Effects of a Novel Dual Lipid Synthesis Inhibitor and Its Potential Utility in Treating Dyslipidemia and Metabolic Syndrome


Running Title: Effects of a Dual Lipid Synthesis Inhibitor

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Abstract

We have identified a novel ω-hydroxy-alkanedicarboxylic acid, ESP 55016, that favorably alters serum lipid variables in an animal model of diabetic dyslipidemia. In obese female Zucker (fa/fa) rats, ESP 55016 reduced serum nonHDL-C, triglyceride, and non-esterified fatty acid levels, while elevating serum HDL-C and β-hydroxybutyrate levels in a dose-dependent manner. ESP 55016 reduced fasting serum insulin and glucose levels while also suppressing weight gain. In primary rat hepatocytes, ESP 55016 increased the oxidation of 14C-palmitate in a dose- and carnitine palmitoyl transferase-I (CPT-1)-dependent fashion. Furthermore, in primary rat hepatocytes ESP 55016 not only inhibited fatty acid synthesis from 14C-glucose, 14C-pyruvate, 14C-acetate, and 3H2O, but inhibited sterol synthesis as well, with IC50s in the micromolar range for both pathways; inhibition occurred within five minutes. Sterol synthesis inhibition occurred at a step prior to mevalonate formation in the sterol biosynthetic pathway. The physiological relevance of the in vitro findings with ESP 55016 was confirmed in vivo, demonstrable by inhibition of both hepatic fatty acid and sterol synthesis within two hours of administration. The “dual inhibitor” activity of ESP 55016 in primary hepatocytes, was unlikely due to activation of the AMP-activated protein kinase (AMPK) pathway since AMPK and acetyl-CoA carboxylase (ACC) phosphorylation states as well as ACC activity were not altered by ESP 55016. Further studies indicated the conversion of ESP 55016 to a CoA derivative in vivo. ESP 55016-CoA markedly inhibited the activity of partially purified ACC. The activity of partially purified HMG-CoA reductase was not altered by the
xenobiotic-CoA. These data suggest that ESP 55016-CoA favorably alters lipid metabolism in animal models of dyslipidemia in part, by initially inhibiting fatty acid and sterol synthesis plus enhancing oxidation of fatty acids through the ACC/malonyl-CoA/CPT-I regulatory axis.
Introduction

Systematic chemical modification of long chain hydrocarbons has been an approach to identify novel lipid regulating compounds for treating human dyslipidemias (1). Over the last thirty years, a variety of compound classes have been identified which demonstrate activity in animal models of dyslipidemia. For example, Parker and coworkers describe a series of alkylxyarylcarboxylic acids of which 5-(tetradecyloxy)-2-furancarboxylic acid (TOFA) has been well studied (2, 3). TOFA showed marked hypolipidemic activity in both rats and monkeys (2). Another series termed MEDICA (β,β'-methyl- α, ω-dicarboxylic acids) has been developed and extensively studied by Bar-Tana and coworkers (4). In particular, MEDICA 16 has been shown to possess hypolipidemic, anti-diabetic, and anti-atherosclerotic activity in relevant animal models (5, 6). In work spanning many years, Berge, Bremer and colleagues have described a series of 3-thia fatty acids that possess properties similar to MEDICA compounds when administered to animal models of dyslipidemia (7-9). In addition, Pill and coworkers described a series of ω-substituted alkyl carboxylic acids that showed insulin sensitizing activity and lipid regulating properties in rodents (10-12). Finally, Bisgaier and coworkers describe a series of carboxyalkylethers with lipid regulating activity, including HDL-C elevation, in rats (13). One of the carboxyalkylethers, PD-72953, also known as CI-1027 or gemcabene, has been administered to humans and shown to have effects on serum lipid levels (14). Currently, no consensus exists on the primary mechanism of action of long-chain hydrocarbon derivatives that eventually leads to the favorable lipid changes in
humans or to the amelioration of metabolic derangements in animal models of
dyslipidemia, diabetes, and obesity. Proposed primary mechanisms of action
include alterations in enzyme activities through allosteric or redox state changes
and modulation of gene expression through activation or inhibition of nuclear
hormone receptors (3, 9, 13, 15-21).

In the current report, we describe a novel ω-hydroxy-alkanedicarboxylic acid, ESP 55016, that favorably alters serum lipid profiles in the Zucker rat, an
animal model of diabetic dyslipidemia. Data from those studies led us to
hypothesize that fatty acid oxidation was enhanced by ESP 55016. We have
used both in vitro and in vivo models to test this hypothesis while focusing our
efforts on identifying the short-term (minutes to hours) metabolic changes
induced by ESP 55016 with the goal of identifying the initial primary
mechanism(s) of action.
Materials and Methods

Materials

Dulbecco's Modified Eagle Media containing 4.5 g/L D-glucose (DMEM), non-essential amino acids, HEPES buffer, sodium pyruvate and gentamicin were obtained from Mediatech, Inc. (Herndon, VA). Fetal bovine serum, Dulbecco’s-phosphate buffered saline (D-PBS), Glutamine and GlutMax-1™ were obtained from Invitrogen Life Technologies (Grand Island, NY). Bovine albumin, fraction V 35% solution, insulin, dexamethasone, 14C-sodium bicarbonate, and zaragosic acid were obtained from Sigma Chemical Company (St. Louis, MO). The following items were obtained from PerkinElmer Life Sciences, Inc. (Boston, MA): [1-14C]-acetic acid sodium salt (40-60 mCi/mmol), R/S-[2-14C]-mevalonolactone (40-60 mCi/mmol), [U-14C]-palmitic acid sodium salt (54.5 mCi/mmol), 3H2O (1 mCi/g), [2-14C]-pyruvic acid (22 mCi/mmol), and D-[1-14C]-glucose (54.5 mCi/mmol). [U-14C]-lauric acid (55 mCi/mmol) and [U-14C]-caprylic acid (53 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Biocoat® type I collagen coated 6-well plates were purchased from Becton Dickinson Labware (Bedford, MA). ScintiVerse™ scintillation cocktail was obtained from Fisher Scientific Chemicals (Fairlawn, NJ).

Synthesis of ESP 55016

8-Hydroxy-2,2,14,14-tetramethyl-pentadecanedioic acid (ESP55016), a white crystalline solid, was prepared by a multistep reaction sequence, as follows: 1,5-dibromopentane was reacted with ethyl lithio-isobutyrate at low temperature (-40 to –10 °C) to produce ethyl 7-bromo-2,2-dimethylheptanoate,
which was further used as an alkylating agent in the reaction with \( p \)-toluenesulfonylmethyl isocyanide (TosMIC). The TosMic adduct thus obtained was subjected to hydrolysis in acidic conditions to yield 8-oxo-2,2,14,14-tetramethyl-pentadecanedioic acid diethyl ester, which was then reduced at the central keto moiety to a hydroxyl group and afford diethyl 8-hydroxy-2,2,14,14-tetramethyl-pentadecanedioate. Hydrolysis of the ester terminal groups of the latter in basic conditions finally produced the desired compound 8-hydroxy-2,2,14,14-tetramethyl-pentadecanedioic acid (ESP 55016).

