was then added to each sample, and the mixtures were shaken vigorously for 2 min then centrifuged at 2,000 g in a bench-top Sorvall for 10 min. After a second petroleum ether extraction, the resultant petroleum ether layers were removed, dried under nitrogen, and dissolved in 500 μl dry pyridine solution. A 500 μl aliquot of TMSI + Pyridine, 1:4 (Sylon TP) was then added to each sample, and derivatization was allowed to continue at room temperature (RT) for 1 h. Derivatized samples were analyzed using an HP-6890 Series gas chromatograph equipped with a 6890 Series GC Injector and interfaced with an HP-5973N mass selective detector. Separation was achieved using a Supelco SAC-5 (15 m × 0.25 mm × 0.25 μm) GC column. The oven temperature was held at 250°C for 0 min, then heated to 300°C at a rate of 2°C/min. The ions monitored using full scan electron ionization (70 eV) corresponded to the molecular ions of the trimethylsilyl derivatives. Retention times of detectable precursor sterols relative to that of cholesterol were cholestanol (dihydrocholesterol), 1.03; 8-dehydrocholesterol, 1.06; desmosterol, 1.07; 7-dehydrocholesterol, 1.07; lathosterol, 1.10; 4-methylsterol, 1.20; 4,4-dimethylsterol, 1.20; lanosterol, 1.42; and dihydrolanosterol, 1.42, similar to those previously reported for C-3 hydroxyl-derivatized sterols (17, 18).

Based on GC/MS analysis, the sterol composition of control rat liver was ~98% cholesterol, with 0.04% cholestanol and 8-dehydrocholesterol, 0.17% desmosterol and 7-dehydrocholesterol, 0.13% lathosterol, 1.11% monomethyl and dimethyl sterols, and 0.49% trimethylsterols (lanosterol and dihydrolanosterol). The sterol composition of control rat plasma was >99% cholesterol, with 0.57% monomethyl and dimethyl sterols and 0.38% trimethylsterols (lanosterol and dihydrolanosterol). Levels of cholestanol, 8-dehydrocholesterol, desmosterol, 7-dehydrocholesterol, and lathosterol in control rat plasma were all below the limits of detection.

**Measurement of CYP51 activity**

The activity of recombinant human CYP51, expressed in TOP3 cells and partially purified by the method of Stromstedt, Rozman, and Waterman (19), was determined by measuring the conversion of lanosterol to 4,4-dimethylcholesta-8,14,24-trien-3β-ol as previously described (19), with the following modifications: Briefly, 25 μl of a 1 mM suspension of lanosterol in a mixture of tyloxapol-acetone (1:1; v/v), dioleylphosphatidylcholine micelles (5 mg/ml), methanol, and 100 mM potassium phosphate buffer (pH 7.4) was added to 5 ml glass tubes to provide a final lanosterol concentration of 50 μM (approximate K_m). Inhibitors, dissolved in methanol for final concentrations ranging between 0.01 and 200 μM, were then added, and the lanosterol/inhibitor mixtures were allowed to dry under nitrogen for 10 min. To the residues were added 20 pmol partially purified recombinant human CYP51, 125 pmol human CYP reductase, and 50 μl rat lipid. After incubation at RT for 10–15 min for enzyme reconstitution, 540 μl of 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.5 mM KCN was added to each tube. Reaction mixtures were preincubated for 2 min at 37°C, then reactions were initiated by the addition of 50 μl of an NADPH regenerating system (final incubation concentration, 10 mM MgCl₂, 0.54 mM NADPH, 6.2 mM DL-isocitric acid, 0.5 U/ml isocitrate dehydrogenase). After 60 min incubation at 37°C, reactions were terminated by addition of a 25 μl volume of ethyl acetate that also contained the internal standard, ergosterol (1 mg/ml), followed by extraction with 5 ml ethyl acetate. After vigorous mixing and centrifugation to facilitate phase separation, 3–4 ml of the ethyl acetate phase was transferred to fresh tubes and evaporated to dryness under nitrogen at 50°C. Samples were reconstituted in 150 μl mobile phase (see below), and 25–50 μl was applied to an HPLC system. Ergosterol (internal standard) and 4,4-dimethylcholesta-8,14,24-trien-3β-ol (reaction product) were separated on a Waters Novapak C18 column (4.0 μM, 150 mm × 3.9 mm), with a mobile phase consisting of methanol-acetonitrile-HPLC-grade water (45:45:10 v/v/v), at a flow rate of 1.5 ml/min. Ergosterol and 4,4-dimethylcholesta-8,14,24-trien-3β-ol were monitored by UV detection at 248 nm using a SpectroMonitor variable wavelength detector (LDC Analytical; Riviera Beach, FL), and the Multichrom™ data acquisition system (Version. 2.11; Fisons Instruments, Beverly, MA) was used for data collection and analysis. Approximate retention times for 4,4-dimethylcholesta-8,14,24-trien-3β-ol and ergosterol were 25 and 30 min, respectively.

**Measurement of human liver glycogen phosphorylase activity**

The activity of recombinant human liver glycogen phosphorylase, expressed in baculovirus, purified to >95% homogeneity, and fully activated by phosphorylase kinase as previously described (20–22), was determined by measuring glycogen synthesis from glucose-1-phosphate by assessing the release of inorganic phosphate (reverse reaction) at 22°C in 100 μl of 50 mM HEPES buffer (pH 7.2) containing 100 mM KCl, 2.5 mM EGTA, 2.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.63 mM glucose-1-phosphate, 1.25 mg/ml glycogen, 9.4 mM glucose, 0.7% DMSO, and up to 2 μg of partially purified, activated human liver glycogen phosphorylase, based on the method of Engers, Shechosky, and Madsen (22), as previously described (9). The inorganic phosphate released during a 60 min incubation was measured at 0.2 nm, 20 min after the addition of 150 μl of 1 M HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green (23).

**Measurement of sterol and fatty acid synthesis in cultured cells**

Sterol and fatty acid synthesis were evaluated in HepG2 cells by measuring incorporation of [2-14C]acetate into cellular lipids as previously described (13, 14), with modifications (15, 16) to allow simultaneous assessment of both sterol and fatty acid synthesis. HepG2 cells grown in T-75 flasks as previously described (13, 14) were seeded into 24-well plates at a density of 1.2 × 10⁵ cells/well and maintained in 1.0 ml of supplemented DMEM (DMEM containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 40 μg/ml gentamicin) for 7 days in a 37°C, 5% CO₂ incubator with medium changes on days 3 and 5. On day 8, the medium was removed and replaced with fresh medium containing 1% DMSO ± effector compounds. Immediately after compound addition, 25 μl of media containing 4 μCi of [2-14C]acetate (56 mCi/mmol) was added to each incubation well. Plates were then sealed with parafilm to prevent evaporation, and cells were incubated at 37°C for 6 h with gentle shaking. After incubation, the samples were saponified by addition to each well of 1 ml of 5 N KOH in MeOH, followed first by incubation for 2 h at 70°C and then by overnight incubation at RT. Mixtures were transferred to glass conical tubes and extracted three times with 4.5 ml hexane. The pooled organic fractions (containing cholesterol, post-squalene cholesterol precursors, and other non-saponifiable lipids) were dried under nitrogen, resuspended in 25 μl chloroform, and applied to 1 × 20 cm channels of Silica Gel 60C TLC plates. Channels containing nonradioactive cholesterol, lanosterol, and squalene 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), air dried, and assessed for radioactivity using a Berthold Linear Radioactivity Analyzer (Oak Ridge, TN) that reports radioactive peak location and integrated peak area. The desmethylsterol peak (R_f = 0.27) was predomin-
nately (>99%) cholesterol and also contained traces of cholestanol, cholesterol, desmosterol, and lathosterol (24). The methylsterol peak (Rt = 0.34) contained lanosterol, dihydrolanosterol, 4-methylsterols, and 4,4-dimethylsterols (24). Squalene dioxide, squalene oxide, and squalene migrated with Rt values of 0.42, 0.65, and 0.88, respectively.

