AMP-activated protein kinase and ATP-citrate lyase are two distinct molecular targets for ETC-1002, a novel small molecule regulator of lipid and carbohydrate metabolism


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Abstract  ETC-1002 (8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid) is a novel investigational drug being developed for the treatment of dyslipidemia and other cardio-metabolic risk factors. The hypolipidemic, antiatherosclerotic, anti-obesity, and glucose-lowering properties of ETC-1002, characterized in preclinical disease models, are believed to be due to dual inhibition of sterol and fatty acid synthesis and enhanced mitochondrial long-chain fatty acid β-oxidation. However, the molecular mechanisms (mediating these activities) remained undefined. Studies described here show that ETC-1002 free acid activates AMP-activated protein kinase in a Ca²⁺/calmodulin-dependent manner, with no detectable changes in adenylyl energy charge. Furthermore, ETC-1002 is shown to rapidly form a CoA thioester in liver, which directly inhibits ATP-citrate lyase. These distinct molecular mechanisms are complementary in their beneficial effects on lipid and carbohydrate metabolism in vitro and in vivo. Consistent with these mechanisms, ETC-1002 treatment reduced circulating proatherogenic lipoproteins, hepatic lipids, and body weight in a hamster model of hyperlipidemia, and it reduced body weight and improved glycemic control in a mouse model of diet-induced obesity. ETC-1002 offers promise as a novel therapeutic approach to improve multiple risk factors associated with metabolic syndrome and benefit patients with cardiovascular disease.

Cardiovascular disease (CVD) remains a leading cause of morbidity and mortality in the Western world. Elevated levels of LDL-cholesterol (LDL-C) have consistently shown a positive association with the development of CVD, justifying the current therapeutic strategies to prevent CVD primarily by the use of statins. Members of this drug class inhibit HMG-CoA reductase (HMGR), the rate-limiting enzyme for de novo cholesterol synthesis, thereby leading to decreased LDL-C (2–4). While the benefits of statins have been documented (2), many individuals on statin therapy still remain at a higher risk of developing CVD. It is possible this residual risk is a result of other metabolic syndrome (MetS) risk factors characterized by dyslipidemia and insulin resistance and is also in part due to statin intolerance (5–7) and noncompliance often related to statin-induced myalgia (8, 9). Statins are effective at decreasing LDL-C and CVD; however, frequent muscle-related side effects limit dosage and impede maximal risk reduction in dyslipidemic patients (10). Furthermore, recent evidence suggests that high-dose statins may increase the risk of developing type 2 diabetes (T2D) (11), further justifying the need for alternative therapeutic interventions that have statin-like effects for lowering LDL-C and are designed to

Supplementary key words  fatty acid synthesis • cholesterol synthesis • fatty acid oxidation • LDL-cholesterol • cardiovascular disease • metabolic syndrome

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beneficially affect other common cardiometabolic risk factors associated with atherosclerosis and MetS.

ETC-1002 (8-hydroxy-2,2,14,14-tetramethylpentadecanedioic acid), also known as ESP55016, is a novel investigational drug being developed for the treatment of dyslipidemia and other cardio-metabolic risk factors. ETC-1002 favorably changes lipid profiles in preclinical models of dyslipidemia (12), benefits glucose homeostasis in mouse models of impaired glycemic control (12, 13), and decreases atherosclerosis in LDL-receptor-deficient mice (14). Importantly, clinical studies have shown that ETC-1002 reduces LDL-C levels in subjects with mild dyslipidemia and has beneficial effects on other relevant cardio-metabolic risk factors, including insulin levels, hsCRP, and blood pressure (15).

In the current study, we demonstrate that these beneficial effects of ETC-1002 on lipid and carbohydrate metabolism are tightly linked to activation of hepatic AMP-activated protein kinase (AMPK), a master kinase controlling whole-body energy homeostasis. Additionally, the CoA thioester of ETC-1002 revealed potent inhibitory activity against hepatic ATP-citrate lyase (ACL), another central enzyme coordinating extra-mitochondrial carbon flux into the synthesis of lipids. The combination of these two distinct molecular mechanisms not only may regulate LDL-C but also may exhibit additional beneficial attributes for the treatment of CVD and provide clinically meaningful efficacy for other related risk factors associated with MetS.

MATERIALS AND METHODS

DMEM, nonessential amino acids, HEPES, PBS, sodium pyruvate, and penicillin/streptomycin were obtained from Invitrogen (Logon, UT). Fetal bovine serum (FBS) was obtained from HyClone (Grand Island, NY). Bovine albumin, fraction V, insulin, hydrocortisone, Triacsin C, Compound C, 5-amino-4-imidazole-Hyclone (Grand Island, NY). Bovine albumin, fraction V, insulin, (Logon, UT). Fetal bovine serum (FBS) was obtained from NuGene, and penicillin/streptomycin were obtained from Invitrogen to ETC-1002 in water. Carboxymethyl cellulose (CMC) and Tween-20 were added to make a final solution containing 0.5% CMC and 0.025% Tween, with a final pH 7–8. Compound concentrations in dosing solutions were based upon a 10 ml/kg body weight dosing volume.

Hepatocyte isolation

Nutritionally staged male Sprague-Dawley [Crl:CD (SD)] rats were anesthetized with isoflurane, and livers were perfused for hepatocyte isolation according to the method of Ulrich et al. (16). Hepatocytes were plated in high-glucose DMEM containing 20% FBS, 14 mM HEPES, 0.2% bovine albumin, 2 mM L-glutamine, 1× MEM nonessential amino acids, 100 nM insulin, 100 µg/ml dexamethasone, and 20 µg/ml gentamicin at a density of 1.5 × 10^5 cells/cm^2 on collagen-coated 6-well plastic dishes. After the attachment period, (3–4 h), cells were washed once and cultured overnight in DMEM containing 10% FBS.

Cell culture and siRNA transfection

HepG2 cells were grown and treated in DMEM containing 1 g/L D-glucose supplemented with 10% FBS. Reverse transfections were performed in 6-well culture plates at 2.5 × 10^5 cells/well using Lipofectamine 2000. Cells were incubated for 48 h with 10 nM silencer siRNA for liver kinase β (LKB)1 or negative control prior to compound treatment.

Nucleotide measurements

Cells were placed on ice, deproteinized with ice-cold 6% perchloric acid, scraped, neutralized with 10 M NaOH, and buffered with 1 M K,HPO4 to precipitate potassium perchlorate. Solutions were transferred to microcentrifuge tubes and centrifuged for 5 min. Supernatant (20 µl) was diluted in cold HPLC-grade water and maintained at approximately 4°C until injection. Diluted sample (20 µl) was injected into the LC-MS/MS system and three m/z transitions were monitored (m/z 348.2 → 136.5 for AMP, m/z 428.1 → 136.5 for ADP, and m/z 508.2 → 136.5 for ATP) on an API-4000 triple-quadrupole mass spectrometer (AB Sciex, Framingham, MA). The relative amounts of AMP, ADP, and ATP were determined for each sample by normalizing triplicate measures of test conditions to vehicle treatment from the same plate. To determine the adenine nucleotide levels in freeze-clamped liver, approximately 500 mg of frozen liver was homogenized in ice-cold methanol and diluted in cold HPLC water before injecting 30 µl into the LC-MS/MS system. AMP, ADP, and ATP concentrations in liver were determined by comparing the sample peak area to the peak area of known calibration standard samples prepared in methanol.

