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Small molecule activation of lecithin cholesterol acyltransferase modulates lipoprotein metabolism in mice and hamsters

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ABSTRACT

The objective was to assess whether pharmacological activation of lecithin cholesterol acyltransferase (LCAT) could exert beneficial effects on lipoprotein metabolism. A putative small molecule activator (compound A) was used as a tool compound in in vitro and in vivo studies. Compound A increased LCAT activity in vitro in plasma from mouse, hamster, rhesus monkey, and human. To assess the acute pharmacodynamic effects of compound A, C57Bl/6 mice and hamsters received a single dose (20 mg/kg) of compound A. Both species displayed a significant increase in high-density lipoprotein cholesterol (HDLc) and a significant decrease in non-HDLc and triglycerides acutely after dosing; these changes tracked with ex vivo plasma LCAT activity. To examine compound A's chronic effect on lipoprotein metabolism, hamsters received a daily dosing of vehicle or of 20 or 60 mg/kg of compound A for 2 weeks. At study termination, compound treatment resulted in a significant increase in HDLc, HDL particle size, plasma apolipoprotein A-I level, and plasma cholesteryl ester (CE) to free cholesterol ratio, and a significant reduction in very low-density lipoprotein cholesterol. The increase in plasma CE mirrored the increase in HDL CE. Triglycerides trended toward a dose-dependent decrease in very low-density lipoprotein and HDL, with multiple triglyceride species reaching statistical significance. Gallbladder bile acids content displayed a significant and more than 2-fold increase with the 60 mg/kg treatment. We characterized pharmacological activation of LCAT by a small molecule extensively for the first time, and our findings support the potential of this approach in treating dyslipidemia and atherosclerosis; our analyses also provide mechanistic insight on LCAT's role in lipoprotein metabolism.

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1. Introduction

Lecithin cholesterol acyltransferase (LCAT) is a plasma enzyme that esterifies the 3β-hydroxyl group of free cholesterol (FC) with the fatty acyl chain from the sn-2 position of phosphatidylcholine (PC) [1]. The majority of circulating LCAT resides in high-density lipoprotein (HDL) particles and is responsible for cholesterol esterification in HDL and HDL maturation from small particles to large, cholesteryl ester (CE)–enriched particles [1-3]. It has therefore been hypothesized that increased LCAT activity can increase HDL cholesterol (HDLc) levels, promote reverse cholesterol transport (RCT), and exert beneficial effects on dyslipidemia and atherosclerosis [1,4-6].

Human LCAT deficiency syndromes are associated with lipid disorders and pathological sequelae, and the full and partial loss-of-function mutations result in familial LCAT deficiency (FLD) and fish eye disease, respectively [7]. Major clinical findings for severe LCAT deficiency include corneal opacification, anemia, proteinuria, and glomerulosclerosis, which is marked by excessive deposition of lipids in the kidneys and often progression into renal failure [7,8]. The lipid disorders associated with FLD and fish eye disease are highly variable but always include HDL deficiency [7,9]. Lecithin cholesterol acyltransferase mutant allele carriers may also have elevated low-density lipoprotein (LDL) [10]. Phenotypes regarding atherosclerotic lesion development have appeared variable. For example, there have been cases where examination of arteries from FLD patients at autopsy revealed early or advanced atherosclerosis [11-13], but also cases in which FLD patients did not manifest premature atherosclerosis [14]. Controlled cohort studies where LCAT natural mutations were found to be associated [10] or not associated [15] with increased lesion development via imaging have both been reported. Possible reasons for the conflicting findings include small cohort size, variable confounding risk factors, and different methodologies for lesion assessment used.

Numerous animal models for LCAT over- or under-expression have been generated, and results from mouse models have been inconsistent. For example, an LCAT knockout (KO) mouse generated on a background of either apolipoprotein (apo) E KO or LDL receptor KO displayed reduced HDLc but paradoxically reduced atherosclerosis, possibly due to concomitant reduction in non-HDLc [16]. Transgenic overexpression of LCAT in mice resulted in an increase in large, CE-enriched HDL but paradoxically accelerated atherosclerosis [17], which was ameliorated when the cholesteryl ester transfer protein (CETP) transgene was introduced [18]. In comparison, in higher species such as rabbit, transgenic overexpression of LCAT resulted in not only a significant increase in HDLc, but also a marked decrease in non-HDLc, triglyceride, and lesion development [19]. Overexpression of LCAT via somatic viral delivery also resulted in an antiatherogenic lipid profile characterized by increased HDLc and decreased apoB and triglycerides (TG) in monkeys [20]. In hamsters, LCAT overexpression resulted in increased HDLc, biliary cholesterol excretion, and hepatic Cyp7a1 messenger RNA (mRNA) [21]. Taken together, animal studies suggest that effects of LCAT on lipid profile and lesion development are highly dependent on animal models and the presence of additional key lipid metabolizing enzymes such as CETP. Studies conducted in hamsters, rabbits, and monkeys, which more closely resemble humans in their lipoprotein metabolism, suggest that increased LCAT functionality likely is beneficial for lipid metabolism and atherosclerosis.