**Obese Female Zucker Rat Study.**

Ten-twelve week (400-500 grams) old obese female Zucker rats Crl: (Zuc)-faBR were obtained from Charles River Laboratories and acclimated to the laboratory environment for 7 days. ESP 55016 was dissolved or suspended in a dosing vehicle consisting of 20% ethanol and 80% polyethylene glycol-200 [v/v] or 1.5% (w/v) carboxymethyl cellulose and 0.2% (v/v) Tween-20 (CMC-Tween); we did not observe any significant differences in the response of compound-treated animals with the two vehicles. Dose volume of ESP 55016 or vehicle was set at 0.25% of body weight. Doses were administered daily by oral gavage for 14 days, approximately between 8-10AM. Animals were fasted for 6 hours prior to blood collection. To measure blood glucose levels, the tail vein was lanced and blood spotted onto a glucometer (Bayer, Model 3952E). Prior to and after 7 days of dosing, a 1.0-2.0 mL sample of blood was collected by administering \( \text{O}_2/\text{CO}_2 \) anesthesia and bleeding from the orbital venous plexus. Following 14 days of dosing, blood was collected by cardiac puncture after
euthanasia with CO₂. All blood samples were promptly processed for separation of serum and stored at -80°C until analysis.

**Serum Analysis**

Serum triglyceride (Triglycerides Kit # 1488872, Boehringer Mannheim Corporation, Indianapolis, IN), β-hydroxybutyrate (β-HBA kit catalog # 310A Sigma Diagnostics, St. Louis, MO) and non-esterified fatty acid (NEFA-C kit catalog # 995-75409 Wako Diagnostics, Richmond, VA) levels were determined with commercially available kits on a Hitachi 912 Automatic Analyzer (Roche Diagnostics Corporation, Indianapolis, IN). Serum insulin was determined using an ELISA kit (Alpco Diagnostics, Windham, NH). Serum total cholesterol was assayed by a colorimetric assay based on the method of Allain et al (22) adapted for the Hitachi 912. Serum lipoproteins were separated by gel-filtration chromatography on a Superose 6HR (1x 30 cm) column equipped with on-line detection for total cholesterol as described by Kieft et al. (23). Total cholesterol content of VLDL, LDL and HDL lipoproteins were calculated by multiplying the independent values determined for serum total cholesterol by the percent area of the lipoprotein in the respective profiles.

**Hepatocyte Isolation**

Male Sprague-Dawley, Crl:CD (SD) rats were fed Purina Certified Rodent Chow pellets #5001 (Ralston-Purina Co., St. Louis, MO) and nutritionally staged for hepatocyte isolation by fasting for 48 hrs (used in studies with ¹⁴C-acetate, ¹⁴C-pyruvate, ¹⁴C-mevalonolactone, ³H₂O, and ¹⁴C-fatty acids) or fasting for 48 hours and re-feeding a high carbohydrate diet for 48 hours (used in study with
Rats were anesthetized with intraperitoneal injection of sodium pentobarbital. Livers were perfused and cells were isolated according the method of Ulrich (24). Hepatocytes were plated in DMEM with glucose and serum (DMEM containing 4.5 g/L D-glucose supplemented to 20% fetal bovine serum, 14 mM HEPES, 0.2% bovine albumin, 2 mM L-glutamine, 1X MEM non-essential amino acids, 100 nM insulin, 100 µg/mL dexamethasone and 20 µg/mL gentamicin) at a density of 1.5 x 10^5 cell/cm^2 on collagen-coated 6-well plastic dishes. Following attachment (3-4 hours) serum-free media was utilized. In all metabolic labeling experiments, radiolabeled compounds and ESP 55016 or vehicle were added to cultures at the same time.

**In Vitro Measurement of Mitochondrial β-Oxidation**

Hepatocyte cultures were incubated in the presence of 14C-palmitic acid, 14C-lauric acid, or 14C-caprylic acid and assayed for production of acid-soluble products. Experiments were performed using DMEM without D-glucose. All radiolabeled fatty acids were at final concentrations of 200 µM and specific activities of 1 mCi/mmol. Vehicle contained 0.3% DMSO. Fatty acid oxidation rates were measured over 4 hours. Incubations were stopped by addition of perchloric acid followed by extraction with hexane. The acidic aqueous phase was assayed for radioactive content and represented acid soluble products.

**In Vitro Measurement of Lipid Synthesis**

Hepatocyte cultures were incubated in the presence of 14C-acetate, 14C-pyruvate, 14C-glucose, or 3H2O. Experiments were performed in DMEM with glucose plus the appropriate metabolic precursor as follows: 1 µCi/mL 14C-
acetate, 0.5 µCi/mL \(^{14}\)C-pyruvate, 5 µCi/mL \(^{14}\)C-glucose or 0.8 mCi/mL \(^{3}\)H\(_2\)O. In the study utilizing \(^{14}\)C-glucose, D-glucose was not included in the culture media. Cells were treated with compound or vehicle (0.3% DMSO) for up to 4 hours followed by lipid isolation.

**In Vivo Measurement of Lipid Synthesis with \(^{14}\)C-Acetate**

Rats were fasted for 48 hrs and then refed for 48 hrs with the last 2 hrs of refeeding involving exposure to ESP 55016 and subsequent metabolic labeling. Specifically, twenty-five male Sprague-Dawley (Crl: CD (SD) IGSBR) rats (5 per group) weighing approximately 125-150 grams were fasted for 48 hours and subsequently refed for 48 hours a purified diet containing 58% sucrose and 14% corn starch as the carbohydrate and 19% casein as the protein sources (Diet # D01121101B, Research Diets, Inc., New Brunswick, NJ). Near the end of refeeding, rats in each group received a single dose of 3, 10, 30, or 100 mg/kg ESP 55016 or the CMC-Tween vehicle by oral gavage. One hour after dosing each rat received an intraperitoneal injection of 0.3 mL of saline containing 40 µCi of \([1-{^{14}}\)C\] acetate. One hour later, animals were sacrificed, a blood (for serum) and liver samples were obtained and used to quantify radiolabeled saponified and non-saponified lipid levels. This scheme resulted in a total 2 hour exposure to ESP 55016.