De novo lipid synthesis assay

Rates of lipid synthesis were assessed in cultured primary rat hepatocytes using [14]acate or [14]citrate. Experiments were performed in DMEM with 4.5 g/l glucose. Cells were treated with compound or vehicle (0.1% DMSO) for up to 4 h followed by lipid isolation. After metabolic labeling, saponified and nonsaponified lipids were extracted from cells essentially as described by Slayback et al. (17).

ETC-1002 formulation /
ETC-1002-CoA synthesis

For in vitro assays, ETC-1002 was formulated using aseptic technique at 30 and 100 mM in sterile dimethylsulfoxide (DMSO) and stored in sterile microcentrifuge tubes at 4°C for up to four weeks (stability was assessed). Working solutions of ETC-1002 were prepared in serum-free DMEM containing 12 mM HEPES, 10,000 U/ml penicillin, and 100 µg/ml streptomycin. ETC-1002-CoA was synthesized using rat liver microsomes essentially as described by Cramer et al. (12). For in vivo experiments, ETC-1002 dosing solutions were formulated by preparing a disodium salt aqueous solution using 2:1 molar ratio of NaOH to ETC-1002 in water. Carboxymethyl cellulose (CMC) and Tween-20 were added to make a final solution containing 0.5% CMC and 0.025% Tween, with a final pH 7–8. Compound concentrations in dosing solutions were based upon a 10 ml/kg body weight dosing volume.
Glucose production assay

Glucose production was measured in primary rat hepatocyte cultures. Cells were cultured in glucose- and phenol red-free DMEM, containing 10 mM lactate, 1 mM pyruvate, and nonessential amino acids (glucose production buffer, GPB). To assess the effects of ETC-1002 on glucagon-stimulated glucose production, cells were incubated with and without 0.3 mM glucagon (Sigma, St. Louis, MO) with various concentrations of ETC-1002 (0.1 to 100 μM). Media was sampled over time. Following specified treatments, cells were washed twice in GPB. Cells were then incubated for an additional hour to assess glucose production by adding GPB containing equivalent glucagon concentrations without ETC-1002. Cells were incubated for 1 h, and the concentration of glucose in the media was determined using a glucose oxidase assay kit (catalog #GAGO20-IKT; Sigma Chemicals).

ATP-citrate lyase enzyme activity assay

The activity of recombiant human ACL was carried out essentially as described in (18). Briefly, 7.5× compounds were added to a 96-well PolyPlate containing 60 μl of Buffer (87 mM Tris, pH 8.0, 20 mM MgCl2, 10 mM KCl, 10 mM dithiothreitol) per well with substrates CoA (200 μM), ATP (400 μM), and [14C]-citrate (specific activity: 2 μCi/μmol) (150 μM). Reaction was started with 4 μl (300 ng/well) ACL, and the plate was incubated at 37°C for 3 h. The reaction was terminated by the addition of 3.5 μl 500 mM EDTA. MicroScint-O (200 μl) was then added to the reaction mixture and incubated at room temperature overnight with gentle shaking. The [14C]acetyl-CoA signal was detected (5 min/well) in a TopCount NXT liquid scintillation counter (Perkin-Elmer, Waltham, MA).

Western blots

Hepatocyte cell lysates were prepared using approximately 150–400 μl 1× lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM PMSF, and 1× phosphatase inhibitor cocktail (Sigma). Total lysate protein concentrations were determined using the BCA Protein Assay (Bio-Rad Laboratories, Hercules, CA). Protein concentrations were adjusted and diluted in 4× LDS (liothyd dodecyl sulfate gel sample buffer) containing 50 mM dithiothreitol. Proteins were separated using SDS-PAGE (4–12%) Bis/Tris, MOPS running buffer (Invitrogen, Logan, UT). Separated proteins were electrophoretically transferred to PVDF membranes. Nonspecific binding was blocked, and membranes were probed with antibodies against β-actin, total and phosphorylated ACC, AMPKα, hepatocyte nuclear factor (HNF)-4α, FOXO1, and LKB1 (Cell Signaling Technologies, Danvers, MA), PGC-1α (BioVision, Mountain View, CA), cyclin synthase, phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G6Pase) (Abcam, Cambridge, MA), and HMGR-set872 (Millipore, Billerica, MA).

Short-chain acyl-CoA and citrate measurements

For in vitro studies, cell culture samples were prepared as previously described for nucleotide measurements. For in vivo studies, freeze-clamped liver was homogenized in ice-cold methanol, and then samples were prepared by liquid-liquid extraction using chloroform and 0.1% formic acid in water. A portion of the aqueous phase was transferred and injected into the LC-MS/MS system monitoring five m/z transitions (808.0 → 461.2 for acetyl-CoA, 191.0 → 111.3 for citrate, 766.0 → 408.4 for CoASH, 910.0 → 408.2 for HMG-CoA, and 852.0 → 808.4 for malonyl-CoA) on an API-4000 triple-quadrupole mass spectrometer (AB Sciex, Framingham, MA). Acetyl-CoA, citrate, CoASH, HMG-CoA, and malonyl-CoA concentrations were determined by comparing the sample peak area to the peak area of known calibration standard samples prepared in methanol.

In vivo studies

Wistar rats. Male Wistar Han [Crl:WI] rats (Charles River Laboratories) weighing 225–250 g were acclimated to the laboratory environment for seven days, housed 2–3 per cage in a temperature-controlled room, and maintained on a 12 h light and dark cycle with ad libitum access to food and water. Prior to single-dose ETC-1002 administration, rats were fasted for 48 h and refed a high-carbohydrate diet for an additional 48 h. For two-week assessment, rats were maintained on standard chow diet (Purina 5001) and dosed by oral gavage with ETC-1002 at 30 mg/kg/day for two weeks in the morning. Following nutritional staging and/or dosing, food was withdrawn 2 h prior to last the oral dose of vehicle control or ETC-1002. Blood and liver were collected from isoflurane-anesthetized animals 2 or 8 h after the last dose, blood was collected from the subclavian vein, and liver tissue was harvested by freeze clamp. The freeze-clamped liver samples were held frozen in liquid nitrogen immediately following excision and stored at −70°C. Plasma triglycerides, β-hydroxybutyrate (β-HBA), and total cholesterol levels were measured with commercially available kits (Wako Diagnostics, Richmond, VA) adapted to a 96-well format. All animal procedures were conducted in accordance with protocols approved by an Institutional Animal Care and Use Committee at Michigan Life Science and Innovation Center.

Golden Syrian hamsters. Male golden Syrian hamsters were obtained from Charles River (Montreal, QC) at 8–10 weeks of age and weighed 100–120 g. Animals were maintained on ProLab RMH 1000 standard rodent chow diet (PMI Nutrition International, St. Louis, MO) during a seven-day quarantine period. Following randomization into treatment groups (n = 6), hyperlipidemia was induced by feeding high-fat, high-cholesterol (HFHC) Prolab RMH 1000 diet containing: 11.5% coconut oil, 11.5% corn oil, 5% fructose, and 0.5% cholesterol. During the study, animals were individually housed in an environmentally controlled room with a 12 h light and dark cycle. Following two weeks on HFHC diet, hamsters were dosed by oral gavage once daily with vehicle (0.5% carboxymethyl cellulose and 0.025% Tween-20, pH 7–8) or vehicle plus ETC-1002 (30 mg/kg) for three weeks. Body weights were recorded every two days at the beginning of dosing, and food consumption was measured every four days. Blood samples were collected by administering isoflurane anesthesia and bleeding from the orbital venous plexus in lithium heparinized tubes during the study and by cardiac puncture under anesthesia at the end of the study. Plasma samples were analyzed for triglycerides, total cholesterol, nonesterified fatty acids, and β-hydroxybutyrate on an automated chemistry analyzer. Liver and epididymal fat were collected, weighed, frozen in liquid nitrogen, and stored at −80°C until processing. All hamster procedures were conducted in accordance with the current guidelines for animal welfare at the Hospital for Sick Children and were in compliance with National Institutes of Health Publication 86-23, 1985; Animal Welfare act, 1966, as amended in 1970, 1976, and 1985, 9 CFR Parts 1, 2, and 3.