Recombinant LCAT infusion as a potential therapeutic approach for treating dyslipidemia and atherosclerosis has gained attention in recent years. Examples include recombinant LCAT infusion studies in mice [22] and rabbits [23]. In an effort to identify therapeutically relevant strategies to stimulate LCAT activity, recent reports have described a class of small-molecule activators of LCAT [24,25]. The therapeutic implications of a small-molecule activator are promising; however, information regarding the pharmacology and mechanism of action of these putative LCAT activators (including compound A, Fig. 1) are lacking. The purpose of the current study was to characterize the pharmacology and mechanism of action of compound A using both in vitro and in vivo models of LCAT biology.

2. Methods

2.1. *Compound A*

Compound A was prepared as described previously [25]. Briefly, to a cooled (0°C) solution of 5-(ethylthio)-1,3,4-thiadiazole-2-thiol (2.56 g, 14.3 mmol) in benzene (24 mL) and DMF (N, N-dimethylformamide) (24 mL) was added sodium hydride (0.631 g, 60% by weight, 15.8 mmol). After 15 minutes, 3-chloropyrazine-2-carbonitrile (2.00 g, 14.3 mmol) was added; and the resulting mixture was heated at 80°C. After 3 hours, the reaction mixture was allowed to cool to ambient temperature and then was poured into saturated aqueous NH_4Cl. The resulting mixture was extracted with EtOAc. The organic phase was separated and concentrated in vacuo. Purification by silica gel chromatography (Isco CombiFlash [Teledyne Isco, Lincoln, NE], 120-g column, 0% to 30% EtOAc in hexanes) provided compound A (2.22 g, 7.89 mmol, 55% yield) as a light-yellow solid: LC/MS m/z 282.2 $[M + H]^+$; ^1H NMR (500 MHz, $CDCl_3$) δ 8.57 (d, J = 2.0 Hz, 1 H), 8.53 (d, J = 2.0 Hz, 1 H), 3.42 (q, J = 7.5 Hz, 2 H), 1.50 (t, J = 7.5 Hz, 3 H).

2.2. *Animals*

All procedures described below were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee (Rahway, NJ) and were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the US National Institutes of Health. Animals were maintained in a 12-hour/12-hour light-dark cycle with free access to food and water in group housing conditions in a temperature-controlled environment (22°C).

Male C57BL/6 mice obtained from Taconic Farms (Germantown, NY) were maintained on regular rodent chow (7012; Teklad, Madison, WI; 5% dietary fat; 3.75 kcal/g). Mice were assigned into 7 groups (n = 6 per group). One group of mice was dosed with vehicle (30% Captisol [CyDex, Lenexa, KS], intraperitoneally); mice from this group were sacrificed, and