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 (containing protonated fatty acids) 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 60 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), air dried, and visualized for radioactive fatty acids by analysis using a Berthold Linear Radioactivity Analyzer that reports radioactive peak location and integrated peak area. Sterol and fatty acid synthesis are expressed as dpm [2,14C]acetate incorporated into either cholesterol, post-squalene cholesterolgenic intermediates, or saponifiable lipids during the 6 h incubation at 37°C.

Measurement of sterol biosynthesis in ob/ob mice

Hepatic incorporation of R,S-[2,14C] mevalonolactone into cholesterol and precursor sterols in C57BL/6J-6/ob/ob mice was assessed as previously described for hepatic incorporation of R,S-[2,14C] mevalonolactone into cholesterol and precursor sterols in golden Syrian hamsters (13), with the following modifications: Five- to eight-week-old male C57BL/6J-6/ob/ob mice were housed in groups of five animals each and were given food and water ad libitum for 1 week. After the 1 week acclimation period, animals weighing ~50 g were administered by oral gavage 0.2 ml of either vehicle or vehicle containing CP-320626. One hour after compound administration, animals received an intraperitoneal injection of 0.1 or vehicle containing CP-320626. One hour after compound administration, animals were euthanized by pentobarbital injection, and two 0.5 g liver pieces were removed, and 5.0 ml aliquots were dried under nitrogen at 10°C, triturated, or recrystallized, as specified. Unless otherwise specified, reversed-phase HPLC (RP-HPLC) was performed with 214 nm detection on a 250 × 4.6 mm Ramin Microsorb (Woburn, MA) C18 column eluted isocratically by a two-pump/mixer system supplying the indicated mixture of acetonitrile and aqueous pH 2.1 (with H3PO4) 0.1 M KH2PO4, respectively, at 1.5 ml/min, with sample injected in a 1:1 mixture of acetonitrile and pH 7.0 phosphate buffer (0.025 M in each Na2HPO4 and KH2PO4). Percent purity was generally that determined from a 10–15 min run time. Reagents and starting materials, where the preparation is not given, were used as obtained from commercial sources; dimethylformamide and CH2Cl2 used as reaction solvents were the anhydrous grade supplied by Aldrich Chemical Co. (Milwaukee, WI). Microanalyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, NY, or by Quantitative Technologies, Inc., Whitehouse, NJ. A reaction temperature of 0–20°C indicates that the reaction vessel was initially cooled in an insulated ice bath that was allowed to warm overnight.

Procedure A (amide formation using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride)

A 0.1–0.3 M solution of the amine (1.0 equivalent, or ammonium hydroxide and 1.0–1.1 equiv of triethylamine) in CH2Cl2 or dimethylformamide (as specified) was treated sequentially at 0°C (unless other temperature specified) with 1.0–1.1 equivalent of the specified carboxylic acid, 1.5 equivalent hydroxybenzotriazole hydrate, and, after several minutes, 1.0–1.1 equivalent (corresponding in mole ratio to the carboxylic acid) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC), and the mixture was stirred for 14–20 h at RT (if initially cooled, allowed to warm to RT). If the product contained ionizable amine functionality, the acid wash was omitted. Exceptions in the use of Procedure A are noted individually. Reactions conducted at 0–25°C were conducted with initial cooling of the vessel in an insulated ice bath that was allowed to warm to RT over several hours. The mixture was diluted with ethyl acetate, washed twice with 2N NaOH and twice with 2N HCl, dried over MgSO4, and concentrated, giving a crude product that was purified by chromatography on silica gel, trituration, or recrystallization, as specified.

Synthesis of inhibitors

The syntheses of CP-320626 [1], [3], and [5] are reported (10). For all other inhibitors, unless otherwise specified, NMR spectra were recorded at 20–21°C on a Varian XL-300 (Cary, NC) or a Bruker (Billerica, MA) AM-300 spectrometer at 300 MHz for proton and 75.4 MHz for carbon nuclei. NMR spectra at 400 MHz were obtained on a Varian UNITY400 spectrometer at 20–21°C. Chemical shifts are expressed in ppm downfield from trimethylsilylane (external reference). Routine mass spectral data were obtained using either a Hewlett-Packard (Cambridge, MA) 5989 MS Engine operated with a particle beam interface and ammonia chemical ionization (designated PBMS) or a Fisons Instruments Trio-1000 spectrometer operated with a thermospray ionization interface (ammonia, designated TSPMS). The cited ion was the base peak (100% relative intensity) unless otherwise specified. Where chloride- or bromine-containing ions are described, the 35Cl- or 85Br-containing ions were also observed in the expected ratio. High-resolution mass spectra (HRMS) were obtained on a Micromass LCT-TOF spectrometer (Waters, Millford, MA) using API electrospray ionization, acetoniatriile-water-0.01% formic acid inlet mobile phase gradient, and internal calibrants (1-tryptophan and leucine enkephalin). Unless otherwise specified, reversed-phase HPLC (RP-HPLC) was performed with 214 nm detection on a 250 × 4.6 mm Ramin Microsorb (Woburn, MA) C18 column eluted isocratically by a two-pump/mixer system supplying the indicated mixture of acetonitrile and aqueous pH 2.1 (with H3PO4) 0.1 M KH2PO4, respectively, at 1.5 ml/min, with sample injected in a 1:1 mixture of acetonitrile and pH 7.0 phosphate buffer (0.025 M in each Na2HPO4 and KH2PO4). Percent purity was generally that determined from a 10–15 min run time. Reagents and starting materials, where the preparation is not given, were used as obtained from commercial sources; dimethylformamide and CH2Cl2 used as reaction solvents were the anhydrous grade supplied by Aldrich Chemical Co. (Milwaukee, WI). Microanalyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, NY, or by Quantitative Technologies, Inc., Whitehouse, NJ. A reaction temperature of 0–20°C indicates that the reaction vessel was initially cooled in an insulated ice bath that was allowed to warm overnight.
uct was purified by chromatography on silica gel eluted with 50, 75, and 100% ethyl acetate in hexanes, giving the title substance [171 mg, 65%; HPLC (60/40) 4.23 min (97%)]. This material was identical to [1] by HPLC and NMR. For [2]: *H NMR (CDCl3) δ 9.20 (br, 1H), 7.57 (d, 1H, J = 2 Hz), 7.33 (d, 1H, J = 8 Hz), 7.3–7.2 (m, 2H), 7.14 (m, 2H), 6.97 (m, 2H), 6.85 (m, 1H), 5.34 (m, 1H), 4.05–3.80 (m, 2H), 3.7–3.3 (m, 1.5H), 3.25 (m, 1H), 3.10 (m, 2H), 2.93 (m, 0.5H), 1.9–1.7 (m, 2.5H), 1.45 (m, 2H), 1.15 (m, 0.5H); TSPMS 444/446 (MH+, 100%).

1-[5-(chloro-indole-2-carboxylic acid)-3-(4-fluorophenyl)-i-alanyl]4-hydroxy-piperidine [4]. N-(t-Boc)-p-phenylalanine (3.8 g, 11.0 mmol) and 4-hydroxyproline (2.18 g, 21.5 mmol) were coupled in dichloromethane (40 ml) using 3.30 g (21.5 mmol) hydroxymethylbenzotriazole hydrate (HBT) and 3.03 g (15.8 mmol) at 0–25°C according to Procedure A (but washed with acid first, then base), giving 1-[5-(t-Boc)-p-phenylalanyl]4-hydroxyproline, which was used without further purification [4.7 g, 94%; HPLC (60/40) 3.52 min (98%)]. This amide (4.35 g, 12.5 mmol) was dissolved in 4 M HCl-dioxane at 0°C, and the resulting suspension was stirred at 25°C for 1 h. The mixture was concentrated and the residue was triturated with ether and dried, giving 1-[p-phenylalanyl]-4-hydroxyproline hydrochloride (3.44 g, 97%). According to Procedure A, this hydrochloride (870 mg, 3.1 mmol) and 5-chloro-1H-indole-2-carboxylic acid (600 mg, 3.4 mmol) were coupled in dichloromethane (10 ml) (0–25°C reaction temperature, 60 h reaction time), and the crude product was purified by chromatography on silica gel eluted with 50, 75, and 100% ethyl acetate in hexanes followed by trituration with 1:1 ether-hexanes, giving the title product [1.14 g, 86%, HPLC (60/40) 4.06 min (98%)]. For [4]: *H NMR (CDCl3) δ 9.38 (s, 0.5H), 9.34 (s, 0.5H), 7.60 (d, 1H, J = ~2 Hz), 7.47–7.1 (m, 7H), 6.86 (m, 1H), 5.4–5.3 (m, 1H), 4.0–3.9 (m, 1H), 3.9–3.75 (m, 1H), 3.6–3.4 (m, 1.5H), 3.35–3.2 (m, 1H), 3.2–3.1 (m, ~5H), 2.9–2.75 (m, 0.5H), 1.9–1.3 (m, ~3.5H), 1.0–0.8 (m, 0.5H); PMR 426/428 (MH+, 100%). Anal. calc. for C29H26Cl2N2O4 + 0.25 H2O: C, 64.18; H, 5.74; N, 9.76. Found: C, 64.28; H, 5.94; N, 9.41.