Diet-induced obesity in mice. Male C57BL/6N mice were obtained from Taconic (Germantown, NY) at 8 weeks of age and singly housed on α-dri paper bedding on a normal 12 h light and dark cycle (6 AM to 6 PM). Upon arrival mice, were fed a high-fat diet (HFD) containing 60% kcal fat (D12492; Research Diets,
ETC-1002 activates AMPK and inhibits ATP-citrate lyase

New Brunswick, NJ) for 12 weeks. Mice were randomized into two treatment arms at 20 weeks of age based on 4 h fasted blood glucose and body weight and received oral dosing of either CMC/Tween vehicle or 30 mg/kg/day ETC-1002 q.d in the morning for an additional two weeks. Body weight and food consumption were monitored throughout the study. Following the two-week dosing period, food was removed at 8 AM, and bedding was changed 2 h prior to oral administration of ETC-1002. Two hours post dose, fasting samples were collected. Fasting blood glucose levels were measured immediately prior to anesthesia using a hand-held Alphatrak glucometer (Abbott, Chicago, IL), with blood collected by unrestrained tail snip. For insulin determinations, blood was collected under isoflurane anesthesia via retro-orbital sinus into EDTA-coated tubes, and plasma was isolated by centrifugation. Plasma insulin levels were measured with a commercially available ELISA (Crystal Chem Associates, Downers Grove, IL).

Lipoprotein profiles and size exclusion chromatography

Plasma samples were transferred to autosampler vials and maintained at 4°C until injection onto the FPLC system (Waters Alliance 2695 Separations Module) utilizing size-exclusion chromatography with a Superose 6 10/300GL column (GE Healthcare Biosciences, Uppsala, Sweden) and 0.9% sodium chloride/0.02% sodium azide in water. Postcolumn effluent and CHOL CHOD-PAP cholesterol reagent (Roche Diagnostics, Indianapolis, IN) were mixed in-line and reacted in a 37°C heated knitted coil prior to monitor at 490 nm (Waters 2996) PDA. Very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) ratios were determined by calculation of the peak area for each protein as a percentage of the total peak area of all proteins detected in the sample.

Liver extraction and HPLC-ELSD procedure for lipid measurements

Approximately 100 mg of frozen liver was homogenized in a glass screw-top vial in 0.50 ml 150 mM sodium chloride (NaCl)/5 mM 3-(N-morpholino)propanesulfonic acid (MOPS)/1 mM sodium azide in water. Postcolumn effluent and CHOL CHOD-PAP cholesterol reagent (Roche Diagnostics, Indianapolis, IN) were mixed in-line and reacted in a 37°C heated knitted coil prior to monitor at 490 nm (Waters 2996) PDA. The aqueous layer (top, approximately 0.8 ml) from the extraction procedure described above was injected into the HPLC-ELSD system utilizing an Alltima C8 5 \( \mu \)m, 100 x 4.6 mm ID HPLC column (Alltech Associates, Deerfield, IL) running a 15-40% acetonitrile in 25 mM potassium hydrogen phosphate (pH 7.0) gradient before being UV detection at 254 nm on a G1314A detector (Agilent Technologies, Santa Clara, CA).

ETC-1002-CoA concentrations were determined by comparing the sample peak area to the peak area of a ETC-1002-CoA calibration standard.

RESULTS

ETC-1002 has been previously shown to inhibit de novo sterol and fatty acid synthesis in primary rat hepatocytes in vitro and in vivo, with equal potency (12). In these studies, ETC-1002-CoA thioester was identified as the primary active form of ETC-1002 and was shown to inhibit partially purified ACC (IC\(_{50} = 29 \mu M\)) without activating the AMPK pathway in vitro (12). In a follow-up study reported here, we unexpectedly found a marked and sustained increase in AMPK (T172) (358.3 ± 48.14; \( P = 0.0007 \)) and ACC (S79) phosphorylation (164.7% ± 12.39; \( P = 0.001 \)) (Fig. 1) in rat livers following two weeks of treatment with ETC-1002. Interestingly, in liver extracts, the ETC-1002 free acid concentration was approximately 110:1 molar ratio compared with the CoA thioester indicating that previously uncharacterized free acid may be involved in regulating ETC-1002-mediated metabolic activities. Furthermore, while ACC inhibition has been attributed to ETC-1002-CoA, this only explains the inhibition of fatty acid synthesis, leaving the mechanism for the equipotent inhibition of sterol synthesis unidentified.

To obtain better insight into the molecular targets for ETC-1002 free acid and the CoA thioester, we first characterized the temporal nature of ETC-1002 uptake and CoA

![Fig. 1. ETC-1002 activates liver AMPK in chow fed rats. Phospho-AMPK\(_{\alpha}\) (T172) (P-AMPK), total AMPK\(_{\alpha}\) (T-AMPK), phosphor-ACC (S79) (P-ACC), total ACC (T-ACC), and \( \beta \)-actin were determined by Western blotting of freeze-clamped liver homogenates from chow-fed rats dosed with vehicle or vehicle containing 30 mg/kg/day ETC-1002 for 14 days. Phospho/total protein ratios were calculated and are expressed as mean % vehicle ± SEM; n = 5. Comparisons were made using an unpaired Student \( t \)test; *\( P < 0.05 \).](http://www.jlr.org/content/suppl/2012/11/01/jlr.M030528.DC1.html)
thioesterification in primary rat hepatocytes. Treatment with ETC-1002 resulted in rapid uptake and CoA thioesterification (Fig. 2A), which was associated with immediate inhibition (≤ 5 min) of de novo lipid synthesis (Fig. 2B) and transient increases in phosphorylation of AMPK (T172), ACC (S79) and HMGR (S182) (Fig. 2C). These data revealed that ETC-1002 uptake in primary rat hepatocytes is closely linked to CoA thioesterification and, unlike in vivo, results in an approximately 1:1 to 2:1 molar ratio (ETC-1002 free acid:ETC-1002-CoA) and only transient AMPK activation (Fig. 2C). The identification of relatively low intracellular ETC-1002 free acid concentrations and lack of sustained AMPK activation in primary rat hepatocytes further suggested that, in vivo, the ETC-1002 free acid may indeed be linked to the AMPK activation, while ETC-1002-CoA may mediate its effects through a mechanism distinct from AMPK.