**In Vitro Measurement of Sterol Synthesis with \(^{14}\)C-mevalonate**

Lipid synthesis experiments were performed in hepatocytes cultured in DMEM with glucose and further supplemented with 100 µM R/S mevalonolactone plus the appropriate metabolic precursor as follows: \(^{14}\)C-acetate (1.0 µCi/mL,
specific activity 0.2 Ci/mol) and \(^{14}\)C-mevalonate (1.0 µCi/mL, specific activity 8.4 Ci/mol). Cells were treated with vehicle (1.0% DMSO), ESP 55016, or 200 nM zaragosic acid for 1 hour.

**In Vivo Measurement of Sterol Synthesis \(^{14}\)C-mevalonate**

Twenty male Sprague-Dawley (Crl: CD (SD) IGSBR) rats weighing approximately 125-150 grams were divided into four groups of five and acclimated for a minimum of 7 days to an alternating cycle of 12 hours of light (4 PM to 4 AM) and darkness (4 AM-4 PM) while having free access to water and standard Purina Laboratory Rodent Chow® (5001). Following the acclimation phase animals were given a Purina 5001 chow diet supplemented with 2.5% cholestyramine for an additional 5 days while maintaining the same light/dark cycle. Cholestyramine feeding prevents reabsorption of intestinal bile acids thereby inducing hepatic HMG-CoA reductase activity several fold (25). The light cycle used transfers the natural peak of the diurnal cholesterol biogenesis cycle to approximately 10 AM (26). By the end of the acclimation and cholestyramine feeding phases rats weighed an average of 280g. Animals were administered vehicle (CMC-Tween) or vehicle plus 30 mg/kg ESP 55016 by oral gavage. One hour later, animals received a single intraperitoneal injection of either sodium [1-\(^{14}\)C]-acetate (40 µCi/rat) or R/S-[2-\(^{14}\)C]-mevalonolactone (4 µCi/rat). Following 1 hour of isotope exposure animals were euthanized with CO\(_2\). Blood was collected by cardiac puncture and processed for separation of serum and a portion of the liver was removed and frozen in liquid nitrogen to determine isotope incorporation into nonsaponified (sterols) lipids.
Isolation of Saponifiable (Fatty Acids) and Nonsaponifiable (Sterol) Lipids

Following metabolic labeling in vitro and in vivo, saponified and nonsaponified lipids were extracted from serum, liver, and cell suspensions essentially as described by Slayback et al. (27).

Methods for Acetyl-CoA Carboxylase and AMP-activated Protein Kinase Phosphorylation State

Crude hepatocyte lysates for ACC and AMPK phosphorylation state assessments were isolated with a digitonin-based lysis buffer containing 20 mM Tris (pH 7.5), 50 mM NaCl, 0.25 M sucrose, 50 mM NaF, 30 mM Na Pyrophosphate, 2 mM DTT, 10 µM ZnCl₂, 0.1 mM Na vanadate, 0.4 mg/mL Digitonin and 1X Phosphatase Inhibitor Cocktail I (Sigma-St. Louis, MO). A 35% ammonium persulfate cut was obtained from the crude lysate and re-suspended in digitonin-free lysis buffer. Protein concentrations within each sample were subsequently determined using the BCA protein assay (Pierce-Rockford, IL).

Thirty to ninety micrograms of protein from primary rat hepatocytes treated with DMSO or 10-300 µM ESP 55016, was separated by size via a 4% stacking/7.5% resolving SDS PAGE and transferred to PVDF membrane. For ACC phosphorylation, the membrane was probed with a rabbit polyclonal IgG directed against phosphorylated acetyl-CoA carboxylase at serine 79 (Upstate Biotech-Lake Placid, NY). For AMPK phosphorylation, the membrane was probed with a rabbit polyclonal IgG directed against phosphorylated AMP-activated Protein Kinase at threonine 172 (Cell Signaling Technologies- Beverly, MA). For both
proteins, bound primary antibody was captured and detected with a horseradish peroxidase conjugated rabbit polyclonal IgG (Jackson ImmunoResearch Laboratories, Inc.-West Grove, PA), and ECL + plus Western Blot Development Substrate (Amersham Pharmacia Biotech-Piscataway, NJ) followed by exposure to Biomax ML film (VWR Scientific Products-Chicago, IL).

**Synthesis, Detection and Identification of Xenobiotic-CoA derivative**

Rat liver microsomes were used to generate a 55016-CoA conjugate by the method of Pande and Meade (28). The reaction products were separated using a Waters Alliance 9960 high pressure liquid chromatograph (HPLC) fitted with an Intersil C:18, 5 µm, 250 x 4.60 mm column (MetaChem, Lake Forest, CA). Peaks were detected at 254 nm using a Waters 2996 photodioarray detector. Elution peaks in which appearance was both time and dose dependent in the microsomal assay, were collected as ESP 55016-CoA candidates and subjected to mass spectrometry. HPLC fractions were taken up in standard electrospray ionization (ESI) positive ion buffer [water:methanol:acetic acid (48:48:4)] to a concentration of ~ 0.01 mg/mL acyl-CoA, as determined by OD<sub>254</sub>, and infused at 60 µL/hr into the Apollo ESI source of a Bruker Apex III FTMS instrument operating in the positive ion detection mode. Ions were accumulated for 1.5 seconds in the hexapole segment before injection into the cell. Transients (8-128) were accumulated using the standard SIMPLE.EXP pulse program. The resulting free induction decays (FID) (512K points) were apodized using a squared sine bell function, Fourier transformed, and the absolute magnitude
signal was displayed. The ESP 55016-CoA species was easily detected and identity confirmed by molecular mass.

**Isolation of partially purified Acetyl-CoA Carboxylase (ACC) and Assessment of Enzyme Activity**

ACC was partially purified from rat liver using strepavidin-agarose and the ACC reaction performed essentially as described (29, 30). The reaction involved preincubation of the partially purified enzyme at 37°C for 30 minutes in the presence of 50 mM Tris (pH 7.5), 10 mM potassium citrate, 10 mM MgCl₂, 1 mM DTT and 0.075 mg/mL BSA (Fatty Acid-Free) to activate ACC followed by addition of 4 mM ATP, 0.5 mM acetyl-CoA and 12.5 mM ¹⁴C-sodium bicarbonate (7.2 mCi/mMol) (Sigma-St. Louis, MO). Incorporation of ¹⁴C into acid stable products (¹⁴C-malonyl-CoA) was determined by liquid scintillation spectroscopy. Data were transformed into µmol of malonyl-CoA formed as a function of time to determine the reaction rate (1 Unit of activity is defined as one µmol malonyl-CoA formed per minute). Characterization of partially purified ACC under these reaction conditions indicated that generation of malonyl-CoA was linear for up to 10 minutes with 0.5 ng/µL of affinity-purified protein. Further characterization indicated that the Kₘ values for key variables were as follows: acetyl-CoA, 26 µM; ATP, 0.2 mM; citrate, 4 mM. The Vₘₐₓ under saturating conditions was approximately 4 U/mg.
Results