1-[5-(fluorooxindole-2-carboxylic acid)-3-(4-fluorophenyl)-i-alanyl]4-hydroxy-piperidine [6]. 1-[3-(4-fluorophenyl)-3-alamine]-4-hydroxyproline hydrochloride (10) (159 mg, 0.52 mmol) and 5-fluoro-1H-indole-2-carboxylic acid (760 mg, 2.5 mmol) were coupled in dichloromethane (8 ml) (0–25°C reaction temperature, 20 h reaction time), and the crude product was purified by chromatography on silica gel eluted with chloroform-methanol, 8:1, giving the title product (1.26 g, 94%). This amide (1.26 g, 3.3 mmol) was dissolved in ethyl acetate and cooled to 0°C. The solution was saturated with HCl and the resulting suspension was stirred at 0–25°C for 1 h. The mixture was concentrated to approximately one-half its volume and diluted with ether, and the solid was collected and dried, giving (5)-2-amino-3-(2-fluoro-phenyl)-1-(4-hydroxy-piperidin-1-yl)-propan-1-one (0.76 g, 100%). According to Procedure A, this hydrochloride (760 mg, 2.5 mmol) and 5-chloro-1H-indole-2-carboxylic acid (760 mg, 2.5 mmol) were coupled in dichloromethane (6 ml) (0–25°C reaction temperature, 20 h reaction time), and the crude product was purified by chromatography on silica gel eluted with chloroform-methanol, 8:1, giving the title product (0.815 g, 97%). Mp 127–129°C. For [8]: *H NMR (CDCl3, 400 MHz) δ 9.40 (s, 0.5H), 9.37 (s, 0.5H), 7.57 (8, 1H), 7.43 (d, J = 7.1 Hz, 1H), 7.29–7.13 (m, 5H), 7.04–6.98 (m, 2H), 6.83 (s, 1H), 5.42 (m, 1H), 4.05–3.85 (m, 2H), 3.8–3.7 (m, 0.5H), 3.7–3.6 (m, 0.5H), 3.56–3.11 (m, 4H), 1.86–1.60 (m, 4.5H), 1.55–1.45 (m, 1.5H), 1.25–1.20 (m, 1H); PMBS 444 (MH+, 100%). HRMS m/e 444.1504 (calcd for C29H26ClN2O4FCl + H, 444.1490).
acetic acid in hexanes, giving the title product (362 mg) in 86% yield. For [10]: HPLC (60/40) 5.06 min (97%); mp 227–229°C; TSPMS 460/462 (MH+, 100%). Anal. calcd for C₇₀H₇₅N₇O₇: C, 60.01; H, 5.04; N, 9.13. Found: C, 59.83; H, 5.18; N, 9.16.