ETC-1002-CoA inhibits de novo sterol and fatty acid synthesis via direct inhibition of ACL

To characterize the link between ETC-1002-CoA thioesterification and equipotent inhibition of sterol and fatty acid synthesis, mass changes in intermediates of lipid synthesis were analyzed with a radiosotope tracer-independent LC-MS/MS method. Free CoA (CoASH), malonyl-CoA, HMG-CoA, acetyl-CoA, and citrate were selected to provide greater insight into ETC-1002-related effects occurring between mitochondrial citrate production and the entry of acetyl-CoA into the fatty acid and sterol synthesis pathways. The treatment of primary rat hepatocytes with ETC-1002 resulted in concentration-dependent reductions in acetyl-CoA, malonyl-CoA, and HMG-CoA, with concomitant increases in citrate (Fig. 3A and Table 1) and ETC-1002-CoA (Fig. 3B and Table 1), whereas no significant effect on CoASH concentration was observed (Fig. 3A and Table 1). Remarkably, these effects occurred within 5 min of treatment (data not shown). Conversely, the human hepatoma cell line HepG2 served as a negative control, showing the absence of ETC-1002-CoA formation (Fig. 3B). Importantly, Triacsin C, an inhibitor of multiple long-chain acyl-CoA synthetase (ACS) isoforms, not only reduced intracellular concentrations of ETC-1002-CoA but also attenuated the effects of ETC-1002 on metabolic intermediates (Table 2) and de novo lipid synthesis (Fig. 3C). These data demonstrate that the conversion of ETC-1002 to a CoA thioester is required for equipotent inhibition of de novo sterol and fatty acid synthesis and that inhibition occurs after citrate formation, at or before ACL-dependent acetyl-CoA production. Indeed, hepatic knockdown of ACL expression...
ETC-1002 activates AMPK and inhibits ATP-citrate lyase

Fig. 3. ETC-1002-CoA thioesterification and inhibition of total lipid synthesis. (A) Primary rat hepatocytes were treated for 2 h with the indicated concentration of ETC-1002, and intracellular CoASH, malonyl-CoA, HMG-CoA, acetyl-CoA, and citrate were measured by LC-MS/MS. (B) Hepatocytes and HepG2 cells were treated with the indicated concentration of ETC-1002-CoA was determined by HPLC. (C) [14C]acetate incorporation into total lipids was measured in hepatocytes treated for 4 h with 10 μM ETC-1002 and 1, 3, or 10 μM Triacsin C. Data from (A, C) were normalized to vehicle controls and expressed as mean percent ± SEM of triplicate wells. Data from (B) are expressed as pmol/well and shown as mean ± SEM of triplicate wells. Data are representative of multiple experiments. Comparisons were made with (C) using one-way ANOVA; *P < 0.05.

Sterol regulatory element binding proteins (SREBP) activity, resulting in the upregulation of key enzymes involved in fatty acid synthesis. Consistent with in vitro data, in nutritionally staged (lipogenic) rats treated with a single oral dose of 30 mg/kg of ETC-1002, the CoA thioester was detected in liver extracts within 2 h after dosing. Furthermore, ETC-1002 treatment was associated with a reduction in hepatic intermediates of lipid synthesis, including acetyl-CoA, malonyl-CoA, and HMG-CoA, and an increase in citrate levels (Fig. 4B). The effects on these intermediates of lipid synthesis were sustained when assessed 8 h after dosing, whereas citrate returned to levels comparable to vehicle-treated rats (data not shown).

While the ability of ETC-1002-CoA to directly inhibit ACL may explain, at least in part, the inhibition of de novo lipid synthesis both in vitro and in vivo, further understanding of the molecular mechanism for the ETC-1002 free acid should provide crucial information as to how these two active forms might cooperate to mediate multiple beneficial effects in preclinical disease models.

### TABLE 1. ETC-1002 induces concentration-dependent reductions in metabolic precursors of sterol and fatty acid biosynthesis

<table>
<thead>
<tr>
<th>ETC-1002</th>
<th>CoASH</th>
<th>Malonyl-CoA</th>
<th>HMG-CoA</th>
<th>Acetyl-CoA</th>
<th>Citrate</th>
<th>ETC-1002-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>449.0 ± 12.7</td>
<td>61.3 ± 4.1</td>
<td>21.9 ± 1.5</td>
<td>309.8 ± 19.3</td>
<td>4683.3 ± 300.5</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1 μM</td>
<td>463.7 ± 51.0</td>
<td>23.9 ± 0.5*</td>
<td>19.7 ± 0.3</td>
<td>201.0 ± 5.1*</td>
<td>5616.7 ± 788.1</td>
<td>115.3 ± 5.5</td>
</tr>
<tr>
<td>3 μM</td>
<td>463.7 ± 36.5</td>
<td>10.2 ± 1.6*</td>
<td>7.5 ± 0.7</td>
<td>145.3 ± 31.6*</td>
<td>5866.7 ± 1438.6</td>
<td>294.7 ± 19.1</td>
</tr>
<tr>
<td>10 μM</td>
<td>458.8 ± 24.5</td>
<td>3.0 ± 0.3*</td>
<td>3.9 ± 0.4</td>
<td>94.7 ± 12.5*</td>
<td>11166.7 ± 1166.7*</td>
<td>545.7 ± 10.5</td>
</tr>
<tr>
<td>30 μM</td>
<td>422.0 ± 54.2</td>
<td>0.8 ± 0.1*</td>
<td>3.1 ± 0.4*</td>
<td>106.3 ± 5.8*</td>
<td>10333.3 ± 1922.1</td>
<td>406.0 ± 113.0</td>
</tr>
<tr>
<td>100 μM</td>
<td>392.3 ± 4.9</td>
<td>BD ± N/A</td>
<td>2.3 ± 0.2*</td>
<td>57.3 ± 4.8*</td>
<td>10150.0 ± 2350.0</td>
<td>690.7 ± 11.8</td>
</tr>
</tbody>
</table>

Concentrations of CoASH, malonyl-CoA, HMG-CoA, acetyl-CoA, citrate, and ETC-1002-CoA are shown in primary rat hepatocytes exposed to 1, 3, 10, 30, and 100 μM ETC-1002 for 2 h. Data are expressed as percentage of vehicle in Fig. 3. Data are representative of multiple experiments and shown as mean (pmol/well) ± SEM of triplicate values.

*P < 0.05 compared with vehicle treatment (one-way ANOVA).
ETC-1002 activates the AMPK pathway via a calcium- and energy-independent mechanism

Unlike primary rat hepatocytes, HepG2 cells fail to generate measurable amounts of ETC-1002-CoA thioester (Fig. 3B), making this cell line a viable model for characterizing the potential AMPK-activating properties of ETC-1002 free acid. Consistent with the apparent association between ETC-1002 uptake and CoA thioesterification, HepG2 cells required higher media concentrations of the compound to achieve intracellular levels comparable to those measured in primary rat hepatocytes.

### TABLE 2. Triacsin C attenuates ETC-1002-dependent reductions in multiple biosynthetic precursors of sterol and fatty acid synthesis

<table>
<thead>
<tr>
<th>Analyte (pmol/well)</th>
<th>Vehicle</th>
<th>ETC-1002</th>
<th>Triacsin C</th>
<th>ETC-1002 + Triacsin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoASH</td>
<td>1250.0 ± 108.9</td>
<td>1185.0 ± 50.0</td>
<td>1368.3 ± 67.7</td>
<td>1271.7 ± 179.5</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>59.9 ± 7.0</td>
<td>11.2 ± 1.3</td>
<td>75.8 ± 4.9</td>
<td>32.2 ± 1.7</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>12.8 ± 1.5</td>
<td>6.5 ± 0.3</td>
<td>15.3 ± 2.3</td>
<td>13.6 ± 2.2</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>351.2 ± 25.7</td>
<td>158.8 ± 39.5</td>
<td>448.7 ± 22.6</td>
<td>322.3 ± 35.1</td>
</tr>
<tr>
<td>Citrate</td>
<td>1417.5 ± 775.0</td>
<td>14816.7 ± 164.1</td>
<td>9933.3 ± 990.5</td>
<td>11783.3 ± 277.4</td>
</tr>
<tr>
<td>ETC-1002-CoA</td>
<td>BD ± n/a</td>
<td>259.7 ± 11.1</td>
<td>BD ± n/a</td>
<td>129.7 ± 4.9</td>
</tr>
</tbody>
</table>

Primary rat hepatocytes were pretreated for 30 min with vehicle or 3 μM Triacsin C prior to the addition of 3 μM ETC-1002 for 2 h. Intracellular concentrations of CoASH, malonyl-CoA, HMG-CoA, acetyl-CoA, and citrate were measured by LC-MS/MS, ETC-1002-CoA was measured by HPLC. BD, below detection; n/a, non applicable. Data are shown as mean (pmol/well) ± SEM of triplicate values.