Effect of ESP 55016 in Obese Female Zucker Rats

The obese female Zucker rat has a mutation in the leptin receptor that leads to a human-like non-insulin dependent diabetes mellitus (NIDDM) with associated dyslipidemia. We assessed the effects of oral daily administration of ESP 55016 on serum lipid changes after one and two weeks at doses up to 100 mg/kg (Figure 2). After one week of dosing, nonHDL-C was reduced at almost all doses tested (except at 10 mg/kg). After two weeks, nonHDL-C increases of 1.38 and 1.33-fold were observed at 10 and 30 mg/kg, respectively, likely reflecting an increase in cholesterol biosynthetic enzymes and mRNAs which have been observed with cholesterol synthesis inhibitors in rodents (31). However, at 100 mg/kg there was an 81% and 80% decrease after both one and two weeks of dosing likely indicating a sufficient concentration of inhibitor to suppress the increased cholesterol biosynthetic enzyme mass. A similar but less pronounced compensation (compare one week vs. two week data) was reflected in the triglyceride response however, a typical dose response was observed with decreases of 30, 52, 78, and 91% at 3, 10, 30, and 100 mg/kg after two weeks of treatment. HDL-C levels increased in a time and dose-dependent manner with increases of 1.57, 2.45, and 2.79-fold at 10, 30, and 100 mg/kg, respectively, after two weeks. ESP 55016 caused similar changes in nonHDL-C, triglycerides, and HDL-C in other animal models including the chow-fed rat, cholesterol-fed rat,
and LDL-receptor knockout mouse (data not shown). Therefore, ESP 55016 favorably alters lipids in models with either normal or deficient leptin signaling.

Non-esterified fatty acids (NEFA) were reduced after ESP 55016 treatment and generally mirrored by a concomitant rise in serum \( \beta \)-hydroxybutyrate (\( \beta \)-HBA) levels (Figure 3). At two weeks NEFA levels were reduced by 30, 28, 39, 44, and 47% at 1, 3, 10, 30, and 100 mg/kg of ESP 55016, respectively. Vehicle treated control animals displayed elevated serum \( \beta \)-HBA over the two week time course of the study consistent with the progression of diabetes. At doses of 10 mg/kg or higher, there was an increase in serum \( \beta \)-HBA in the ESP 55016-treated animals above the elevations observed in vehicle treated controls. After two weeks of dosing at 10, 30, and 100 mg/kg, ESP 55016 increased serum \( \beta \)-HBA levels by 3-4 fold over pretreatment values.

At an ESP 55016 dose of 100 mg/kg, serum levels of glucose and insulin were markedly reduced in the fasted animal (Table 1). Glucose levels were reduced by 17% and 14% after one and two weeks of treatment, respectively, while insulin levels were reduced by 57% and 47% during the same time period; no significant changes were observed in vehicle-treated controls. ESP 55016 treated animals also gained less weight over the two week study period. In comparison to pretreatment weight, vehicle treated animals gained 9% body weight while ESP 55016-treated animals gained only 3% body weight. Long-term studies in Zucker rats demonstrated ESP 55016-treatment dramatically reduced weight gain over a 12-week period despite no differences in food intake compared to untreated animals (data not shown). Note that it typically takes 2
days of fasting for Zucker rats to reduce serum insulin by 50% (32). These data indicate that ESP 55016 favorably alters serum lipid variables by reducing nonHDL-C, triglycerides, and nonesterified fatty acids while raising HDL-C. Furthermore, ESP 55016 appears to also possess the ability to improve insulin sensitivity while suppressing body weight gain.

**Effect of ESP 55016 on Fatty Acid Oxidation in Primary Rat Hepatocytes**

Since ESP 55016 treatment of Zucker rats elevated serum β-HBA, we hypothesized that the compound stimulated hepatic fatty acid oxidation. ESP 55016 dose-dependently (3 µM to 30 µM) stimulated \(^{14}\text{C}\)-palmitic acid oxidation in primary rat hepatocytes after 4 hours reaching a level 71% greater than control at 30 µM (Figure 4A). ESP 55016-stimulated oxidation of \(^{14}\text{C}\)-palmitic acid was enhanced further by L-carnitine, a substrate for CPT-I, an essential enzyme in the transport of long-chain fatty acids into the mitochondria (Figure 4A). ESP 55016-stimulated oxidation of \(^{14}\text{C}\)-palmitic acid was completely blocked by R-aminocarnitine a CPT-I inhibitor (Figure 4A). These data suggest that ESP 55016 increases mitochondrial β-oxidation of long chain fatty acids through a CPT-I-dependent pathway.

To test if ESP 55016 alters β-oxidation pathways within the mitochondria, we measured oxidation of medium-chain fatty acids in primary hepatocytes. Lauric (C12:0) and caprylic (C8:0) acid do not require CPT mediated translocation into the mitochondria and thus allow assessment of intra-mitochondrial β-oxidation capacity. ESP 55016 neither altered the oxidation of \(^{14}\text{C}\)-lauric nor \(^{14}\text{C}\)-caprylic acid under any condition including the presence or
absence of L-carnitine or R-aminocarnitine (Figure 4B). These data suggest that ESP 55016 does not alter intra-mitochondrial β-oxidation pathways, but rather expedites the CPT-I-dependent import of long-chain fatty acids into the mitochondria.

**Effect of ESP 55016 on Lipid Synthesis in Primary Rat Hepatocytes**

β-oxidation and fatty acid synthesis are opposing metabolic processes. We used four different metabolic precursors to determine if ESP 55016 inhibited fatty acid synthesis in primary rat hepatocytes. As shown in Figure 5, ESP 55016 inhibited fatty acid synthesis in a dose-dependent manner when using $^3$H$_2$O, $^{14}$C-glucose, $^{14}$C-pyruvate, and $^{14}$C-acetate as tracers. The IC$_{50}$s were similar ranging from 2-13 µM. Furthermore, ESP 55016 also inhibited sterol synthesis from all four precursors with IC$_{50}$s ranging from 2-10 µM (Figure 5). Inhibition of lipid synthesis occurred rapidly with similar levels of inhibition observed when cells were exposed to ESP 55016 for times ranging from 5 minutes to 4 hours (data not shown). ESP 55016 at 30 µM also inhibited triglyceride secretion by >80% in treated hepatocytes (data not shown). The rapid inhibition of fatty acid and sterol biosynthesis (minutes) suggested that ESP 55016 or its metabolites altered key enzyme activities of these pathways through a non-transcriptional mechanism.