1-[[5-(Chlorinolide-2-carbonyl)-3-[4-methoxyphenyl]-]t-alanyl[24]-4-hydroxypiperidine [11]]. N-[t-Boc]-O-methyl]-t-tyrosine (2.5 g, 8.47 mmol) and 4-hydroxypiperidine (0.94 g, 9.3 mmol) were coupled in dichloromethane (50 ml) using 1.15 g (8.5 mmol) HBT and 2.1 g (11 mmol) DEC at 0–25°C according to Procedure A, giving (S)-[1-(4-methoxy-benzyl)-2-(4-hydroxypiperidin-1-yl)-2-oxo-ethyl]-carbamic acid tert-butyl ester, which was used without further purification (3.1 g, 98%). This amide (3.1 g, 8.2 mmol) was dissolved in ethyl acetate and cooled to 0°C, the solution was saturated with HCl, and the resulting suspension was stirred at 0–25°C for 1 h. The mixture was concentrated to dryness, and the residue was triturated with ether. The solid was collected and dried, giving (S)-2-amino-3-[4-methoxy-phenyl]-1-(4-hydroxypiperidin-1-yl)-propan-1-one hydrochloride (1.25 g, 48%). This hydrochloride (630 mg, 2.0 mmol), 5-chloroindole-carboxylic acid (391 mg, 2.0 mmol), triethylamine chloride (1.25 g, 48%). This hydrochloride (630 mg, 2.0 mmol), 5-chloroindole-2-carboxylic acid (126 mg, 0.65 mmol) according to Procedure A was performed using triethylamine (69 mg, 0.65 mmol), HBT (149 mg, 0.98 mmol), and DEC (130 mg, 0.68 mmol) in dichloromethane (4 ml) at 0–25°C reaction temperature but substituting the following workup: The reaction mixture was diluted with ethyl acetate, the resulting solution was washed with 1N NaOH (2 ml), the aqueous layer was extracted three times with ethyl acetate, and the combined organic extracts were washed with 1N HCl, dried, and concentrated. The residue was purified by chromatography on silica gel eluted with 1, 2, 4, and 16% ethyl acetate-hexanes followed by ethyl acetate, giving a colorless foam. A portion of this material (1.11 g, 0.20 mmol) was dissolved in dimethylformamide containing diethylamine (20 µl, 0.20 mmol) for 75 min at RT and concentrated. The residue was chromatographed on silica (15 g in 3, 10, 20, and 30% ethanol-dichloromethane containing 0.5% (v/v) concentrated NH₄OH, giving 1-[N-(Fmoc)-t-lysyl]-4-hydroxypiperidine hydrochloride [14]. 4-Hydroxypiperidine (227 mg, 2.24 mmol) and N-(Fmoc)-N-[t-Boc]-l-lysine (1.00 g, 2.13 mmol) were coupled according to Procedure A using HBT (490 mg, 3.2 mmol) and DEC (429 mg, 2.24 mmol) in dichloromethane, and the crude product was purified by chromatography on 29 g silica packed with 1:1 ethyl acetate-hexanes and eluted with 3:1 ethyl acetate-hexanes followed by ethyl acetate, giving a colorless foam. A portion of this material (111 mg, 0.20 mmol) was dissolved in dimethylformamide containing diethylamine (20 µl, 0.20 mmol) for 75 min at RT and concentrated. The residue was chromatographed on silica (15 g in 3, 10, 20, and 30% ethanol-dichloromethane containing 0.5% (v/v) concentrated NH₄OH, giving 1-[N-(t-buty1)-4-hydroxypiperidine as an oily solid [25 mg, 38%, m/e 330 (MH+)]. This material (0.076 mmol) and 5-chlorinolide-2-carboxylic acid (15 mg, 0.076 mmol) were coupled according to Procedure A in dichloromethane (2 ml), giving an oil (50 mg), which was chromatographed on 5 g silica eluted with 1, 2, and 4% ethyl acetate in dichloromethane to give the amide as a foam [36 mg, HPLC (60/40) 3.6 min, (99.6%), and having consistent NMR and mass spectra]. This material (31 mg) was dissolved in 4 M HCl-dioxanes, stirred at RT for 1.5 h, concentrated, and dried, giving 18 mg of a red-orange solid, which was triturated with ether [HPLC (50/50) 2.19 min, (94%)]. 1H NMR (D₉O) δ 7.74 (s, 1H), 7.49 (d, 1H, J = ~7 Hz), 7.32 (d, 1H, J = ~7 Hz), 5.1 (m, 1H), 4.1–3.9 (m, ~2H), 3.5–2.9 (m, ~5H), 2.1–1.8 (m, 4H), 1.8–1.6 (m, 2H), 1.6–1.4 (m, 4H); TSPMS 407 (MH+, 100%). HMRMS m/e 407.1855 (calcd for C₂₄H₂₆N₃O₄ClH).
diethylamine (0.10 ml) for 1 h at RT, evaporated, and the residue suspended in ether-dichloromethane and filtered. The filtrate was concentrated (328 mg) and chromatographed on 8 g silica eluted with 1, 2, 4, 10, 20, and 50% ethanol-dichloromethane containing 0.5% (v/v) concentrated NH₄OH and the requisite fractions concentrated, giving [1-[L-aspartyl(ω-Bu)]-4-hydroxypiperidine (217 mg, 80%). This material (213 mg, 0.78 mmol) was coupled to 5-chloroindole-2-carboxylic acid (153 mg, 0.78 mmol) in dichloromethane (5 ml) according to Procedure A, and the crude product (373 mg) was purified by chromatography on 9 g silica eluted with 25, 40, 50, 75, and 100% ethyl acetate-hexanes, giving [1-[N-(5-chloroindole-2-carbonyl)-l-aspartyl(ω-Bu)]-4-hydroxypiperidine as a colorless foam [350 mg, 94% yield, HPLC (60/40) 4.18 min (97%), TSPMS 450 (MH⁺)]. This ester (293 mg, 0.65 mmol) was dissolved in trifluoroacetic acid at RT for 2 h and concentrated to a yellowish foam, which was partitioned between 2 M NaOH and ethyl acetate. The aqueous layer was separated and extracted twice with ethyl acetate, then stirred in an ice bath with ethyl acetate and acidified to pH 2 with 6N HCl. The organic layer was separated, the aqueous layer extracted with ethyl acetate, and the organic layers were combined and dried, giving the title product as a colorless solid in quantitative yield. For [15]: HPLC (DMSO-d₆) δ 11.85 (br, 1H), 7.47 (d, 1H, J = 8.7 Hz), 7.05 (d, 1H, J = 8.7 Hz), 6.79 (m, 1H), 5.45–5.15 (m, 2H), 2.98 (s, 3H), 1.80 (s, 6H), 0.42 (s, 9H). HRMS m/e 517 mg, 35%, HPLC (50/50) 4.75 min, (97%). 1H NMR (CDCl₃) δ 6.91 (m, 2H), 7.07 (d, 1H, J = 8.7 Hz), 7.03 (d, 1H, J = 8.7 Hz), 7.00 (s, 1H), 5.42 (br, 1H), 4.9 (m, 1H), 4.1–3.6 (m, 3H), 3.3–3.0 (m, 2H), 2.85 (dd, 1H), 2.78 (dd, 1H), 2.45 (s, 3H), 1.8–1.4 (m, 5H), 1.38 (s, 9H). This material was dissolved in 4 M HCl-dioxanes (3 ml), stirred at RT for 1 h, concentrated, and triturated with ether, giving 314 mg of 1-[L-aspartyl]-4-hydroxypiperidine dihydrochloride, 1H NMR (D₂O partial) δ 8.72 (d, 1H, J = 9 Hz), and 7.44 (d, 1H, J = 9 Hz). The dihydrochloride (200 mg, 0.73 mmol) and 5-chloroindole-2-carboxylic acid (135 mg, 0.69 mmol) were coupled using triethylamine (74 mg, 0.73 mmol), HBT (168 mg), and DEC (140 mg, 0.73 mmol) in dichloromethane (4 ml) according to Procedure A but omitting the acid wash, and the crude product was chromatographed on silica (7 g eluted with 5, 10, and 20% ethanol-dichloromethane containing 0.5% (v/v) concentrated NH₄OH, to give the title substance as a colorless powder [292 mg, 81%, HPLC (40/60) 2.57 min, (98%)]. For [17]: 1H NMR (DMSO-d₆) δ 11.8 (br, 1H), 8.87 (d, 0.5H), 8.83 (d, 0.5H), 7.72 (d, 1H, J = 2 Hz), 7.53 (m, 1H), 7.40 (d, 1H, J = 8.6 Hz), 7.23 (m, 1H), 7.18 (dd, 1H, J ≈ 2, 8.7 Hz), 6.79 (m, 1H), 5.25–5.15 (q, 1H, 4.8 (m, 1H), 4.1–3.6 (m, 3H), 3.2–3.1 (m, 1H), 3.0–2.85 (m, 2H), 1.7–1.5 (m, 2H), 1.3–1.15 (m, 2H). HRMS m/e 416.1479 (calc for C₁₆H₂₆N₂O₄Cl + H, 416.1489). 1-[N-(5-chloroindole-2-carbonyl)-l-alanyl]-4-hydroxypiperidine [18]. 4-Hydroxypiperidine (101 mg, 1.0 mmol) and Boc-t-alanine (189 mg, 1.0 mmol) were coupled according to Procedure A using EDC (200 mg, 1.05 mmol) and HBT (229 mg, 1.5 mmol) in dichloromethane (5 ml) giving 1-[N-(t-Boc)-l-alanyl]-4-hydroxypiperidine (118 mg, 44%). This material (118 mg, 0.43 mmol) was dissolved in 4 M HCl-dioxane (2 ml) and stirred at 25°C for 2 h, concentrated, and the residue triturated with ether, giving 1-(t-alanyl)-4-hydroxypiperidine dihydrochloride (90 mg, 100%). Coupling of this hydrochloride (58 mg, 0.28 mmol) and 5-chloro-piperidine-2-carboxylic acid (55 mg, 0.28 mmol) according to Procedure A was performed using triethylamine (37 µl, 0.28 mmol), EDC (56 mg, 0.29 mmol), and HBT (51 mg, 0.33 mmol) in dichloromethane (5 ml) at 25°C overnight. The product was purified by radial chromatography (ethyl acetate) and obtained as a foam (81 mg, 83%). For [18]: PBMS 350/352 (MH⁺, 100%). 1H NMR (DMSO-d₆, 400 mHz) δ 11.75 (br, 1H), 8.74 (d, 0.5H, J = 8.7 Hz), 8.72 (d, 0.5H, J = 8.5 Hz), 7.75 (s, 1H), 7.37 (m, 1H), 7.15 (m, 1H), 7.13 (m, 1H), 4.95 (m, 1H), 4.71 (m, 1H), 4.0 (m, 0.5H), 3.8–3.6 (m, 2.5H), 3.3–3.25 (m, 0.5H), 3.2–3.05 (m, 1H), 2.9–2.8 (m, 0.5H), 1.8–1.6 (m, 2H), 1.3–1.2 (m, 2H), 1.24 and 1.23 (d, J = 5–6 Hz, 3H total). HRMS m/e 350.1271 (calc for C₁₀H₁₃NO₄Cl + H, 350.1271). 1-[N-(5-chloroindole-2-carbonyl)-l-valyl]-4-hydroxypiperidine [19]. 4-Hydroxypiperidine (101 mg, 1.0 mmol) and t-Boc-t-valine (217 mg, 1.0 mmol) were coupled according to Procedure A using EDC (200 mg, 1.05 mmol) and HBT (229 mg, 1.5 mmol) in dichloro-
romethane (5 ml), giving 1-[N-(t-Boc)-L-valyl]-4-hydroxypiperidine (213 mg, 73%). This material (205 mg, 0.68 mmol) was dissolved in 4 M HCl-dioxane (4 ml) and stirred at 25°C for 1.5 h, concentrated, and the residue triturated with ether, giving 1-(t-valyl)-4-hydroxypiperidine hydrochloride (160 mg, 100%). Coupling of this hydrochloride (83 mg, 0.35 mmol) and 5-chloro-1H-indole-2-carboxylic acid (68 mg, 0.35 mmol) according to Procedure A was performed using triethylamine (45 µl, 0.35 mmol), EDC (70 mg, 0.38 mmol), and HBT (64 mg, 0.42 mmol) in DMF (4 ml) at 25°C. The product was purified by radial chromatography (1:1 hexanes-ethyl acetate, then ethyl acetate), giving 1-[(t-Boc)-L-valyl]-4-hydroxypiperidine hydrochloride (83 mg, 0.35 mmol) and 5-chloro-1H-indole-4-hydroxypiperidine hydrochloride (10) (124 mg, 0.50 mmol) and the product was purified by chromatography on silica eluted with 4:1 ethyl acetate-hexanes followed by ethyl acetate, giving the title substance (170 mg, 86%), HPLC (50/50 acetonitrile-0.1% aqueous formic acid, 5 µM Kromasil C-18 50 × 4.6 mm, 1.0 ml/min) retention time 3.6 min (99% purity, 280 nM detector wavelength). ¹H NMR (DMSO-d₆, 400 MHz) δ 8.72 (d, 0.5H, J = 8.5 Hz), 8.70 (d, 0.5H, J = 8.5 Hz), 7.78 (d, 2H, J = 7.5 Hz), 7.49–7.42 (m, 1H), 7.40–7.38 (m, 2H), 7.27–7.12 (m, 5H), 5.07 (q, 1H), 4.67 (m, 1H), 3.95–3.85 (m, 0.5H), 3.8–3.75 (m, 1H), 3.75–3.5 (m, 1.5H), 3.3–3.15 (m, 0.5H), 1.15–1.10 (m, 0.5H). HRMS m/e 353.1892 (calcd for C₂₁H₂₄N₂O₃ + 0.33 H₂O: C, 69.54; H, 6.24; N, 7.03. Found: C, 69.70; H, 6.26; N, 6.50.