\( ^a P < 0.05 \) compared with vehicle (one-way ANOVA).
\( ^b P < 0.05 \) compared with ETC-1002 (one-way ANOVA).
\( ^c P < 0.05 \) compared with Triacsin C (one-way ANOVA).

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Fig. 4. ETC-1002-CoA inhibits recombinant human ACL in a cell-free system and reduces lipid synthesis intermediates downstream of ACL in vivo. (A) Inhibition of ACL by ETC-1002-CoA is concentration-dependent and competitive for CoASH. The controls, vehicle, hydroxycitric acid (HCA), and ETC-1002-FA (free acid) are shown. Data are representative of multiple experiments and shown as mean percentage of vehicle ± SEM of triplicate wells. (B) Normal fasted/refed Wistar rats were treated with a single 30 mg/kg ETC-1002 dose. Acetyl-CoA, malonyl-CoA, HMG-CoA, and citrate were measured in freeze-clamped liver samples 2 h post dose and expressed as ng/g liver (wet weight). Data are mean ± SEM, n = 5. Comparisons were made using unpaired Student’s t-test; *P < 0.05.
In primary rat hepatocytes and rat liver. No significant increases in markers of cell viability including LDH release and caspase 3/7 activity could be linked to compound exposure (data not shown).

Consistent with ETC-1002-induced AMPK activation observed in rat liver (Fig. 1), HepG2 cells treated with ETC-1002 revealed a sustained and concentration-dependent increase in AMPK (T172) and ACC (S79) phosphorylation comparable to the AMPK-activating effect of metformin (1,000 μM) (Fig. 5A). To further characterize the mechanism leading to AMPK activation by ETC-1002, HepG2 cells were pretreated with STO-609, an AMPK kinase Ca2+/calmodulin-dependent kinase (CamKKβ)-specific inhibitor. STO-609 did not significantly reduce AMPK or ACC phosphorylation in ETC-1002- or metformin-treated cells, indicating that AMPK activation is not dependent on intracellular Ca2+ signaling (Fig. 5B). Intriguingly, while the ATP analog and AMPK inhibitor, compound C, significantly reduced AMPK and ACC phosphorylation by metformin, it did not inhibit ETC-1002-dependent AMPK activation (Fig. 5B). To determine whether ETC-1002-dependent AMPK activation is associated with reductions in AEC, intracellular ATP, ADP, and AMP concentrations were measured in HepG2 cells treated with vehicle, rotenone (10 μM), or ETC-1002 (100 μM). Treatment with rotenone (complex I inhibitor) resulted in increased AMP and ADP levels and in reduced ATP levels and AEC compared with vehicle treatment, while ETC-1002 had no effect (Fig. 5C). These data suggest that the activation of the AMPK pathway by ETC-1002 may be independent of reductions in energy production.

Many immortalized and highly proliferative cell lines, such as HepG2, are known to synthesize the majority of their ATP from glycolysis despite fully functional mitochondria and sufficient oxygen availability (21). Therefore, to ensure that the apparent energy-independence of AMPK activation by ETC-1002 was not an artifact of compensatory anaerobic metabolism, HepG2 cells were grown in normal glucose-containing media or glucose-free media supplemented with galactose (22). Intracellular ATP was measured in cells treated with ETC-1002, rotenone, or CCCP (mitochondrial uncoupler). While HepG2 cells grown in the presence of glucose showed an approximately 50% reduction in ATP content when exposed to rotenone or CCCP, ATP levels dropped significantly further when galactose was substituted for glucose (Fig. 5D). Remarkably, treatment with up to 300 μM ETC-1002 in either growth medium did not alter ATP content, further indicating that AMPK activation by ETC-1002 was independent of effects on mitochondrial-dependent oxidative energy production. These intriguing findings in HepG2 cells suggest that ETC-1002 does not likely affect AMPK through the disruption of calcium homeostasis or energy production but through a distinct mechanism.

ETC-1002 decreases glucagon-dependent glucose production in hepatocytes in vitro

Hepatic glucose production is regulated by multiple factors, including nutrients, energy availability (ATP availability and AMPK signaling), hormone signaling (e.g., insulin and glucagon), and substrate flux. As ETC-1002 can potentially mediate its effects on lipid metabolism through AMPK- and ACL-related pathways, glucagon-stimulated glucose production was assessed in primary rat hepatocytes exposed to ETC-1002 or insulin. Glucagon treatment alone induced an approximately 2-fold increase in glucose levels in media conditioned by hepatocytes overnight, which was reduced by ~50% in cells treated with 10 μM ETC-1002 or 100 nM insulin (Fig. 6A). Additionally, inhibition of glucose production by ETC-1002 was concentration-dependent, with IC50 = 3.6 μM (Fig. 6B). Further investigation into the underlying mechanism(s) mediating these effects showed that ETC-1002 decreased glucagon-induced PEPCK and G6Pase protein expression (Fig. 6C). Furthermore, unstimulated hepatocytes treated overnight with metformin or ETC-1002 showed reductions in total FOXO1 levels by ~30% and ~40%, respectively (Fig. 6D), consistent with its role in hepatic glucose production. These data demonstrate that ETC-1002 reduces hepatocyte glucose production in primary rat hepatocytes through a mechanism associated with reductions in FOXO1, PEPCK, and G6Pase protein levels.

LKB1 is required for ETC-1002-mediated activation of the AMPK pathway

In liver, physiological activation of AMPK is considered to be mediated primarily through LKB1-dependent phosphorylation. To determine whether the activation of AMPK by ETC-1002 was LKB1-dependent, we used small inhibitory RNA (siRNA) interference to reduce endogenous LKB1 protein levels in HepG2 cells and assessed the effects of ETC-1002 on ACC (S79) phosphorylation along with AEC, intracellular lipids, and the effectors of gluconeogenesis, FOXO1, and HNF-4α.

HepG2 cells were transfected with negative control “mock” or LKB1 siRNA and treated for 24 h with vehicle, ETC-1002 (100 μM), or metformin (1,000 μM). HepG2 cells transfected with LKB1 siRNA resulted in a 75% (P = 0.0119) reduction in LKB1 protein compared with cells transfected with mock siRNA (Fig. 7A). While mock-transfected cells showed an ETC-1002- and metformin-dependent increase (ETC-1002 = +780% ± 35; P = 0.0374; metformin = +730% ± 80; P = 0.0187) in ACC (S79) phosphorylation and reductions in HNF-4α protein levels (Fig. 7B, C), these effects were abolished in cells transfected with LKB1 siRNA, demonstrating that both ETC-1002 and metformin activate AMPK in an LKB1-dependent fashion. Interestingly, FOXO1 protein levels were not changed with ETC-1002 treatment, metformin treatment, or LKB1 knockdown, suggesting the LKB1-AMPK axis does not control FOXO1 expression under these conditions (data not shown). Consistent with inhibition of mitochondrial respiration, the treatment of HepG2 cells with metformin resulted in a reduction in AEC, which was further decreased by LKB1 knockdown (Fig. 7D and supplementary Table 1). Contrary to the activities of metformin and consistent with energy-independent nature of AMPK activation by ETC-1002, no changes in
AEC in either mock or LKB1 siRNA-treated cells were detected in cells treated with ETC-1002. Importantly, LKB1 knockdown also prevented ETC-1002- and metformin-induced reduction in intracellular total cholesterol and TG levels (Fig. 7E, F). These data demonstrate that LKB1 activity is required for ETC-1002-mediated AMPK activation via a pathway that does not alter energy production in HepG2 cells.