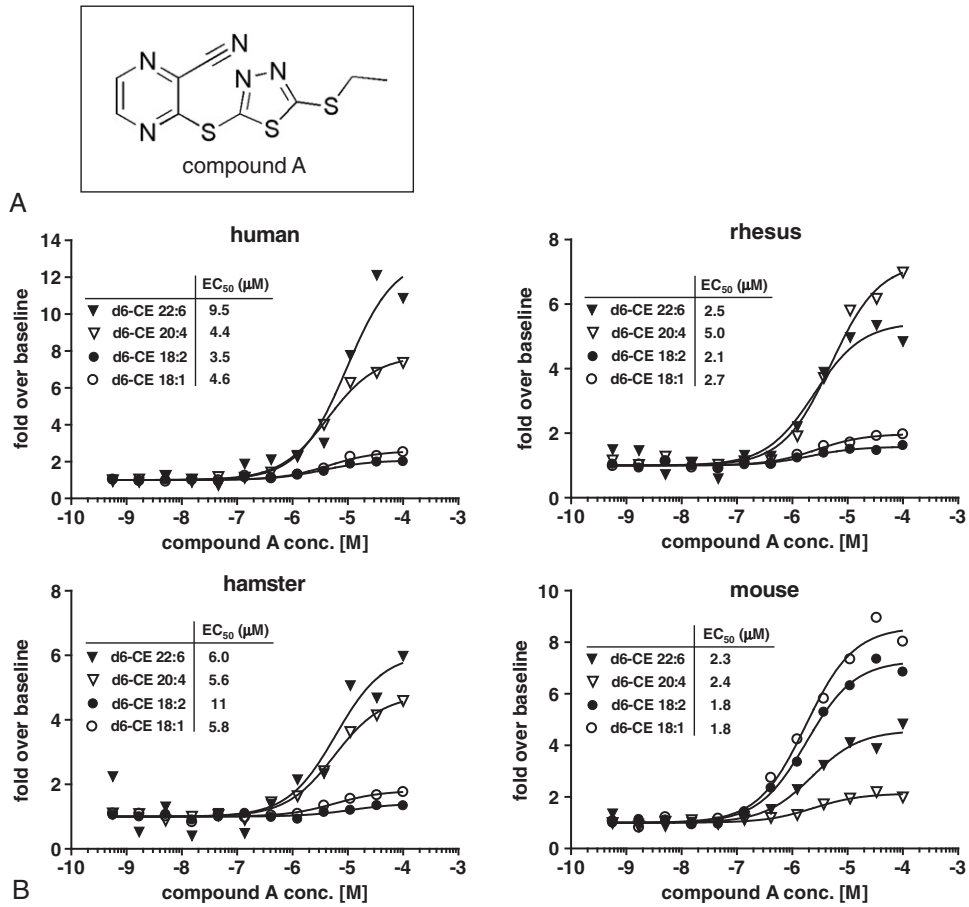

Fig. 1 – Compound A stimulated plasma LCAT activity from multiple species in vitro. A, Structure of compound A. B, Compound A at different concentrations was spiked into plasma from human, rhesus monkey, hamster, and C57Bl/6 mouse. Samples' LCAT activity was then measured by the LC/MS assay. LCAT is calculated as the ratio of the signal of each d6-labeled CE product to the signal of internal standard and then expressed as fold difference (y-axis) over baseline activity for the corresponding CE product. x-Axis is the concentration of compound A in plasma. EC_{50} is shown in the graph.

blood was collected 1 hour following dosing. Mice from the remaining groups were dosed with compound A (20 mg/kg, intraperitoneal injection); and mice from 1 of each of the 6 groups were sacrificed at 1, 3, 6, 10, 24, or 48 hours, respectively, following dosing. Blood was collected in EDTA via cardiac puncture, and plasma was reserved for various analyses. Livers were collected and snap-frozen in liquid nitrogen and stored at –80°C for gene expression analysis.

Male Syrian golden hamsters (Harlan Laboratories, Madison, WI) were placed on a high-fat diet (D08092301; Research Diets, New Brunswick, NJ; 45% kcal from fat/lard, 0.12% cholesterol) for 3 weeks before treatment. In the acute treatment study (single dose), hamsters were treated with either vehicle (30% Captisol) or compound A at 20 mg/kg (intraperitoneal injection). Blood was collected at 1, 5, 24, and 48 hours following treatment via jugular vein and at 72 hours via cardiac puncture. Plasma in EDTA was reserved for various analyses. In the chronic treatment study, hamsters were separated into 3 treatment groups, randomized by body weight (n = 8 per group). Hamsters were treated daily with either vehicle (0.5% methyl cellulose) or compound A at 20 and 60 mg/kg via oral gavage. After 14 days of treatment, blood was collected via cardiac puncture; and plasma was isolated for determination of effect on lipids. Gallbladder bile was collected by aspiration. All samples were stored at –80°C until analyzed.

2.3. *LCAT activity assay*

LCAT was measured using a modified liquid chromatography/mass spectrometry (LC/MS) assay based on the originally described proteoliposome substrate–thin layer chromatography method [26], with the incorporation of stable isotope-labeled FC in the substrate, to allow LC/MS detection of newly synthesized CE species. Briefly, a proteoliposome substrate that contained egg yolk PC (Sigma-Aldrich, St Louis, MO), deuterium-labeled FC (cholesterol-2,2,3,4,4,6-6 deuterium [d6]; Isotec, St Louis, MO), and purified human apoA-I (Academy Biomedical, Houston, TX) at a molar ratio of 250:12.5:0.8 was prepared by the cholate dialysis technique. Plasma LCAT activity assay reaction was carried out in a 50-μL system on a 96-well plate that included the following

components: 1.5 μL plasma, 10 μL proteoliposome substrate (250 nmol PC, 12.5 nmol d6-FC, 0.8 nmol apo1A-I), 0.5% bovine serum albumin, and 5 mmol/L β-mercaptoethanol. The reaction was allowed to proceed at 37°C for 2 hours and then mixed with 50-μL stop solution (5 μmol/L internal standard [CE 17:0; MP Biomedicals, Solon, OH] and 2% formic acid in methanol). Fifty microliters of pentanol was then added to the reaction mixture to extract lipids. Cholesteryl esters in the extracted lipids were analyzed using positive electrospray ionization and multiple reaction monitoring on a Waters Acquity UPLC and Xevo-TQMS mass spectrometer (Waters, Milford, MA). The peak areas of the major, newly formed CE species (d6-CE 18:1, d6-CE 18:2, d6-CE 20:4, d6-CE 22:6) were normalized to the peak area of the CE 17:0 internal standard to provide relative quantitation of newly formed CE species, and this measure was used to represent LCAT activity of the corresponding plasma sample. All plasma samples were assayed in duplicate, with the average used for subsequent analysis.

2.4. *Lipids, apolipoproteins, lipoproteins, and bile acids*

In unfractionated whole plasma samples, total cholesterol (TC), FC, HDLc, non-HDLc, and TG were measured by standard biochemical methods using commercially available enzymatic colorimetric kits (Wako Diagnostics, Richmond, VA). Plasma TGs (where indicated) were also quantitated by LC/MS (methods below).