N,N-dimethyl-2-(5-chloroindole-2-carbonyl)-3-(2-fluorophenyl)-L-alaninamide [20]. N-(t-Boc)-L-3-(2-fluorophenyl)alanine (0.280 g, 1.0 mmol) and HBT (0.16 mg, 1.2 mmol) were coupled in dichloromethane (5 ml) using triethylamine (51 mg, 0.50 mmol), HBT (115 mg, 0.75 mmol), and DEC (101 mg, 0.525 mmol), and the product was purified by chromatography on silica eluted with 4:1 ethyl acetate-hexanes followed by ethyl acetate, giving the title substance (170 mg, 86%), HPLC (50/50 acetonitrile-0.1% aqueous formic acid, 5 µM Kromasil C-18 50 × 4.6 mm, 1.0 ml/min) retention time 4.7 min (99% purity, 280 nM detector wavelength). ¹H NMR (DMSO-d₆, 400 MHz) δ 8.81 (d, 0.5H, J = 8.3 Hz), 8.72 (d, 1H, J = 7.5 Hz), 7.62 (d, 1H, J = 8.3 Hz), 7.58 (m, 1H), 7.43 (t, 1H, J = 7.7 Hz, 7.29 (t, 1H, J = 8 Hz), 7.25–7.35 (m, 4H), 7.30–7.10 (m, 1H), 5.12 (q, 1H), 4.68 (d, 1H, J = 4 Hz), 3.95–3.89 (m, 0.5H), 3.8–3.75 (m, 1H), 3.7–3.5 (m, 1.5H), 3.3–3.15 (m, 0.5H), 1.3–2.9 (m, 3.5H), 1.65–1.55 (m, 1.5H), 1.15–1.45 (m, 0.5H), 1.3–1.0 (m, 2H). Anal. calcd for C₂₉H₂₃N₂O₄ClF + 0.33 H₂O: C, 69.54; H, 6.24; N, 7.03. Found: C, 69.70; H, 6.26; N, 6.50.

RESULTS

Antihyperlipidemic effects of CP-320626 in ob/ob mice

Evidence that CP-320626 exhibits antihyperlipidemic properties was first obtained in the diabetic ob/ob mouse, where significant reductions in plasma cholesterol were observed following 10 mg/kg b.i.d administration for 15 days (12). These reductions were observed 2 days after administration for 15 days (12). These reductions were observed in the absence of changes in plasma insulin, glucagon, free fatty acids, and plasma triglycerides.

The plasma cholesterol lowering observed in experimental animals was not a consequence of interference by CP-320626 with the cholesterol oxidase-based cholesterol detection system, because the signal produced for a 400 mg/dl cholesterol standard was not altered by concentrations of CP-320626 between 0.1 µg/ml and 12 µg/ml (27 µM), concentrations that bracket concentrations observed at efficacious doses.
fatty acids, or β-hydroxybutyrate (12), indicating that the observed reductions in plasma triglycerides and cholesterol were not a tertiary consequence of secondary alterations in glycemic control mechanisms induced by the hypoglycemia resulting from glycogen phosphorylase inhibition.

**Hypolipidemic effects of CP-320626 in rats and dogs**

The antihyperlipidemic properties of CP-320626 observed in ob/ob mice were confirmed in a series of two week studies in normoglycemic rats and dogs in which treatment at doses between 30 and 400 mg/kg (rats) and 3 and 30 mg/kg (dogs) was associated with substantial reductions in serum cholesterol and triglycerides (Tables 1 and 2) in the absence of notable glucose lowering (not shown). For example, male rats treated with CP-320626 displayed a dose-dependent up to 60% decrease in serum cholesterol and triglycerides for doses between 30 and 400 mg/kg (Table 1). The magnitude of these reductions was related to exposure to CP-320626, with significant reductions occurring at peak plasma drug levels (C max) in excess of 3 μM (Table 1). Female rats treated with CP-320626 displayed similar effects (not shown).

Dogs were even more sensitive to the hypolipidemic effects of CP-320626. As shown in Table 2, both male and female dogs treated with CP-320626 displayed a dose-dependent up to 90% decrease in plasma cholesterol and an up to 50% reduction in plasma triglycerides for doses between 3 and 30 mg/kg. Again, the unanticipated magnitude of cholesterol and triglyceride reduction was related to exposure to CP-320626, with significant reductions occurring at peak plasma drug levels (C max) in excess of 2 μM (Table 2). These results, together with the absence of glucose lowering by CP-320626 in these normoglycemic animals, suggest that CP-320626 affects a second target that results directly in the observed hypolipidemic effects.

**Direct inhibition of cholesterol biosynthesis by CP-320626**

To determine whether the plasma cholesterol and triglyceride lowering observed in experimental animals was a consequence of direct inhibition of de novo sterol and/or tri-

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**TABLE 1.** Lipid-lowering effects of 2 week CP-320626 treatment in male rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Plasma C max (µM)</th>
<th>Plasma AUC (µM × h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male rats</td>
<td>0</td>
<td>62 ± 4</td>
<td>72 ± 11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>55 ± 4</td>
<td>48 ± 7</td>
<td>3.2 ± 0.9</td>
<td>18 ± 8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>42 ± 2</td>
<td>50 ± 5</td>
<td>11.2 ± 2.8</td>
<td>112 ± 82</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>35 ± 1</td>
<td>29 ± 2</td>
<td>34.3 ± 6.8</td>
<td>420 ± 100</td>
</tr>
</tbody>
</table>

AUC, area under the curve. Male Sprague Dawley rats (250 g) were maintained on RMH 3200 laboratory meal and administered either vehicle (0.1% Pluronic P-105 in 10% DMSO) or vehicle containing the indicated doses of CP-320626 twice daily for 11 days. Plasma samples were obtained after an overnight fast, 3 h after the final dosing, and were assessed for total cholesterol, triglycerides, and drug levels as outlined in Experimental Procedures.

Values represent the mean ± SD for n = 10 rats per group.