**Effect of ESP 55016 on Lipid Synthesis in vivo with $^{14}$C-acetate**

To confirm the physiological relevance of the *in vitro* findings, we tested whether ESP 55016 inhibited hepatic fatty acid and sterol synthesis *in vivo* in the fasted-refed rat. Figure 6 indicates that ESP 55016 dose-dependently reduced
the incorporation of $^{14}$C-acetate into both fatty acids and sterols in serum and liver. The IC$_{50}$ appeared to be between 3-10 mg/kg for both the liver and serum.

Effect of ESP 55016 on $^{14}$C- mevalonolactone Incorporation into Sterols in vitro

To further define the step(s) in the cholesterol biosynthetic pathway that may be inhibited by ESP 55016, we measured sterol synthesis in primary rat hepatocytes with the radiolabeled metabolic tracer $^{14}$C-mevalonolactone. $^{14}$C-mevalonolactone is readily taken up by cells and converted to $^{14}$C-mevalonate for cholesterol biosynthesis. Mevalonate is the product of the reaction catalyzed by HMG CoA reductase. Therefore, if ESP 55016 does not inhibit the incorporation of $^{14}$C-mevalonate into sterols, we would deduce that the compound blocks a pathway step prior to mevalonate formation.

ESP 55016 had only a minor inhibitory effect (less than 20% inhibition at 30 µM) on incorporation of $^{14}$C-mevalonolactone into sterol lipids (Figure 7) however, as previously demonstrated (Figure 5) and as shown here (Figure 7), the compound inhibited the incorporation of $^{14}$C-acetate into sterols with an IC$_{50}$ of <10 µM. Zaragosic acid, a squalene synthetase inhibitor, as expected, reduced incorporation of $^{14}$C-mevalonate into sterol lipids by 80% (Figure 7). These data suggest that in vitro, ESP 55016 inhibits sterol synthesis at one or more steps prior to mevalonate synthesis.

Effect of ESP 55016 on $^{14}$C- mevalonolactone Incorporation into Sterols in vivo
To confirm the physiological relevance of our *in vitro* findings, we used the cholestyramine-primed rat model to induce hepatic cholesterol synthesis (33). ESP 55016 reduced the amount of radiolabeled sterols in serum by 86% compared to vehicle-treated controls when $^{14}$C-acetate was used as the metabolic tracer (Figure 8) confirming our earlier findings (Figure 6). However, when $^{14}$C-mevalonate was used as the metabolic tracer there was no difference in the amount of radiolabeled sterol in the serum or liver between ESP 55016-treated and vehicle-treated animals (Figure 8).

**Effect of ESP 55016 on AMP-activated Protein Kinase (AMPK) and Acetyl-CoA Carboxylase (ACC)**

Previous work by Henin et al demonstrated that activation of AMP-activated protein kinase (AMPK) by AICAR, an AMP analogue, rapidly inhibited both fatty acid and cholesterol synthesis in primary rat hepatocytes by reducing ACC and HMG-CoA reductase activity (34). AMPK-catalyzed phosphorylation of key amino acid residues in either ACC or HMG-CoA reductase results in reduced enzyme activity (35, 36). Regarding ACC specifically, phosphorylation of threonine 172 of AMPK results in enhanced activity which then leads to AMPK-catalyzed phosphorylation of ACC at serine 79. ESP 55016 did not affect the phosphorylation states of either ACC or AMPK in treated-hepatocytes; AICAR, as expected (37), enhanced phosphorylation of both enzymes (Figure 9). ACC activity was also unchanged in these same extracts after ESP 55016 treatment (data not shown). In addition, extensive *in vivo* studies with a prodrug of ESP 55016, over a wide range of doses and times, also did not demonstrate activation
of the AMPK pathway as assessed by phosphorylation states or activities of AMPK or ACC (data not shown). Furthermore, there were no changes in AMP, ADP, or ATP levels when hepatocytes were treated with ESP 55016 (data not shown). These data strongly suggest that ESP 55016 does not inhibit lipid synthesis by activating the AMPK pathway.

**Conversion of ESP 55016 to its CoA derivative**

Since phosphorylation of ACC did not appear to be responsible for reduced fatty acid synthesis *in vitro*, we tested whether ESP 55016 or intracellular metabolites of ESP 55016 might inhibit ACC directly. Some naturally occurring fatty acid-coenzyme A (CoA) molecules inhibit ACC (38). Therefore, our initial aim was to determine whether ESP 55016 could be converted to a xenobiotic-CoA derivative using rat microsomes. Separation of reaction products by HPLC resulted in a peak with a retention time of about 8 minutes; the appearance of which was both incubation time and ESP 55016 (parent) concentration-dependent (Figure 10A). Ultraviolet spectrophotometric analysis of the reaction product at 8 minutes revealed a characteristic absorption spectra for a CoA containing molecule (Figure 10A, inset). Analysis of that peak by mass spectrometry definitively identified this chemical species as ESP 55016-CoA (Figure 10B). To confirm that ESP 55016-CoA is formed *in vivo*, hepatic lipids were isolated 1.5 hr after dosing rats with 100 mg/kg ESP 55016. ESP 55016-CoA was again clearly detected in this preparation as confirmed by mass spectrometry (data not shown). The estimated ESP 55016-CoA concentration in the liver under these dosing conditions was approximately 2.5 nmol/mg protein.
That liver concentration when converted to aqueous molarity as described by Bronfman et al is roughly equivalent to 0.5 mM (39).

**Effect of ESP 55016-CoA on ACC and HMG-CoA reductase activity in a cell-free assay**

To test the inhibitory effects of ESP 55016-CoA on ACC directly, partially purified ACC was prepared. ESP 55016-CoA markedly inhibited ACC activity in a concentration dependent-manner (Figure 11). Data from several experiments indicated that the IC$_{50}$ for ESP 55016-CoA was 29 ± 5 µM (mean ± SEM, n=7) under assay conditions that were saturating for citrate, acetyl-CoA, bicarbonate, and ATP. Similar results were obtained when either chemically or microsomal synthesized ESP 55016-CoA were tested in this system (data not shown). The IC$_{50}$ estimate (29 µM) was within two-fold of the upper range for inhibition of fatty acid synthesis in hepatocyte cultures (13 µM) with significant overlap in the 95% confidence intervals of those measurements. The parent, non-CoA derivative, ESP 55016 had essentially no effect on ACC activity (Figure 11). Palmitoyl-CoA, a known inhibitor, displayed an IC$_{50}$ of 2 µM under these assay conditions (data not shown).