Lipid composition characterization in lipoprotein particles was conducted by gradient gel electrophoresis followed by high-resolution LC/MS. The lipoprotein separation from 25-μL plasma samples was carried out on the Lipoprint gradient gel electrophoresis system (Quantimetrix, Redondo Beach, CA) according to the manufacturer's instructions. Following electrophoresis, the gel bands that contained different lipoprotein fractions (very low-density lipoprotein [VLDL], LDL, and HDL) were excised and homogenized in phosphate-buffered saline. Lipids in each fraction were then extracted using the Bligh and Dyer method [27]. Major lipid classes (CE, TG, PC, lysophosphatidylcholine [LysoPC]) in each lipoprotein fraction were then analyzed via an Acquity UPLC system coupled to a high-resolution LC/MS system (Synapt G2 HDMS QT of, hybrid quadrupole orthogonal time of flight mass spectrometer [Waters]) operated in positive ion electrospray mode. Detailed methods are described in previous publications [28,29]. Cholesteryl esters, TG, PC, and LysoPC in unfractionated whole plasma samples where indicated were also extracted by the Bligh and Dyer method and quantitated by LC/MS using the same procedures. Gallbladder bile was diluted 665-fold using 20% acetonitrile with 0.1% formic acid and 80% water with 1% formic acid. The diluted bile acid sample was centrifuged at 10 000 rpm at 4°C for 10 minutes, and the supernatant was collected and transferred to a 96-deep well microplate for LC/MS analysis using electrospray negative ion mode ionization.

Fast protein liquid chromatography (FPLC) analysis was carried out as described previously [28].

Plasma apoB and apoA-I were quantitated via an LC/MS assay as described previously [30].

2.5. *Liver mRNA isolation and quantitative reverse transcriptase polymerase chain reaction*

Liver mRNA was isolated, and quantitative reverse transcriptase polymerase chain reaction (PCR) for target genes was performed using standard procedures. Briefly, frozen liver tissues were homogenized in RLT buffer (Qiagen, Valencia, CA). Total RNA was extracted from the homogenized tissue using RNeasy Mini Kit (Qiagen), with complementary DNA subsequently generated using RT^2 First Strand kit (SABiosciences, Frederick, MD). Real-time PCR was performed on the 7900HT PCR System (Applied Biosystems, Foster City, CA) with 2× SYBR PCR Master Mix and mouse-specific PCR primers (SABiosciences). Expression levels of target genes (ATP-binding cassette subfamily G member 5 [Abcg5], ATP-binding cassette subfamily G member 8 [Abcg8], and cytochrome P450 subfamily 7A polypeptide 1 [Cyp7a1]) were normalized to the average expression level of 6 control genes (mouse β-actin [Actb], glyceraldehyde 3-phosphate dehydrogenase [Gapdh], beta-glucuronidase [Gusb], hypoxanthine-guanine phosphoribosyltransferase [Hprt1], peptidylprolyl isomerase A [cyclophilin A; Ppia], and ribosomal protein 113a [Rp113a]) in each sample.

2.6. *Data analysis*

Unless otherwise indicated, all results from in vivo studies are presented as mean ± SEM of the group. For the acute single-dose study in C57Bl/6 mice and in hamsters, Student *t* test was performed between the compound A (20 mg/kg)–treated group and the control group. For the chronic hamster study, Dunnett multiple comparison test was performed between the compound A (20 or 60 mg/kg)–treated groups and the control group. *P* values that were < .05 were considered statistically significant.

3. Results

3.1. *Compound A activated plasma LCAT from multiple species in vitro*

Compound A was spiked into plasma from C57Bl/6 mouse, hamster, rhesus monkey, and human at different concentrations. The final concentration of the vehicle (dimethyl sulfoxide) in all plasma samples was 2%. Lecithin cholesterol acyltransferase activity in each sample was then analyzed by an internally developed LC/MS assay that uses stable isotope (six deuterium [d6])–labeled proteoliposome as substrate. Compound A stimulated LCAT activity for all major CE products (d6-CE 22:6, d6-CE 20:4, d6-CE 18:2, and d6-CE 18:1) in a concentration-dependent manner in each species. For all 4 species and all CE products, compound A displayed an EC_{50} in the range of 1 to 10 μmol/L, demonstrating that compound A can activate plasma LCAT from all 4 species. In plasma from human, rhesus, and hamster, the activation window for d6-CE 22:6 and d6-CE 20:4 (5- to 12-fold over baseline) was larger than the activation window for d6-CE 18:2 and d6-CE 18:1 (~2-fold over baseline). In contrast, in C57Bl/6 mouse plasma, the activation window for d6-CE 18:2 and d6-CE 18:1 (~8-fold) was

greater than the activation window for d6-CE 22:6 and d6-CE 20:4 (2- to 4-fold) (Fig. 1B). The potency of compound A from this LC/MS method appeared very comparable to the potency from independent in vitro assays that used radioisotope-labeled–thin layer chromatography method (data not shown).