Fig. 5. ETC-1002 activates the AMPK pathway independent of energy depletion and calcium signaling in HepG2 cells. (A) HepG2 cells were treated for 4 h with vehicle (Veh) or the indicated concentration of ETC-1002 or metformin, and the phosphorylation of AMPKα (T172) and acetyl-CoA carboxylase (ACC) (S79) were assessed from cell lysates by Western blotting. Data are expressed as a ratio of phospho/total and presented as mean percentage of vehicle ± SEM of two wells. (B) HepG2 cells were pretreated with vehicle, 40 μM Compound C (Cmpd C), or 2.5 μM STO-609 prior to exposure to 100 μM ETC-1002 or 1,000 μM metformin for 4 h. Cell lysates were blotted for phospho-AMPKα (T172) (P-AMPK), total AMPKα, phospho-ACC (S79) (P-ACC), total ACC, and β-actin. (C) HepG2 cells were treated with 100 μM ETC-1002 or 10 μM rotenone for 4 h. ATP, ADP, and AMP levels were determined using LC-MS/MS. ADP/ATP, AMP/ATP, and adenylate energy charge (AEC) were calculated. (D) HepG2 cells were grown in glucose or galactose media and exposed to vehicle, 3, 10, 30, 100, 300 μM ETC-1002, 10 μM CCCP, or 10 μM rotenone for 24 h. Relative intracellular ATP concentrations were determined using the ATPlite Luminescence ATP detection assay system (PerkinElmer) according to manufacturer’s directions. Representative data from multiple experiments are shown. Data (A, D) were normalized to vehicle treatment and expressed as mean percentage of vehicle ± SEM of duplicate wells. Data (C) are expressed as mean ± SEM triplicate wells, and ADP/ATP, AMP/ATP, and AEC were calculated. Comparisons were made by one-way ANOVA; *P < 0.05 compared with vehicle treatment in same medium and †P < 0.05 between media within same compound treatment.
ETC-1002 modulates plasma and tissue functional biomarkers of AMPK and ACL activity in vivo

To determine whether modulation of AMPK and ACL activities by ETC-1002 could be linked to physiological responses in vivo, plasma and liver samples from chow-fed Wistar rats treated with ETC-1002 for two weeks at 30 mg/kg were examined for functional biomarkers of alterations in lipid and carbohydrate metabolism. Safety studies in the Wistar rat orally dosed with ETC-1002 at 30 mg/kg for four weeks showed plasma exposures equivalent to clinical exposures with no meaningful drug-related effect on safety parameters, including liver markers of injury alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fig. 8A). Activation of hepatic AMPK with ETC-1002 treatment from the two-week Wistar rat study was not associated with altered hepatic AEC (Fig. 8B and Table 3); however, ETC-1002 treatment coincided with a 70% reduction in hepatic TG, while low basal levels of CE and FC remained insensitive to treatment (Fig. 8B). Importantly, reductions in liver TG content was accompanied by 51% increase in plasma β-HBA, indicating enhanced liver fatty acid β-oxidation (supplementary Table II). Likewise, reductions in plasma TG (−34%) and non-HDL-C (−21%), along with a greater than 2-fold increase in plasma HDL-C (supplementary Table I), were consistent with previously published effects of pharmacological...
ETC-1002 corrects dyslipidemia in golden Syrian hamsters and improves glycemic control in a mouse model of diet-induced obesity

To evaluate the therapeutic effects of ETC-1002 on metabolic disease in vivo, a series of pharmacology studies were undertaken in dyslipidemic golden Syrian hamsters and in the mouse model of diet-induced obesity (DIO). The human-like lipoprotein metabolism of the golden Syrian hamster characterized by comparable LDL receptor regulation (24), plasma cholesteryl ester transfer protein activity (25), and hepatic secretion of full-length apoB (apoB100) containing VLDL particles (26) make it an attractive model for preclinical evaluation of hypolipidemic agents. Furthermore, feeding hamsters a HFHC diet increases weight gain and induces dearrangements in lipoprotein metabolism, resulting in hypertriglyceridemia and hypercholesterolemia with a human-like dyslipidemic lipoprotein profile and activation of AMPK in rats (23). Furthermore, ETC-1002 treatment increased levels of hepatic PGC-1α (68.4% ± 25.6; P = 0.0240) and mitochondrial citrate synthase (105.6% ± 25.2; P = 0.0030), indicating the upregulation of hepatic oxidative metabolism (Fig. 8C). Although the chow-fed rat is not a model for hyperglycemia or dyslipidemia, the reduced basal levels of liver FOXO1 and HNF-4α (61.4% ± 10.0; P = 0.0003 and 54.7% ± 12.2; P = 0.002, respectively) associated with ETC-1002 treatment further supports a potential mechanistic link to the regulation of carbohydrate metabolism.

This mechanistic translation into a normal chow-fed rat model provides further evidence for the AMPK-activating properties of ETC-1002 observed in vitro. Importantly, these data support the AEC-independent activation of AMPK by ETC-1002, which was associated with multiple markers of AMPK activation at the levels of signal transduction, transcription factors, hepatic lipid mass, and lipid biomarkers.

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ETC-1002 activates AMPK and inhibits ATP-citrate lyase

An excess of visceral adipose tissue coupled with hyperglycemia and hyperinsulinemia makes the mouse model of DIO an attractive in vivo tool for studying mechanisms of impaired glycemic control and for the characterization of novel therapeutic agents (33–38). To determine whether ETC-1002 improves glucose homeostasis in vivo, mice were kept on HFD for 12 weeks prior to ETC-1002 treatment. Administration of ETC-1002 at 30 mg/kg for 14 days resulted in a 9% (P < 0.05) reduction in body weight (Fig. 10) while daily food consumption remained unchanged. Consistent with decreased glucose production by hepatocytes in vitro (Fig. 6), two weeks of treatment with ETC-1002 was sufficient to significantly reduce fasting plasma glucose levels by 13% (P < 0.05). Likewise, plasma insulin levels in animals treated with ETC-1002 were reduced by 42% (P < 0.05) (Fig. 10) further supporting the beneficial effects of ETC-1002 on regulation of glucose homeostasis in vivo.

Based on these data generated in a hamster model of dyslipidemia and a mouse model of DIO, ETC-1002 treatment has a broad range of favorable metabolic effects, consistent with the activation of AMPK and inhibition of hepatic ACL. While the relative contributions of AMPK activation and ACL inhibition in vivo are not addressed in these studies, the robust effects of ETC-1002

**TABLE 3. Hepatic ATP, ADP, and AMP levels in Wistar rats treated with ETC-1002**

<table>
<thead>
<tr>
<th></th>
<th>ATP (ng/mg liver)</th>
<th>ADP (ng/mg liver)</th>
<th>AMP (ng/mg liver)</th>
<th>ADP/ATP</th>
<th>AMP/ATP</th>
<th>AEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>9378.0 ± 1124.0</td>
<td>4666.0 ± 148.6</td>
<td>5558.0 ± 451.1</td>
<td>0.5232 ± 0.05837</td>
<td>0.6463 ± 0.1252</td>
<td>0.5926 ± 0.0334</td>
</tr>
<tr>
<td>ETC-1002</td>
<td>9264.0 ± 791.4NS</td>
<td>4714.0 ± 416.0NS</td>
<td>6166.0 ± 878.8NS</td>
<td>0.5086 ± 0.00528NS</td>
<td>0.6501 ± 0.0481NS</td>
<td>0.5796 ± 0.0128NS</td>
</tr>
</tbody>
</table>

Freeze-clamped liver samples from Wistar rats treated with ETC-1002 (30 mg/kg/day) for 14 days (same animals as in Fig. 1) were analyzed for ATP, ADP, and AMP levels using LC-MS/MS. ADP/ATP, AMP/ATP, and AEC were calculated for each animal. Data are expressed as mean ± SEM; n = 5. NS P > 0.05 using unpaired Student t-test.