To test the effect of ESP 55016 and its CoA derivative on HMG-CoA reductase, microsomal and soluble forms of the enzyme from rat liver were prepared (40). Under conditions in which palmitoyl-CoA inhibited HMG-CoA reductase, ESP 55016 or ESP 55016-CoA did not inhibit enzyme activity (data not shown). In addition, hepatic microsomes from ESP 55016-treated
hepatocytes and dosed rats displayed the same HMG-CoA reductase activity compared to untreated controls (data not shown).
Discussion

We present data describing the pharmacologic activity of a novel ω-hydroxy-alkanedicarboxylic acid compound, ESP 55016, in obese female Zucker rats. ESP 55016 reduced nonHDL-C and triglycerides while elevating HDL-C. Of particular note, ESP 55016 markedly reduced serum NEFA which was associated with a rise in the serum level of the ketone body, β-hydroxybutyrate.

The ESP 55016 mechanism of action studies were initially guided by the finding that β-hydroxybutyrate levels were elevated. Since ketone bodies are synthesized as a result of excessive acetyl-CoA in the liver, we hypothesized that ESP 55016 enhanced hepatic oxidation of fatty acids. Indeed, ESP 55016 enhanced oxidation of 14C-palmitate in primary rat hepatocytes. Since mitochondrial oxidation of long-chain fatty acids is mediated through CPT-I, we tested if L-carnitine, a CPT-I reaction substrate, or R-aminocarnitine, a CPT-I inhibitor, could modulate the ESP 55016-induced oxidation effects. ESP 55016 in the presence of L-carnitine enhanced palmitate oxidation beyond rates observed with ESP 55016 alone. In contrast, R-aminocarnitine prevented ESP 55016 enhanced oxidation of palmitate. The palmitate oxidation in the presence of R-aminocarnitine is presumably due to peroxisomal oxidation; ESP 55016 under these conditions did not appear to affect the peroxisomal oxidation pathway for palmitate. Since peroxisome oxidation pathways show preference for fatty acids greater than chain lengths of 16 carbons (41), it is possible that peroxisomal oxidation was also enhanced. Furthermore, an increase in peroxisomal catalyzed chain shortening of palmitate could have occurred.
followed by mitochondrial oxidation of the resultant acyl-CoA. However, we did not test fatty acids greater than C16 in this study nor monitor palmitate chain shortening outside the mitochondria. Nonetheless, these data indicate that active CPT-I is essential for the ESP 55016 stimulation of fatty acid oxidation.

McGarry and coworkers have demonstrated the intimate linkage between rates of mitochondrial fatty acid oxidation and fatty acid biosynthesis (42, 43). Malonyl-CoA is the key intracellular metabolite in that linkage and is produced through the carboxylation of acetyl-CoA catalyzed by acetyl-CoA carboxylase (ACC), widely regarded as the rate limiting step of long-chain fatty acid biosynthesis (44). Malonyl-CoA is a potent inhibitor of CPT-I, thus inhibition of fatty acid synthesis from acetyl-CoA reduces malonyl-CoA levels and stimulates fatty acid oxidation through derepression of CPT-I. We hypothesized that ESP 55016 inhibited fatty acid synthesis by inhibiting ACC. In primary hepatocytes, ESP 55016 inhibited fatty acid synthesis at concentrations which stimulated palmitate oxidation. Although these data suggest that ESP 55016 stimulates fatty acid oxidation by reducing malonyl-CoA levels, it does not rule out the possibility that the xenobiotic-CoA of ESP 55016 can overcome the malonyl-CoA suppression of CPT-I; fatty acyl-CoAs have been shown to relieve the inhibition of CPT-I by malonyl-CoA (45, 46).

In the course of the fatty acid biosynthesis studies, we observed that ESP 55016 also inhibited sterol synthesis from various metabolic precursors, suggesting that ESP 55016 affected a common or multiple biochemical control
point(s) shared by both the fatty acid and sterol biosynthetic pathways. One such control point could have involved AMP-activated protein kinase (AMPK).

AMPK can phosphorylate ACC and HMG-CoA reductase at key amino acid phosphorylation sites to reduce enzyme specific activity and inhibit the synthesis of fatty acids and sterols, respectively (34-36, 47). Recently, particular emphasis has focused on the AMPK and ACC tandem due to their demonstrated roles in disorders of metabolic syndrome including obesity, insulin resistance, and dyslipidemia (48, 49). The AMPK pathway can be activated by hormones such as leptin and adiponectin and by the pharmacologic agents metformin and 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) (37, 50-52). These diverse agents have been shown to activate AMPK, stimulate phosphorylation of ACC, reduce fatty acid synthesis, and enhance fatty acid oxidation. When we tested the ability of ESP 55016 to activate this pathway in primary hepatocytes, no activation was observed.

To further define the mechanism of action for ESP 55016 we focused on ACC and its regulation. ACC is regulated by long-term control mechanisms involving changes in enzyme levels and gene expression as well as short-term control mechanisms including covalent modification (phosphorylation / dephosphorylation) and allosteric effectors (53, 54). Time course studies with ESP 55016 indicated that short-term mechanisms were primarily responsible for the inhibition effects. Since covalent modification of ACC phosphorylation sites (35) did not appear to be affected, we tested and confirmed that ESP 55016-CoA can inhibit ACC directly. That the xenobiotic-CoA is relevant to the mechanism...
of action was further confirmed by identification of millimolar levels of xenobiotic-CoA in liver samples 1.5 hours after a 100 mg/kg dose which is within the timeframe where we observed inhibition of fatty acid synthesis in vivo; this concentration is well within the range we identified for inhibition of ACC (29 µM). These data suggest that ESP 55016 ultimately inhibits fatty acid synthesis by following a pathway which includes its conversion to a xenobiotic-CoA and direct inhibition of ACC by that xenobiotic-CoA. The generation of xenobiotic-CoA derivatives from carboxylic acid containing compounds is well documented (55). For example, MEDICA 16 forms a CoA derivative and that metabolite inhibits ACC in a cell-free assay (20). In summary, our interpretation leads to the following scenario regarding the ESP 55016 mechanism of action with regard to fatty acid synthesis: ESP 55016 is converted to ESP 55016-CoA which directly inhibits ACC and reduces malonyl-CoA levels, perhaps locally near CPT-I, and results in derepression of CPT-I, elevated transfer of long-chain fatty acids, and enhanced fatty acid oxidation. The continuation of this state with daily dosing of ESP 55016 in animals leads to adaptations resulting in altered lipid and insulin levels as well as the suppression of weight gain.