3.2. *Acute single-dose study with compound A in C57Bl/6 mice*

To evaluate the acute effect of compound A on plasma LCAT activity in vivo, compound A was administered intraperitoneally at 20 mg/kg to C57Bl/6 mice. At different time points (1, 3, 6, 10, 24, 48 hours) postdosing, mice were terminated for plasma analyses, with a control group treated with vehicle (30% Captisol); and blood was collected at 1 hour postdosing for comparison as a "baseline" control. Fig. 2 delineates ex vivo plasma LCAT activity from the different time points. From 1 to 6 hours postdosing, LCAT activity on all d6-CE products exhibited a robust increase compared with the vehicle-dosed control; and the peak level was present at 1 hour (Fig. 2). LCAT diminished to a level close to baseline at 10 hours and became indistinguishable from baseline at 24 hours.

Plasma HDLc reached a peak level of approximately 2-fold above baseline at 3 hours and returned to baseline at 24 hours (Fig. 3A). The plasma CE/FC ratio was increased acutely approximately 3-fold above baseline at 1 hour and returned to baseline at 10 hours (Fig. 3B). A leftward shift in the HDL peak from FPLC analysis suggested that the HDL particle size was increased at 1 to 6 hours and returned toward baseline particle size at 10 hours (Fig. 3C). Plasma non-HDLc was reduced by 66% at 1 hour and returned to baseline at 10 hours. This was followed by an elevation in the 24- to 48-hour window (Fig. 3D). Plasma TG was reduced at 1 hour, reached the lowest level of 34% of baseline TG level at 10 hours, and was at baseline levels by 48 hours (Fig. 3E).

Key liver genes involved in cholesterol elimination, Abcg5, Abcg8, and Cyp7a1, also displayed an acute increase postdosing. Induction of Cyp7a1 occurred after induction of Abcg5 and Abcg8 and was with the largest fold change compared with the vehicle control (~6-fold at 10 hours) (Fig. 3F). In summary, a single dose of compound A in C57Bl/6 mice resulted in an acute increase in HDLc, HDL particle size, CE/FC ratio, and liver Abcg5/g8 and Cyp7a1 mRNA, and an acute decrease in non-HDLc and TG. These changes corresponded with the acute increase in ex vivo LCAT activity.

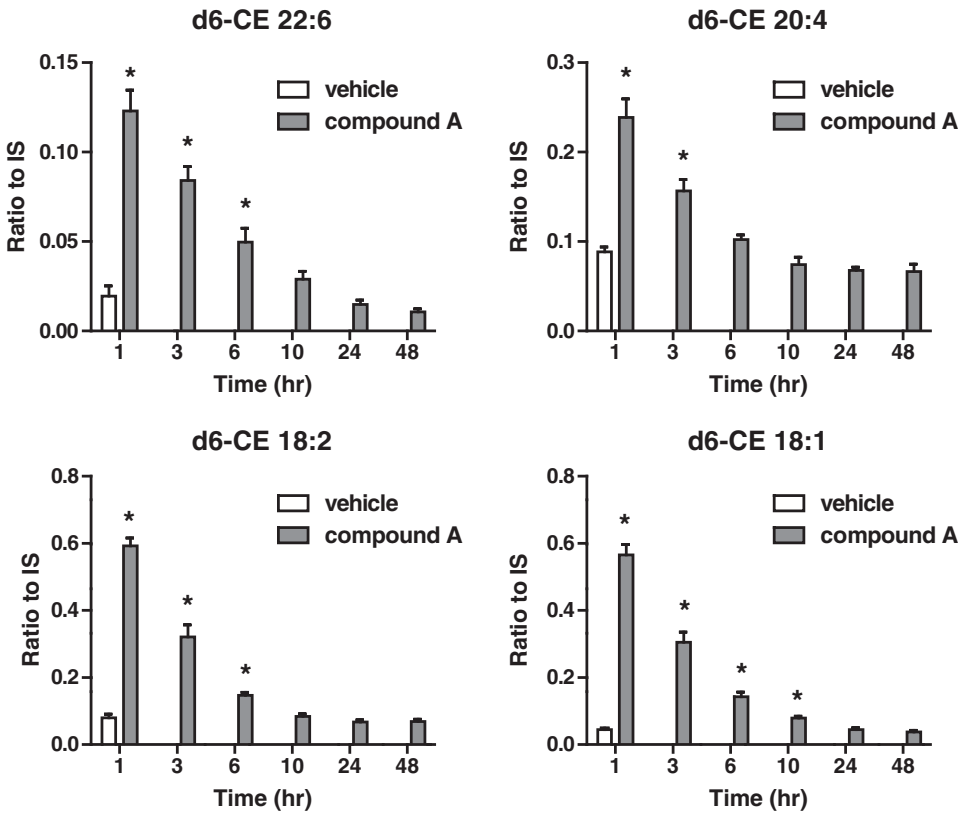

Fig. 2 – Compound A increased LCAT activity in C57Bl/6 mouse in the acute window postdosing. Seven groups of chow diet–fed C57Bl/6 mice were used in this study (n = 6 per group). One group of mice was dosed with vehicle (30% Captisol) intraperitoneally and sacrificed at 1 hour after dosing; the remaining 6 groups were dosed with compound A (20 mg/kg) intraperitoneally and sacrificed at 1, 3, 6, 10, 24, or 48 hours postdosing, respectively. Plasma was collected from all mice at the time of termination for analysis of various end points. Shown is the ex vivo LCAT activity expressed as ratio of the signal of each d6-labeled CE product to the signal of internal standard. *P < .05 compared with vehicle. IS indicates interval standard.