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**TABLE 2.** Lipid-lowering effects of 2 week CP-320626 treatment in dogs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Plasma C max (µM)</th>
<th>Plasma AUC (µM × h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female dogs</td>
<td>0</td>
<td>135 ± 13</td>
<td>29 ± 3</td>
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<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>62 ± 16</td>
<td>30 ± 4</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>39 ± 2</td>
<td>15 ± 5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>14 ± 8</td>
<td>15 ± 3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Male dogs</td>
<td>0</td>
<td>139 ± 17</td>
<td>26 ± 4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>91 ± 32</td>
<td>19 ± 3</td>
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<tr>
<td></td>
<td>10</td>
<td>36 ± 10</td>
<td>14 ± 1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>28 ± 7</td>
<td>12 ± 2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>All dogs</td>
<td>0</td>
<td>137 ± 9</td>
<td>27 ± 2</td>
<td>—</td>
<td>—</td>
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<tr>
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<td>3</td>
<td>77 ± 16</td>
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<tr>
<td></td>
<td>10</td>
<td>38 ± 8</td>
<td>15 ± 2</td>
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<td>6.0 ± 7.0</td>
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<tr>
<td></td>
<td>30</td>
<td>21 ± 4</td>
<td>14 ± 1</td>
<td>24.0 ± 9.7</td>
<td>112.9 ± 56.7</td>
</tr>
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</table>

Male and female beagle dogs (8–10 kg), were fed Agway Respond 2000 laboratory dog chow twice daily for 2 weeks, and just prior to each meal received a 10 ml aqueous bolus of either vehicle (0.1% Pluronic P-105 in 10% DMSO) or vehicle containing the indicated amounts of CP-320626. Plasma samples were obtained after an overnight fast, 3 h after the final dosing, and were assessed for total cholesterol, triglycerides, and drug levels as outlined in Experimental Procedures. Values represent the mean ± SEM for n = 3 dogs per sex per group.

Numbers represent mean ± SD for male plus female combined data (n = 6/group).

a P < 0.01.

**Site of cholesterol synthesis inhibition is CYP51**

To determine the site of cholesterol synthesis inhibited by CP-320626, we asked whether cholesterol production was from generalized effects on intermediary metabolism. To determine whether CP-320626 inhibited cholesterol synthesis in a dose-dependent fashion, exhibiting an IC 50 of 4 mg/dl, indicating that the reduction in cholesterol formation from generalized effects on intermediary metabolism, was evaluated the ability of CP-320626 to inhibit cholesterol and fatty acid synthesis in cultured HepG2 (human liver) cells. As shown in Fig. 2, when CP-320626 and [2-14C]acetate were added simultaneously to near-confluent cultures of HepG2 cells, and simultaneous incorporation of radiolabel into sterols and fatty acids was permitted to proceed for 6 h as outlined in Experimental Procedures, CP-320626 inhibited cholesterol synthesis in a dose-dependent fashion, exhibiting an IC 50 of 4 μM. Fatty acid synthesis was not substantially affected by CP-320626 at concentrations that markedly inhibited cholesterol synthesis (Fig. 2), indicating that the reduction in cholesterol formation was not a consequence of altered acetate pools resulting from generalized effects on intermediary metabolism.
As shown in Fig. 3, reduction of cholesterol synthesis from [2-14C]acetate by CP-320626 occurred concomitantly with an enhanced formation of isotopically labeled methylsterols. Consistent with this observation, the chromatographic pattern of accumulated cholesterol precursors appeared identical to that observed following treatment of HepG2 cells with the known CYP51 inhibitor, ketoconazole (not shown), suggesting that cholesterol synthesis inhibition by CP-320626 was potentially a consequence of inhibition of the choles terolgenic enzyme, CYP51.

When [3H]lanosterol was included in nonsaponifiable lipid fractions prior to TLC analysis, the [14C]-labeled cholesterol precursor accumulating following both ketoconazole and CP-320626 treatment comigrated with authentic [3H]lanosterol (Fig. 4). Because monomethylsterols and dimethylsterols migrate with Rf values between those of cholesterol and the trimethylsterol, lanosterol, by silica gel TLC (24), the 1:1 comigration of the accumulating methylsterol with authentic lanosterol suggests that the accumulating methylsterol is lanosterol, further suggesting that CP-320626 is a direct inhibitor of CYP51.

### Cholesterol synthesis inhibition and lanosterol accumulation in ob/ob mice

To determine whether the effects of CP-320626 noted in HepG2 cells also occur in vivo, the ability of CP-320626 to inhibit cholesterol synthesis and induce accumulation of lanosterol was evaluated acutely in ob/ob mice. Unlike rats and CD1 mice in the untreated state, where the distribution of radiolabeled sterols formed acutely from [14C] acetate is ~85% cholesterol, 10% methylsterols, and 5% squalene (13), in the ob/ob mouse, the cholesterol and methylsterol peaks were nearly equivalent (Fig. 5, upper panel). Consistent with these observations, the monomethylsterol and dimethylsterol levels were ~10-fold greater in ob/ob mouse liver (5.6% of the hepatic cholesterol level) than in rat liver (0.57% of the hepatic cholesterol level), whereas the trimethylsterol levels in ob/ob mouse and rat liver were essentially the same (0.48% of the respective hepatic cholesterol levels), suggesting a pathway bottleneck at the cholesterolgenesis steps of sterol 4-demethylation in ob/ob mice.

Similar to the observations made in Hep-G2 cells, inhibition of hepatic cholesterol synthesis and accumulation of methylsterols was also noted in ob/ob mice 1 h after a single 30 mg/kg oral dose of CP-320626 (Fig. 5, upper panel). Both hepatic cholesterol synthesis inhibition and methylsterol accumulation were dose responsive between 3 mg/kg and 30 mg/kg (Fig. 5, lower panel), suggesting that, as in HepG2 cells, cholesterol synthesis inhibition in ob/ob mice was a consequence of inhibition of CYP51. The 1:1 comigration of the accumulating sterol with lanosterol was again confirmed by cochromatography with authentic [3H]lanosterol (not shown).
Fig. 4. Comigration of the accumulating sterol with lanosterol. HepG2 cells were incubated for 6 h at 37°C in supplemented DMEM containing 5 mM glucose, either 1% DMSO (control; top panel), 1% DMSO containing CP-320626 (final inhibitor concentration of 10 μM; center panel), or 1% DMSO containing ketoconazole (final inhibitor concentration of 1.0 μM; bottom panel), and 4 μCi of [2-14C]acetate. After incubation, samples were saponified, and the nonsaponifiable lipids were extracted with hexane, mixed with an authentic [3H]lanosterol chromatographic standard (15.8 nmol, 86 mCi/mol), and separated by silica gel TLC as described in Experimental Procedures. Channels were then dried, sectioned into 2.0 mm slices, immersed in 5 ml of Aquasol-2 liquid scintillation fluid (New England Nuclear, Boston, MA), and assessed for radioactivity using a dual-channel program of a Beckmann LS6500 liquid scintillation counter. Shown are the [14C]cpm (open circles) and [3H]cpm (lanosterol standard; solid circles) as a function of distance from the bottom of the chromatogram, with cholesterol migrating to 58 mm (R_f = 0.26) and lanosterol migrating to 69 mm (R_f = 0.33).

Fig. 5. Effect of CP-320626 and ketoconazole on sterol synthesis in ob/ob mice. Male C57BL/6J-ob/ob mice (5–7 weeks old; 50–60 g) were administered a 0.2 ml oral bolus of either vehicle, or vehicle containing either CP-320,626 or ketoconazole at a concentration sufficient to give a dose of 30 mg/kg (upper panel) or the indicated doses of CP-320626 (lower panel). One hour after compound administration, animals received an intraperitoneal injection of 0.1 ml R,S-[2-14C]mevalonolactone (100 μCi/ml; 58 mCi/mmol). One hour after radiolabel administration, animals were euthanized by pentobarbital injection, and two 0.5 g liver pieces were removed and saponified as outlined in Experimental Procedures. The nonsaponifiable lipids were then extracted with petroleum ether and separated by silica gel TLC, and the cholesterol and cholesterol precursors were quantitated as described in Experimental Procedures. Data for hepatic cholesterol and methylsterol production represent the mean ± SD for n = 6 mice per group and are expressed as dpm [2-14C]mevalonolactone incorporated into cholesterol (black bars, upper panel; closed circles, lower panel) and methylsterols (gray bars, upper panel; open circles, lower panel) during the 1 h interval between [2-14C]mevalonolactone injection and pentobarbital administration.
Accumulation of lanosterol but not other precursor sterols in rat plasma after treatment with CP-320626

To determine whether in addition to lanosterol, other precursor sterols also accumulate after inhibition of cholesterol synthesis by CP-320626, liver and plasma sterols were isolated from rats treated with CP-320626 for two weeks at a dose of 30 mg/kg/day as outlined in Table 1 and separated and quantitated by GC/MS as described in Experimental Procedures. Under these conditions, plasma cholesterol levels were reduced by 16% \[69 \pm 2.5 \text{ mg/dl (control)} \text{ vs.} 59 \pm 2.4 \text{ mg/dl (treated) (SEM; n = 6)}\]. Plasma trimethylsterol (lanosterol + dihydrolanosterol) concentrations were increased by 32%, from 0.37% of the control cholesterol peak to 0.49% of the control cholesterol peak. Plasma monomethyl and dimethyl sterols, however, were increased only slightly (12%) from 0.57% of the control cholesterol peak to 0.64% of the control cholesterol peak. Plasma cholestanol, 8-dehydrocholesterol, desmosterol, 7-dehydrocholesterol, and lathosterol levels in control rat plasma were all below the limits of detection and were not increased to above lower limits of quantitation after treatment with CP-320626. Liver levels of these precursor sterols, however, were either unchanged (desmosterol and 7-dehydrocholesterol), or slightly reduced (cholestanol and 8-dehydrocholesterol, 15% reduction; lathosterol, 8% reduction). Together, these observations are further evidence for CYP51 as the site of cholesterolgenesis inhibition by CP-320626.