**Fig. 8.** Energy independent activation of hepatic AMPK by ETC-1002 is associated with reduced liver triglyceride, FOXO1 and HNF-4α protein levels, and markers of oxidative metabolism. (A) Plasma AST and ALT from male and female Wistar rats treated for four weeks with ETC-1002 (30 mg/kg/day). (B) Liver from the same animals as in Fig. 1 were analyzed for AEC, TG, CE, and FC content, and expressed as mg/g liver wet weight. (C) Protein levels of PGC-1α, citrate synthase, FOXO1, and HNF-4α were measured by Western blot, normalized to β-actin, and expressed as mean percentage of vehicle control ± SEM, n = 5. Comparisons were made using unpaired Student t-test; *P < 0.05.

hepatic steatosis (27–32). To determine whether ETC-1002 beneficially affects tissue and plasma parameters associated with dyslipidemia, HFHC-fed hamsters were treated for three weeks with ETC-1002 (30 mg/kg/day). ETC-1002 administration was associated with detectable levels ETC-1002-CoA in liver extracts (data not shown) and a 14.4% (P < 0.01) decrease in body weight gain, while no significant changes in food consumption or plasma ALT or AST were observed (data not shown). Consistent with enhanced fatty acid β-oxidation, ETC-1002 treatment increased plasma β-HBA (20%; P < 0.05) and reduced plasma NEFA and epididymal fat mass by 34% and 35%, respectively (P < 0.05) (Fig. 9A). Remarkably, ETC-1002 treatment resulted in dramatic reductions in hepatic TG (−64%, P < 0.001), CE (−67%, P < 0.05), and FC (−31%, P < 0.05) content (Fig. 9B), along with reductions in plasma TG (−41%, P < 0.05) and total cholesterol (−41%, P < 0.05) (Fig. 9C). Further insight into the hypolipidemic effects of ETC-1002 was obtained by measuring the distribution of cholesterol among the proatherogenic lipoprotein particles LDL and VLDL. Hamsters treated with ETC-1002 showed a 64% and 62% reduction in LDL-C and VLDL-C, respectively (P < 0.05) (Fig. 9C), while HDL-C levels remained unchanged (data not shown). An excess of visceral adipose tissue coupled with hyperglycemia and hyperinsulinemia makes the mouse model of DIO an attractive in vivo tool for studying mechanisms of impaired glycemic control and for the characterization of novel therapeutic agents (33–38). To determine whether ETC-1002 improves glucose homeostasis in vivo, mice were kept on HFD for 12 weeks prior to ETC-1002 treatment. Administration of ETC-1002 at 30 mg/kg for 14 days resulted in a 9% (P < 0.05) reduction in body weight (Fig. 10) while daily food consumption remained unchanged. Consistent with decreased glucose production by hepatocytes in vitro (Fig. 6), two weeks of treatment with ETC-1002 was sufficient to significantly reduce fasting plasma glucose levels by 13% (P < 0.05). Likewise, plasma insulin levels in animals treated with ETC-1002 were reduced by 42% (P < 0.05) (Fig. 10) further supporting the beneficial effects of ETC-1002 on regulation of glucose homeostasis in vivo.

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on hepatic and plasma lipid parameters along with improved glucose homeostasis suggest that these two activities may work cooperatively.

**DISCUSSION**

These in vitro and in vivo studies support that ETC-1002 works through two distinct mechanisms: i) activation of the hepatic AMPK pathway by the ETC-1002 free acid and ii) direct inhibition of hepatic ACL by the ETC-1002-CoA thioester (Fig. 11). The elucidation of these distinct molecular mechanisms was contingent on the identification of a key difference that exists between our in vitro rat hepatocyte model and intact rat liver. In our in vitro model, primary rat hepatocytes predominantly convert ETC-1002 to a CoA thioester, limiting intracellular exposure to the free acid. In vivo, we show that ETC-1002 free acid is >100-fold more prevalent than the CoA thioester in rat liver and is associated with AMPK activation. This difference between in vitro and in vivo results may explain the absence of AMPK activation observed in previous studies (12). The rapid formation of ETC-1002-CoA and subsequent transient increases in citrate with concomitant reductions in acetyl-CoA, malonyl-CoA, and HMG-CoA in rat hepatocytes is consistent with ACL inhibition (Fig. 10) and dual inhibition of fatty acid and sterol synthesis (Fig. 10). The role of ETC-1002-CoA in inhibition of lipid synthesis was further supported by exploiting the ACS inhibitory activity of Triacsin C, which reduced ETC-1002-CoA formation, and ETC-1002-dependent reductions in acetyl-CoA, malonyl-CoA, and HMG-CoA. Interestingly, cotreatment of hepatocytes with ETC-1002 and Triacsin C trended toward increased citrate levels, which may appear inconsistent with ACL deinhibition. This result could be due to inhibition of fatty acid oxidation by Triacsin C, resulting in a dependence on glucose oxidation with increased acetyl-CoA levels supporting additional citrate production. Inhibition
of ACL was supported in vivo by demonstrating that hepatic ETC-1002-CoA formation was associated with decreases in the ACL product acetyl-CoA, which is the final common substrate for both fatty acid and sterol synthesis. Furthermore, we show that ETC-1002-CoA inhibits recombinant human ACL (rhuACL) directly in a cell-free system.

ACL is a key cytosolic enzyme, which precedes HMGR and ACC in the lipid biosynthesis pathways and catalyzes the cleavage of mitochondrial-derived citrate to cytosolic oxaloacetate and acetyl-CoA (39–41). ACL is highly expressed in lipogenic tissues, such as liver and adipose (42), and its products are acetyl-CoA and oxaloacetate. In liver, oxaloacetate can serve as the rate-limiting substrate for gluconeogenesis. It is interesting to speculate that ETC-1002-CoA-mediated ACL inhibition may contribute to reduced rates of gluconeogenesis.

Pharmacological inhibition of ACL has been shown to limit sterol and fatty acid synthesis (40, 45–46), upregulate LDL receptor activity (47), and reduce plasma triglycerides and cholesterol levels (45, 48). Furthermore, liver-specific ACL abrogation results in reduced levels of liver acetyl-CoA and malonyl-CoA, plasma triglycerides and fatty acids (19), and hepatic lipids (20).

Intriguingly, recent work has identified a novel regulatory role for ACL-derived acetyl-CoA levels, which links energy substrate availability with gene expression through controlling substrate availability for the acetylation of key proteins (49–51). These findings have raised the possibility that extra-mitochondrial acetyl-CoA concentrations may also regulate PGC-1α acetylation through controlling activity of the acetyltransferase GCN5 at the substrate level. Until recently, the regulatory roles of acetyl-CoA have been believed to be primarily mediated within mitochondria through allosteric means, such as controlling the fate of pyruvate toward carboxylation or dehydrogenation. Recently, many extra-mitochondrial regulatory acetylations have been characterized and shown to be dependent on ACL-derived acetyl-CoA pools (50). These findings support a link between energy excess and gene regulation, as sensed through glucose-derived acetyl-CoA-dependent cytosolic/nuclear acetylation reactions (49, 50).