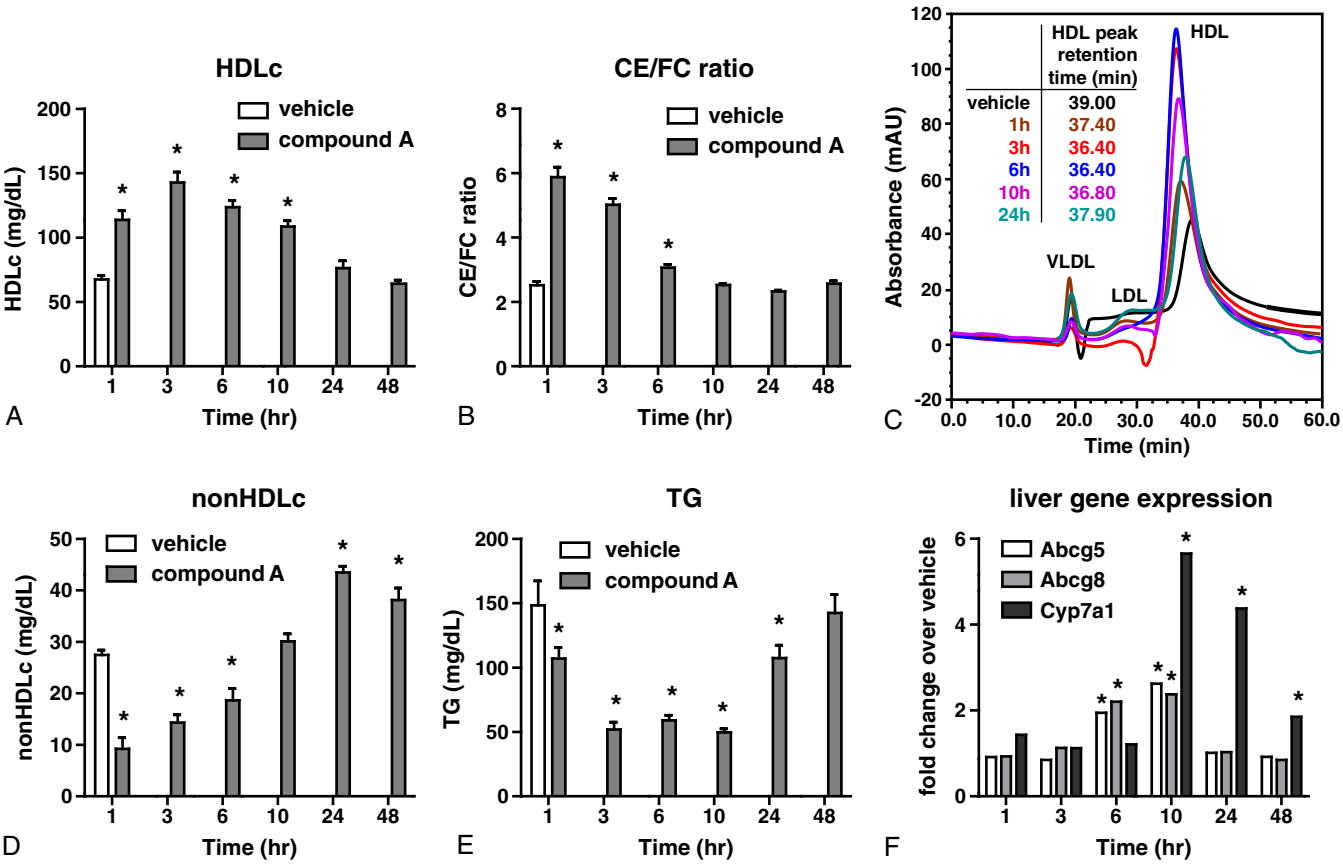

Fig. 3 – Pharmacodynamic effect of compound A in the acute window postdosing in C57Bl/6 mice. Plasma samples from the C57Bl/6 acute single-dose study were subjected to various analyses for pharmacodynamic markers. A, High-density lipoprotein cholesterol level in the animals as measured by the Wako kit. B, Plasma TC and FC were determined by the Wako kits, and plasma CE was derived by subtraction. Shown in the graph is the further derived CE/FC ratio. C, Plasma samples from the vehicle-dosed group and the compound A–dosed 1- to 24-hour groups were subjected to FPLC fractionation of lipoproteins. Each line represents the pooled plasma sample for each group. x-Axis is retention time, and y-axis is absorbance at 600 nm representing TC level in the lipoprotein fractions. D, Plasma non-HDLc as determined by the Wako kit. E, Plasma TG as determined by the Wako kit. F, Liver mRNA from the animals was extracted; and the expression level of Abcg5, Abcg8, and Cyp7a1 was analyzed by standard quantitative reverse transcriptase PCR procedure. Shown is the group average for compound A–treated mice expressed as fold difference over vehicle control, with the level in vehicle control defined as 1-fold. *$P < .05$ compared with vehicle.