Direct inhibition of CYP51 by CP-320626

To demonstrate that inhibition of cholesterol synthesis by CP-320626 was indeed by direct inhibition of CYP51 activity, we assessed the ability of CP-320626 to inhibit recombinant human CYP51 in vitro. As shown in Fig. 6 (upper panel), CP-320626 inhibited recombinant human CYP51 activity with an IC\(_{50}\) of 5.2 \text{ \mu M}, a value similar to that noted for cholesterol synthesis inhibition in HepG2 cells (see above). Inhibition was competitive with respect to the substrate lanosterol and exhibited a \(K_I\) of 0.3 \text{ \mu M} (Fig. 6, lower panel).

Based on difference spectra, CP-320626 demonstrated a pattern of interaction with the enzyme (absorbance maximum at 390 nm, minimum at 420 nm) similar to that observed for the substrate, lanosterol, with a maximum shift occurring at 6 \text{ \mu M}, a value similar to the IC\(_{50}\) of 5.2 \text{ \mu M} shown in Fig. 6 (upper panel). This observation is consistent with the competitive nature of the inhibition shown in Fig. 6 (lower panel) and suggests that CP-320626 interacts with the CYP51 enzyme at the substrate binding pocket near the heme but does not interact coordinately with the heme iron. By contrast, ketoconazole, which, like other azole-containing CYP51 inhibitors, interacts coordinately with the heme iron (25–29), demonstrated a typical Type II pattern of interaction with the enzyme (absorbance maximum at 430 nm, minimum at 394 nm) with a maximal shift at 20 \text{ \mu M}.

CP-320626 also showed specificity for CYP51 inhibition relative to other CYPs, indicating that this inhibition was not simply due to nonspecific interference with the CYP reaction mechanism or with NADPH-CYP reductase. For example, in parallel in vitro evaluations, CP-320626 inhibited CYP51, CYP3A4, CYP2D6, and CYP2C9, with respective IC\(_{50}\) values of 5 \text{ \mu M} (\(K_I = 0.3 \text{ \mu M}\), >50 \text{ \mu M}, 29 \text{ \mu M} (\(K_I = 15 \text{ \mu M}\), and 12 \text{ \mu M} (\(K_I = 10 \text{ \mu M}\).
Structure–activity relationships for CYP51 inhibition within the CP-320626 series

Inhibition of CYP51 by an N-aroyl-α-amino acid derivative such as CP-320626 is novel. We investigated structure–activity relationships of this inhibition by preparing and evaluating other substituted phenylalanines, glycine and other α-amino acids bearing aliphatic, acidic, and basic sidechains, and analogs having the 5-chloroindole moiety replaced by other aryl residues. The syntheses of these compounds were straightforward. The structures and inhibitory activities in HepG2 cell cholesterol synthesis inhibition, recombinant human CYP51 enzyme inhibition, and glycogen phosphorylase enzyme inhibition assays are presented in Tables 3 and 4.

A high degree of correlation between HepG2 cell cholesterol synthesis inhibition and CYP51 inhibition was observed where both activities were measured ($R^2 = 0.77$; Fig. 7), indicating that most (~80%) of the variation in HepG2 cell cholesterol synthesis inhibition is a consequence of CYP51 inhibition. By contrast, there was no correlation between glycogen phosphorylase inhibition and either HepG2 cell cholesterol synthesis inhibition or CYP51 inhibition within the series ($R^2 = 0.029$; not shown). Consistent with this finding is the observation that the two most potent CYP51 inhibitors discovered from within this series, [8] and [9], were only modest glycogen phosphorylase inhibitors (Table 3), whereas, two of the most potent glycogen phosphorylase inhibitors of the series, [2] and [4], showed no appreciable CYP51 inhibition at concentrations of up to 10 μM (Table 3). Furthermore, ketoconazole, a potent inhibitor of CYP51 (IC$_{50}$ = 0.1 μM), did not inhibit human liver glycogen phosphorylase at concentrations up to 10 μM, and the potent glycogen phosphorylase inhibitor, CP-91149 (IC$_{50}$ = 0.13 μM; 9), inhibited CYP51 activity by only 15%, at 10 μM.

The des-fluoro analog 1-phenylalanine congener [3] (10) retains the high CYP51 inhibition of CP-320626. The unnatural α-phenylalanine enantiomers of CP-320626 and [3] ([2] and [4], respectively), were evaluated and found not to inhibit CYP51 at 10 μM. As mentioned above, this structure–activity relationship (SAR) is distinctly divergent from that of glycogen phosphorylase inhibition in N-(5-chloroindole-2-carbonyl)-phenylalanine amides, where such enantiomers have similar activity (10). CYP51 inhibitory activity was also very sensitive to the presence of the benzyl side chain in the compounds examined. Histidine [17], 3-pyridylalanine [12], tyrosine [13], and valine [19] gave somewhat lower levels of inhibition, glycine [16] and alanine [18] were of further reduced inhibitory activity, and the corresponding lysine [14] and aspartic acid [15] derivatives did not show appreciable inhibition at 10 μM. Substitution of the phenylalanine ring at the para position with chloro [10] or methoxy [11] substituents did not diminish CYP51 inhibitory activity, and substitution at the ortho position with either fluoro [8] or chloro [9] substituents produced the most potent inhibitors in the series. The effect of al-

![Table 3](image-url)
tering the 5-chloroindole substituent was also examined. Inhibitory potency was maintained when the chlorine was replaced with hydrogen [7] but not with fluorine [6]. N-methylindole [23] and benzofuran [24] analogs showed little CYP51 inhibitory activity, indicating a possibly critical H-bond contribution from the indole of CP-320626. Benzamide [21] and pyrrole-2-carboxamide [22] analogs also showed marginal inhibitory activity.

**DISCUSSION**

In this report, we describe dual-action glycogenolysis/cholesterolgenesis inhibitors that act through inhibition of both glycogen phosphorylase and CYP51. Inhibition of the activities of glycogen phosphorylase and CYP51 are independent, because CP-320626 and related analogs exhibit divergent structure–activity relationships, with no correlation between the two inhibitory activities across the series of evaluated inhibitors ($R^2/H_11005 = 0.029$). Indeed, the two activities are divergent to the extent that there exist analogs within the series that are potent glycogen phosphorylase inhibitors that are devoid of appreciable CYP51 inhibitory activity [e.g., [2], [4]] and analogs within the series that are potent CYP51 inhibitors but are weak glycogen phosphorylase inhibitors [e.g., [8], [9]]. Furthermore, ketoconazole and azalanstat, which inhibit CYP51 activity with IC$_{50}$ values of 0.1 µM and 0.01 µM, respectively, show no inhibition of glycogen phosphorylase activity at concentrations up to 10 µM, whereas the glycogen phosphorylase inhibitor, CP-91149, which inhibits glycogen phosphorylase activity with an IC$_{50}$ of 0.13 µM (9), showed only slight inhibition of CYP51 activity at a concentration of 10 µM.