The mechanism responsible for inhibition of sterol synthesis is less clear than the mechanism for inhibition of fatty acid synthesis. Our studies with radiolabeled metabolic tracers indicate that inhibition of the sterol biosynthetic pathway occurs after formation of acetyl-CoA but before formation of mevalonate. Thus, three enzymes are potential targets for ESP 55016 or the corresponding CoA: acetoacetyl-CoA thiolase, HMG-CoA synthase, or HMG-CoA
reductase. We focused on HMG-CoA reductase for two reasons. First, reductase activity is directly inhibited by CoA thioesters (40, 56, 57) and secondly, the hypolipidemic compound S-2E is converted to a CoA derivative and S-2E-CoA directly inhibits HMG-CoA reductase in a cell-free assay (58). However, we found that ESP 55016-CoA did not inhibit reductase in a cell-free assay under a variety of conditions. Furthermore, ESP 55016 treatment of hepatocytes and Sprague-Dawley rats followed by isolation of microsomes and ex vivo assay of reductase activity did not reveal any differences compared to untreated controls. It is possible that removal of HMG-CoA reductase from the cellular environment removes the pertinent regulatory mechanism. With respect to HMG-CoA synthase, it seems unlikely that activity would be inhibited by ESP 55016 or its CoA metabolite since ketone body levels are elevated and HMG-CoA synthase is required for the synthesis of HMG-CoA which is then used to produce acetoacetate and subsequently converted to acetone and β-hydroxybutyrate. However, it is conceivable that ESP 55016 or its CoA derivative inhibits acetoacetyl-CoA thiolase which would limit substrate to HMG-CoA synthase resulting in reduced ketone body synthesis. At present, we conclude that ESP 55016 or its CoA derivative inhibits the sterol biosynthetic pathway between acetyl-CoA and the production of mevalonate possibly through an indirect mechanism.

There are alternative mechanisms that may explain or at least contribute to the overall dual inhibitor activity of ESP 55016. First, it is possible that ESP 55016 or its CoA derivative inhibits ATP-citrate lyase, an enzyme that generates
cytosolic acetyl-CoA from mitochondria-derived citrate. Such a conclusion may be valid had we only measured fatty acid and sterol synthesis using $^{14}$C-pyruvate, $^{14}$C-glucose, or $^3$H$_2$O; all of these precursors would generate radiolabeled, mitochondria-derived acetyl-CoA. However, we utilized radiolabeled acetate as well and found very similar IC$_{50}$s for the compound with respect to its dual inhibitor property using all radiolabeled tracers. Several studies have demonstrated that inhibition of ATP-citrate lyase with hydroxycitrate did not reduce the incorporation of $^{14}$C-acetate into fatty acids or sterols suggesting that exogenous $^{14}$C-acetate does not pass through the mitochondria (59-62). Nonetheless, it is conceivable that $^{14}$C-acetate could generate mitochondria-derived $^{14}$C-acetyl-CoA in the liver by one or more pathways. One pathway could involve ligation of acetate to CoA by acetyl-CoA synthetase 1 (AceCS1), transport into the mitochondria, condensation with oxaloacetate to form citrate, followed by regeneration of acetyl-CoA from citrate in the cytosol through the action of ATP-citrate lyase. An alternative mitochondria-based pathway for producing $^{14}$C-acetyl-CoA from $^{14}$C-acetate could involve direct ligation of $^{14}$C-acetate to CoA in hepatic mitochondria. However, this seems unlikely since acetyl-CoA synthase-2 (AceCS2), a mitochondrial-matrix enzyme which is abundant in muscle, is absent in liver (63). In contrast, AceCS1 is abundant in liver and located in the cytosol with the acetyl-CoA generated used for fatty acid and sterol synthesis (64, 65). Clearly, AceCS activity is not needed for the ESP 55016 effects since the pathway from mitochondrial $^{14}$C-pyruvate to $^{14}$C-acetyl-CoA in the cytosol does not require AceCS but rather pyruvate.
dehydrogenase and ATP-citrate lyase (i.e. there is no free acetate generated in this process). Nonetheless, we cannot definitively rule out the possibility that ESP 55016 or ESP 55016-CoA inhibits ATP-citrate lyase to some degree. Indeed, MEDICA 16 has been shown to inhibit both ATP-citrate lyase and ACC (15, 20). In all, our data suggest that ESP 55016 inhibits both pathways at steps after generation of acetyl-CoA.

Another possible mechanism to explain the dual inhibitor activity of ESP 55016 is that the compound increases the cellular pool size of the corresponding “cold” metabolic precursor used in the metabolic labeling studies, thus reducing the specific activity of the exogenously added radiolabeled tracer. However, as noted above, the dual inhibition occurred within minutes, making it unlikely that there were significant changes in intracellular pool sizes of experimentally related metabolites. In addition, the cell culture media contained millimolar concentrations of acetate and pyruvate providing a very large pool which is presumably in equilibrium with cellular levels. Finally, all the radiolabeled tracers used showed similar inhibitory effects including $^3$H$_2$O which does not suffer from pool dilution effects.

It is possible that ESP 55016 sequesters CoA limiting the availability acetyl-CoA for lipid synthesis. This appears to be the case with TOFA at high concentrations (66). However, at concentrations where ESP 55016 inhibited lipid synthesis, fatty acid oxidation was elevated suggesting that CoA was not limiting for generation of palmitoyl-CoA from palmitate. Furthermore, the oxidation rates
of medium-chain fatty acids, caprylic and lauric, were not altered by ESP 55016 treatment again indicating that CoA was not limiting.

Finally, changes in gene expression may be responsible, in part, for long-term (days) effects of ESP 55016 in vivo. The PPAR and SREBP transcription factors regulate expression of key enzymes involved in lipid synthesis and oxidation. Perhaps ESP 55016 metabolites or cellular lipid changes induced by ESP 55016 alter the activities of key transcription factors. Further studies are required to address these possibilities.

ESP 55016 appears to have similar short-term (minutes to hours) biochemical effects as other long-chain hydrocarbon compounds. MEDICA 16, TOFA and 3-thiadicarboxylic acid all enhance fatty acid oxidation and inhibit fatty acid biosynthesis to various degrees (3, 4, 67). In each case, the CoA derivative is formed and in some instances direct effects on ACC have been demonstrated (3, 20, 68). Specifically with respect to ESP 55016, the “mechanistic anchor” contributing to in vivo alterations in lipid metabolism appears to be its ability to function as a “dual inhibitor” of fatty acid and sterol synthesis.