3.3. *Acute single-dose study with compound A in hamsters*

High fat diet–fed hamsters were dosed intraperitoneally with either compound A (20 mg/kg) or vehicle control (30% Captisol). At 1, 5, 24, and 48 hours, intermediate plasma samples were collected; and at 72 hours, animals were sacrificed, and terminal plasma samples were collected for analysis. An ex vivo LCAT activity assay showed that compound A treatment resulted in a significant increase in LCAT activity on d6-CE 22:6 and d6-CE 20:4 product formation compared with the vehicle-treated group at 1 and 5 hours postdosing (Fig. 4A). Product formation for d6-CE 18:2 and d6-CE 18:1 was no different between the 2 groups (not shown). Compound A treatment also resulted in a significant increase in HDLc at all time points postdosing (Fig. 4B), a significant reduction in non-HDLc at 5 and 24 hours postdosing (Fig. 4C), and a significant reduction in plasma TG at 1 and 5 hours postdosing (Fig. 4D).

3.4. *Chronic study with compound A in hamsters*

To assess the effects of chronic activation of LCAT by compound A on lipoprotein metabolism, vehicle control (0.5% methyl cellulose), compound A at 20 mg/kg, or compound A at 60 mg/kg was administered by oral gavage daily into high fat diet–fed hamsters for 14 days. Hamsters were then sacrificed for analysis of plasma parameters. Table 1 provides the results from comprehensive analyses on plasma lipids, lipoproteins, and apolipoproteins after chronic dosing. Compound A treatment resulted in a significant increase in plasma TC and plasma FC, and a significant, dose-dependent increase in plasma CE and plasma CE/FC ratio. Plasma TG showed a trend toward a dose-dependent decrease without reaching