For many years, the position of ACL in lipid biosynthesis has made it an attractive target for pharmacological

Fig. 11. ETC-1002 activates AMPK and inhibits ACL to regulate lipid and carbohydrate metabolism. In liver, ETC-1002 free acid activates the AMPK pathway, which reduces the activities of key rate-limiting enzymes of lipid (HMGR and ACC) and carbohydrate (PEPCK and G6Pase) synthesis. ETC-1002 is also converted to ETC-1002-CoA by ACS and directly inhibits ACL. Inhibition of ACL results in a reduction in cytosolic acetyl-CoA, a common substrate supporting fatty acid and sterol synthesis, as well as reduces HMG-CoA and malonyl-CoA levels. AMPK-dependent inhibitory ACC phosphorylation and reduced enzyme substrate decrease malonyl-CoA levels, deinhibit CPT-1, and increase fatty acid mitochondrial transport for β-oxidation. The combination of AMPK activation and ACL inhibition reduces lipid and glucose synthesis from the substrate and signals transcription levels. ACC, acetyl-CoA carboxylase; ACS, acyl-CoA synthetase; β-Ox, β-oxidation; CPT-1, carnitine palmitoyltransferase-1; G6Pase, glucose 6-phosphatase; HMGR, HMG-CoA reductase; PEPCK, phosphoenolpyruvate carboxykinase; TCA, tricarboxylic acid.
inhibition. However, the emerging role of ACL-derived acetyl-CoA level in linking energy substrate availability with gene expression heightens the relevance of ACL in regulating energy homeostasis.

The underlying mechanism mediating the in vitro and in vivo activation of AMPK by ETC-1002 is particularly intriguing. We show that the ETC-1002 free acid activates AMPK in HepG2 cells independently of changes in the AEC and the calcium-sensing calcium CaMKKβ pathway. Furthermore, compound C, an ATP analog, failed to inhibit activation of AMPK by ETC-1002, consistent with a mechanism independent of AEC. Long-chain fatty acids (LCFA) and their modified analogs have been shown to regulate AMPK activity through direct and indirect mechanisms (52–64). The mechanism most consistent with the activities described for ETC-1002 is that shown by Watt et al., which demonstrates that natural LCFA directly interacts with AMPK, and enhances LKB1-dependent AMPK activation in L6 myotubes independently of AEC (57). This leaves open the possibility that ETC-1002 may increase AMPK activity through a similar direct mechanism. Importantly, the activation of AMPK by natural LCFA is unlikely to yield therapeutic benefit as they also provide substrates for energy production and the formation of DAG (65, 66). Therefore, fatty acid mimetics and/or modifications to LCFA that limit their metabolism may prove to be a viable therapeutic strategy if they retain their AMPK-activating properties.

The AMPK activator metformin is currently the first-line oral therapy for hyperglycemia in individuals with T2D (67, 68). However, monotherapy is capable of maintaining target glycemic control for only a short time (69), and low bioavailability and GI symptoms can limit the treatment dosage. Metformin decreases hepatic glucose production, in part, through inhibition of complex I of the mitochondrial respiratory chain (70, 71), resulting in the activation of AMPK (72). The antidiabetic and antistatotic effects of AMPK activation are primarily mediated through downregulation of the gluconeogenic genes pepck and g6pase (73) and modulation of transcription factors, such as SREBP1, ChREBP, TORC2, HNF-4α, FOXO1, and PGC-1α (72–78). Furthermore, AMPK acutely inhibits fatty acid and sterol de novo synthesis through inhibitory phosphorylation of ACC (79, 80) and HMGR (81), respectively. Interestingly, unlike ETC-1002, metformin does not consistently reduce LDL-C in dyslipidemic and hyperglycemic subjects, which may highlight the beneficial effects of ACL inhibition mediated by ETC-1002-CoA.

The combination of AMPK activation by ETC-1002 and inhibition of ACL by the ETC-1002-CoA thioester would be expected to complement one another, as they affect hepatic lipid synthesis at the signal transduction and substrate-levels, respectively. This unique activity may provide benefit over other AMPK activators (e.g., metformin), which have been shown to provide little benefit for normalizing plasma LDL-C. Furthermore, this dual activity may, in part, account for the pleotropic nature of nonclinical therapeutic benefits observed with structurally related compounds (82–92) and provide further clarity into their respective mechanisms (84, 91, 93–100).

The effects of ETC-1002 on hepatic PGC-1α protein levels are also interesting. In liver, PGC-1α is known to be an important transcriptional coactivator that mediates many metabolic adaptations associated with the fasting and exercise phenotype. PGC-1α activity increases in response to glucagon and low energy signals, resulting in increasing nuclear receptors’ (e.g., PPARα) target gene transcription and increasing fatty acid catabolism (101). Additionally, activation of AMPK in liver has been shown to result in the upregulation of PGC-1α activity and protein levels, which was associated with increased mitochondrial content (102). Although mitochondrial number was not measured in the studies presented here, the increases in PGC-1α and citrate synthase associated with ETC-1002 treatment suggest mitochondrial adaptations consistent with AMPK activation.

PGC-1α also coordinates the hepatic fasting gluconeogenic response through its interactions with transcription factors FOXO1 and HNF-4α. The coordination of these interactions maintains hormone sensitivity during the fasted and fed conditions, ensuring appropriate PEPCk and G6Pase expression levels (76, 103). Interestingly, the activation of AMPK has been shown to directly phosphorylate HNF-4α, resulting in altered nuclear localization, reduced DNA binding, and increased degradation (77, 104). Consistently, ETC-1002 treatment was associated with reduced hepatic HNF-4α protein levels, an activity that was shown in HepG2 cells to be dependent on the LKB1/AMPK axis. Furthermore, pathophysiological conditions associated with hepatic insulin resistance results in the deregulation of posttranscriptional modification (phosphorylation and acetylation) of FOXO1, leading to hyperactive transcriptional activity, which is believed to contribute to hyperglycemia (105). Although reductions in FOXO1 protein levels were observed in rat liver and primary rat hepatocytes, protein levels did not change in response to ETC-1002 treatment in HepG2 cells. Zatara et al. (106) showed that the LCFA analogs of the AMPK-activating MEDIca series reduced liver FOXO1 protein levels in Sprague-Dawley rats and suppressed the nuclear:cytosolic ratio in HepG2 cells in an AMPK-dependent manner. Additional investigations are required to determine whether ETC-1002 modulates FOXO1 nuclear localization in a similar manner or through a distinct mechanism linked to ACL inhibition.

In the present studies, we have described two molecular targets for ETC-1002 and found that the free acid activates AMPK, whereas the ETC-1002-Coa thioester directly inhibits hepatic ACL. We show that these distinct molecular mechanisms are complementary in their beneficial effects on the regulation of lipid and carbohydrate metabolism in vitro and in vivo. Furthermore, we have demonstrated therapeutic relevance by showing that ETC-1002 reduces circulating proatherogenic LDL- and VLDL-cholesterol concentrations, as well as body weight gain and hepatic lipids in a human-like hyperlipidemic hamster model. These unique properties of ETC-1002 can be potentially
utilized for therapeutic interventions designed not only to produce statin-like effects for lowering LDL-C in dyslipidemic subjects but also to reduce other risk factors associated with CVD and MetS.

REFERENCES