The utility of ESP 55016 to treat human dyslipidemias is unclear. De novo fatty acid synthesis (lipogenesis) in humans is considered a quantitatively minor pathway for the synthesis of hepatic-derived fatty acids and triglycerides under normal dietary conditions (69). Nonetheless, de novo fatty acid synthesis in humans may primarily serve a regulatory function to control the rate of fatty acid oxidation, particularly in muscle (69, 70). Whether ESP 55016 will be useful to treat human dyslipidemias and metabolic syndrome related disorders must wait
clinical testing.
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Figure Legends

Figure 1. The structure of ESP 55016.

Figure 2. Serum lipid variables in female obese female Zucker rats. The data summary was compiled from four separate experiments of 4 to 5 animals per treatment group. Data are given as mean ± standard error of the mean of pretreatment (open bars), at 1-week (black bars) and at the end of the 2-week dosing period (gray bars) for daily mg/kg doses of 0 (Control, n=17 animals), 1 (n=8 animals), 3 (n=14 animals), 10 (n=14 animals), 30 (n=16 animals), and 100 (n=8 animals). Comparisons to pretreatment values were made using a paired Student’s t-test, * p<0.05.

Figure 3. Serum nonesterified fatty acid (NEFA) and β-hydroxybutyric acid (β-HBA) in obese female Zucker rats. The same samples described in the legend to Figure 2 were used for these analyses. Comparisons to pretreatment values were made using a paired Student’s t-test, * p<0.05.

Figure 4. Time and dose response of 14C-palmitic acid oxidation to ESP 55016 treatment of primary rat hepatocytes. (A) Experiments with increasing concentrations of ESP 55016 after 4 hrs were performed in DMSO vehicle (open bars), with 500 µM L-carnitine (black bars) and with 500 µM R-aminocarnitine (gray bars). Data are the average of two determinations from a single experiment. (B) Effect of ESP 55016 on oxidation of medium-chain fatty acids.
The oxidation rates of $^{14}$C-caprylic acid and $^{14}$C-lauric acid were assessed using DMSO control incubations (open bar), 500 µM L-carnitine (black bars), or 500 µM R-aminocarnitine (gray bars) in the presence or absence of 10 µM ESP 55016 as indicated. Data represent the average and value ranges of two determinations.

Figure 5. Effect of ESP 55016 on fatty acid and sterol synthesis in primary rat hepatocytes. Hepatocytes from fasted rats ($^{14}$C-pyruvate, $^{14}$C-acetate, $^{3}$H$_{2}$0) or hepatocytes from fasted-refed rats ($^{14}$C-glucose) were exposed to DMEM with 0.1% DMSO or 0.3, 1, 3, 10, and 30 µM ESP 55016 for 4 hrs. in the presence of the various metabolic tracers as noted. Incorporation of radiolabeled tracer into cellular fatty acids (circles) and sterols (squares) were determined. Each data point is the mean ± standard deviation (SD) of three wells.

Figure 6. In vivo inhibition of lipid synthesis by ESP 55016. Rats were fasted for 48 hr followed by refeeding a high carbohydrate diet for 48 hr. On the last day of refeeding, animals received one dose of vehicle or ESP 55016 (100 mg/kg). After one additional hour rats were injected IP with $^{14}$C-acetate and one hr later serum and liver was obtained to determine incorporation into fatty acids and sterols. Five animals were used per group.

Figure 7. Effect of ESP 55016 on incorporation of $^{14}$C-mevalonolactone into sterols in primary rat hepatocytes. Left Panel: Hepatocytes from fasted rats were exposed to DMEM with 0.1% DMSO or 0.3, 1, 3, 10, and 30 µM ESP 55016.
for 4 hrs. in the presence $^{14}$C-mevalonolactone (circles) or $^{14}$C-acetate (squares). Incorporation of radiolabeled tracer into cellular sterols was determined. Right Panel: Zaragosic acid (200 nM), a squalene synthetase inhibitor, was used as a positive control for inhibition of sterol synthesis from $^{14}$C-mevalonolactone. Each data point is the mean of three wells.

Figure 8. Effect of ESP 55016 on incorporation of $^{14}$C-mevalonolactone into sterols in vivo. Rats were fed a cholestyramine diet for five days to induce hepatic cholesterol synthesis. On the last day of feeding, animals received one dose of vehicle or ESP 55016 (100 mg/kg). After one additional hour rats were injected IP with $^{14}$C-acetate or $^{14}$C-mevalonolactone and one hr later serum and liver was obtained to determine incorporation into sterols. Five animals were used per group.

Figure 9. Effect of ESP 55016 on the AMPK pathway in primary rat hepatocytes. Hepatocytes were treated with DMSO, 500 µM AICAR, and various concentrations of ESP 55016 for 1 hr. followed by preparation of cell lysates. The phosphorylation state of ACC and AMPK was determined. Cell lysates were submitted to Western Blot analysis using specific antibodies to phosphorylated serine-79 of rat ACC and phosphorylated threonine-172 of rat AMPK alpha subunit.
Figure 10. Biological synthesis and identification of xenobiotic-CoA of ESP 55016 (A) The HPLC chromatograph analysis of product from microsomal incubation with ESP 55016. The ESP 55016-CoA peak elutes with a retention time of 8.1 minutes. The inset shows the UV-absorption spectra of that peak. (B). A representative negative ion mode mass spectrum showing a m/z [M-H]- of 1092.34862 of the purified 8.1 minute peak, corresponding to the predicted m/z for ESP 55016-CoA. Additional monosodiated and disodiated forms of ESP 55016-CoA are present corresponding to m/z [M-H]- of 1114.32979 and m/z [M-H]- of 1136.32521, respectively.

Figure 11. Xenobiotic-CoA inhibition of partially purified ACC. ESP 55016-CoA was synthesized in a microsomal system, purified, and incubated with partially purified rat liver ACC in a cell-free assay. Parent ESP 55016 was tested at a concentration of 30 µM. This is a representative experiment with several experiments under these assay conditions indicating an IC50 of 29 ± 5 (mean ± SEM, n=7).
Table 1. Effect of ESP 55016 on fasting glucose, fasting insulin, and weight gain in the obese female Zucker rat. Glucose and insulin levels in addition to body weight were determined from the same animals (100 mg/kg dose) used in the experiments depicted in Figures 2 and 3. Numbers in parentheses are the percent increase or decrease compared to the pretreatment value for either the vehicle-treated or ESP 55016-treated group of animals.

* p < 0.05

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<th>Vehicle-treated</th>
<th>ESP 55016-treated (100mg/kg)</th>
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<td>Pretreatment</td>
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<td>Glucose (mg/dL)</td>
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<td>Insulin (ng/mL)</td>
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<td>Weight (g)</td>
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Figure 10