CP-320626 demonstrates a pattern of interaction with CYP51 that suggests that interaction with the enzyme active center is at the substrate binding pocket near the heme but not through coordinate interaction with the heme iron. Thus, CP-320626 acts to inhibit CYP51 exclusively as a substrate mimetic rather than as a combined substrate analog/heme binding agent. This is in stark contrast to theazole-containing CYP51 inhibitors, such as ketoconazole, miconazole, itraconazole, fluconazole, and azalanstat, whose unhindered basic imidazole nitrogen interacts coordinately with the heme iron of CYP51 in a characteristic Type II pattern of interaction, replacing H$_2$O as the native sixth ligand (25–29). This observation is consistent with the absence of any basic atom in CP-320626 that could form a coordinate complex with the heme iron of CYP51. In this regard, Haitao et al. (30) have recently reported a series of nonazole antifungal agents that bind to the active center of Candida albicans CYP51 to inhibit enzymatic activity without coordinate interactions with the active site heme. Whether these observations imply a potential for greater specificity of the indole-2-carboxamide series of CYP51 inhibitors, relative toazole-containing CYP51 inhibitors, remains to be explored.

The ability to treat hyperglycemia and hyperlipidemia simultaneously with a single agent is attractive, especially given that a high percentage of type 2 diabetics but are weak glycogen phosphorylase inhibitors but are weak glycogen phosphorylase inhibitors [e.g., [8], [9]]. Furthermore, ketoconazole and azalanstat, which inhibit CYP51 activity with IC$_{50}$ values of 0.1 µM and 0.01 µM, respectively, show no inhibition of glycogen phosphorylase activity at concentrations up to 10 µM, whereas the glycogen phosphorylase inhibitor, CP-91149, which inhibits glycogen phosphorylase activity with an IC$_{50}$ of 0.13 µM (9), showed only slight inhibition of CYP51 activity at a concentration of 10 µM.

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The ability to treat hyperglycemia and hyperlipidemia simultaneously with a single agent is attractive, especially given that a high percentage of type 2 diabetics also exhibit dyslipidemia (7, 8, 31), are therefore at increased risk of developing cardiovascular disease (7, 8, 31), and are as a consequence often treated with combined therapy to

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**TABLE 4.** SAR of lanosterol 14-demethylase inhibition by analogs of CP-320626

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<th>Number</th>
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<th>Glycogen Phosphorylase Inhibition $IC_{50}$, nM</th>
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The inhibition of [2-14C]acetate incorporation into cholesterol in HepG2 cells produced by 10 µM compound (average of triplicate determinations) and the concentration of compound required to inhibit by 50% the activity of human liver glycogen phosphorylase were determined as outlined in Experimental Procedures.

**Fig. 7.** Correlation between HepG2 cell cholesterol synthesis inhibition and CYP51 inhibition. Inhibition of [2-14C]acetate incorporation into cholesterol in HepG2 cells (average of triplicate determinations) and inhibition of the enzymatic activity of recombinant human CYP51 (average of triplicate determinations), for a series of CP-320626 analogs, were assessed at a concentration of 10 µM as outlined in Experimental Procedures.
improve both the hyperglycemia and the dyslipidemia (7). In this regard, both glycogen phosphorylase inhibitors and CYP51 inhibitors have independently shown favorable efficacy attributes in humans. For example, a prototype glycogen phosphorylase inhibitor, CP-316819, was shown to reduce the postglucagon rise in plasma glucose levels by 54% in healthy human volunteers at a dose of 6 mg/kg (32). In addition, CYP51 inhibitors, such as ketoconazole, have demonstrated significant total and LDL cholesterol-lowering activity in experimental animals (28, 33–35) and in humans, when administered as extended, high-dose therapy for the treatment of prostate cancer (24, 36–39), Cushing’s syndrome (40), or hyperandrogenism (41). Furthermore, the glucose-lowering and cholesterol-reducing activities of CP-320626 occur at approximately the same doses in experimental animals (~5 mg/kg) suggests that the antidiabetic and antihypercholesterolemic properties of this class of agents may occur at approximately equivalent doses in clinic.

However, despite the potential benefits associated with the combined glucose-lowering and lipid-lowering efficacy of these inhibitors, the potential for adverse effects associated with long-term lanosterol accumulation in clinic is untested. Indeed, adverse effects such as lenticular posterior cataracts and ichthyosis have been noted following accumulation of desmosterol in various tissues after treatment with desmosterol reductase inhibitors such as triparanol (42). However, although lanosterol, dihydrolanosterol, and various other methylsterols have been shown to accumulate in the liver and blood of rats treated with ketoconazole (33), in the liver, blood, and various other tissues of hamsters treated with azalanstat (28, 34), and in the blood of humans following prolonged, high-dose treatment with ketoconazole (24, 37, 38), the rapid secretion of these methylsterols into the bile (24, 28) and elimination via the feces (24) may lessen or even prevent similar adverse effects.

In addition, the potential for oxidized lanosterols (43–46), substituted lanosterols (43–46), and CYP51 reaction product intermediates (47, 48) to act secondarily as feedback inhibitors of posttranscriptional processes involved in HMG-CoA reductase synthesis (43, 44, 46, 47, 49) and/or as stimulators of HMG-CoA reductase degradation (43, 48, 49) could further limit the degree of lanosterol accumulation after treatment with these agents.

In this regard, it is noteworthy that although rat plasma levels of lanosterol and dihydrolanosterol were elevated after treatment with CP-320626, their elevation was minimal relative to the observed reduction in plasma cholesterol (16%), representing only ~0.12% of initial plasma cholesterol levels, with no increases in any other sterol precursors noted after treatment. This observation indicates that neither lanosterol nor any other sterol precursor replaces cholesterol in the circulation after treatment with CP-320626, as was reported for desmosterol accumulation after triparanol treatment (34). These observations also suggest that the lanosterol and dihydrolanosterol that could potentially accumulate after CP-320626 treatment are either efficiently eliminated via the bile or are converted to oxidized metabolites that exhibit powerful inhibitory effects on HMG-CoA reductase production or degradation, as outlined above.

An additional concern with CYP51 inhibition as a mechanism for cholesterol lowering is the observation that most CYP51 inhibitors identified to date have shown poor specificity relative to the CYPs involved in adrenal and gonadal steroidogenesis (27, 29, 36, 40). Indeed, it is for this lack of specificity for CYP51 versus the CYPs involved in androgen formation (29, 36, 40) and adrenal steroid formation (29, 36, 40) that high-dose ketoconazole treatment has been effectively utilized in the treatment of prostate carcinomas (24, 36, 39) and Cushing’s syndrome (36). The CYP51 inhibitor azalanstadt has also shown a low specificity for CYP51 relative to these steroidogenic CYPs (27) and has reportedly induced a variety of effects associated with cortisol, mineralocorticoid, and gonadal steroidogenesis inhibition (27, 50). That CP-320626 and related analogs show up to a 50-fold specificity (based on relative Kᵢ values) for CYP51 versus CYP5A4, CYP2D6, and CYP2C9 and that CP-320626 does not coordinate with the heme iron of CYP (see above), together suggest that such specificity for cholesterolgenesis inhibition versus steroidogenesis inhibition is plausible, but would need to be demonstrated preclinically.

Finally, the importance of CYP51 activity in spermatogenesis and in oogenesis through production of the signal sterols FF-MAS and T-MAS in oocytes and testis (51, 52) suggests that systemically available CYP51 inhibitors could also limit these processes. Indeed, inhibition of meiosis by ketoconazole (53, 54) and azalanstat (50) has recently been demonstrated. Clearly, dual-action glycogen phosphorylase/CYP51 inhibitors would need to have their actions restricted to the liver, the primary site for both the glucose-lowering and cholesterol-lowering effects of these compounds, and this could be accomplished through development of inhibitors that are subject to first-pass hepatic metabolism.

In conclusion, dual-action glycogen phosphorylase/CYP51 inhibitors, such as those described in this report, have the potential to favorably affect both hyperglycemia and hypercholesterolemia in patients with type 2 diabetes. However, such agents would need to avoid potential adverse effects associated with cholesterologenesis intermediate accumulation, inhibition of adrenal and gonadal steroidogenesis, and inhibition of spermatogenesis and oogenesis typically associated with long-term treatment with systemically available, poorly selective, CYP51 inhibitors.

REFERENCES