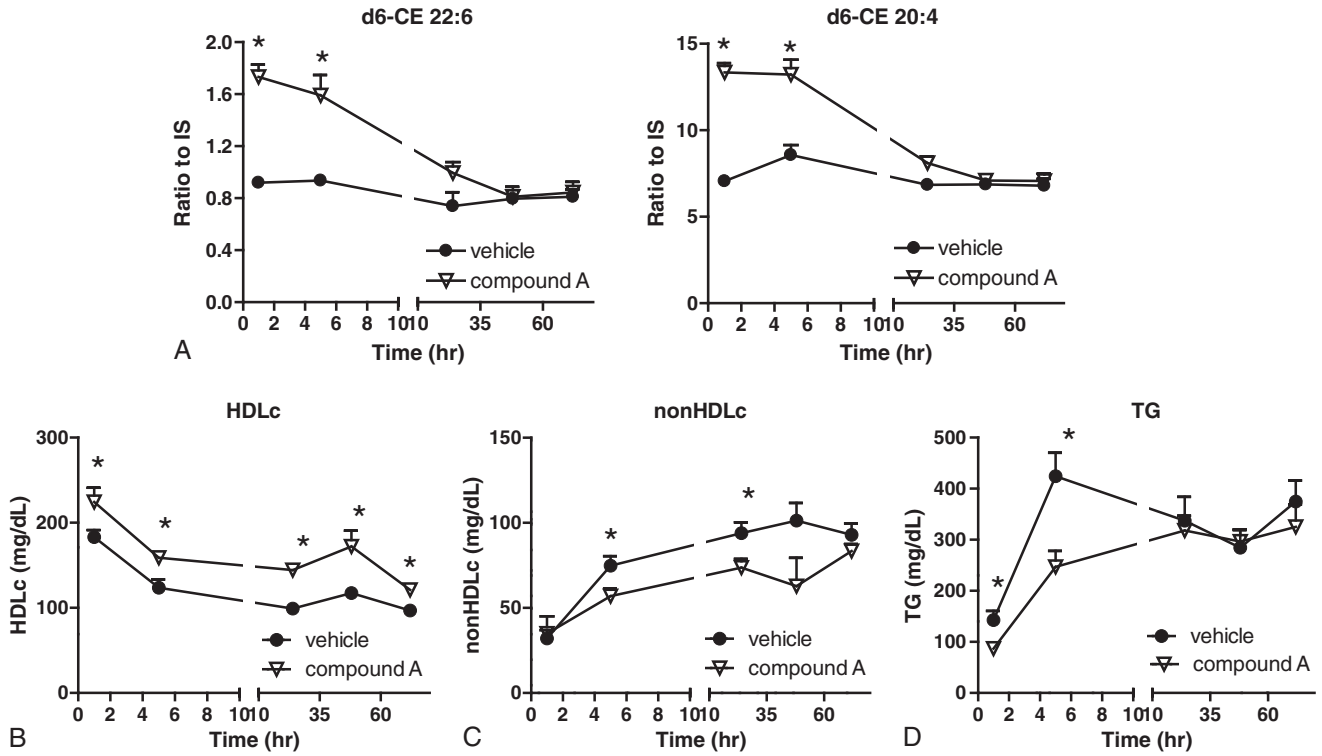

Fig. 4 – Pharmacodynamic effect of compound A in the acute window postdosing in hamsters. Compound A (20 mg/kg) or vehicle control (30% Captisol) was administered intraperitoneally into high fat diet–fed male Syrian golden hamsters (n = 8 per group). Serial bleeds were collected at 1, 5, 24, and 48 hours postdosing; and terminal bleed was collected at 72 hours postdosing. All plasma samples were then subjected to analysis for various end points. A, Ex vivo LCAT activity expressed as ratio of the signal of d6-CE 22:6 or d6-CE 20:4 to the signal of internal standard. D6-CE 18:2 and d6-CE 18:1 formation had no difference between the 2 groups (not shown). B, C, and D, Plasma HDLc, non-HDLc, and TG levels as determined by the Wako kits, respectively. *P < .05 compared with vehicle group of the same time point. IS indicates interval standard.

statistical significance. Plasma PC showed no significant difference for both doses. Plasma LysoPC displayed a significant increase at both doses. Compound A treatment also resulted in a significant increase in HDLc and a significant decrease in VLDL cholesterol (VLDLc) at both doses, whereas LDL cholesterol (LDLc) level remained unchanged. Plasma apoA-I was significantly increased at both doses, whereas plasma apoB was unchanged. The FPLC analysis revealed a dose-dependent leftward shift of the HDL peaks, indicating that compound A treatment resulted in a significant, dose-dependent increase in HDL particle size (Fig. 5).

Because the major site of action of LCAT is on the HDL particle, a detailed analysis of lipids in the lipoprotein particles was carried out with a particular focus on the HDL fraction. Lipoprint polyacrylamide gel electrophoresis was used to separate lipoproteins, as it results in improved separation/resolution of HDL from LDL particles compared with FPLC. The LC/MS analysis of lipoprotein lipid composition showed that compound A treatment resulted in a significant increase in the majority of the CE species in HDL. In HDL, CE 20:4 and HDL CE 22:6 displayed the largest fold difference over vehicle control; and the increase in CE was dose dependent (Fig. 6A). The CE content in VLDL was decreased with compound A treatment, whereas the CE content in LDL in compound A–treated groups was unchanged from vehicle control (not shown). When unfractionated whole plasma samples were subjected to the same CE analysis, however, a pattern of CE increase was observed that was highly similar to that of HDL (Fig. 6B), suggesting that the change in CE in total plasma was driven primarily by the changes in HDL CE.

Acute single-dose treatment with compound A resulted in a significant reduction in plasma TG in both mice (Fig. 3E) and hamsters (Fig. 4D); chronic treatment with compound A in hamsters resulted in a nonsignificant trend of plasma TG reduction (Table 1). The TG class in the lipoprotein particles in this study was therefore further examined. Quantitation from LC/MS revealed that, whereas TG content in LDL remained the same, TG content in both VLDL and HDL trended toward a decrease at both 20 and 60 mg/kg (Fig. 7A). Compositional analysis of TG further revealed that both VLDL TG and HDL TG exhibited a broad pattern of reduction across all TG species, with multiple TG species in each lipoprotein fraction reaching statistical significance (Fig. 7B and C). A comparison between Fig. 7B and C also suggested that TG composition in HDL is highly similar to TG composition in VLDL.

Bile acids content in bile isolated from the gallbladder at study termination exhibited a dose-dependent increase; at 60 mg/kg, compound A resulted in a significant and more than

Table 1 – Plasma lipids, lipoproteins, and apolipoproteins after chronic dosing in hamsters

	Vehicle	20 mg/kg	60 mg/kg
TC, mg/dL	229 ± 6	322 ± 9*	375 ± 16*
CE, mg/dL	163 ± 4	239 ± 8*	284 ± 13*
FC, mg/dL	66 ± 2	83 ± 2*	91 ± 3*
CE/FC ratio	2.49 ± 0.05	2.90 ± 0.06*	3.11 ± 0.05*
TG, mg/dL	110 ± 12	80 ± 17	70 ± 14
PC, mg/dL	86 ± 3	97 ± 3	100 ± 7
LysoPC, mg/dL	16 ± 1	21 ± 1*	21 ± 2*
HDLc, mg/dL	134 ± 5	259 ± 10*	301 ± 22*
LDLc, mg/dL	34 ± 3	34 ± 4	36 ± 4
VLDLc, mg/dL	53 ± 5	33 ± 6*	29 ± 4*
ApoA-I, μmol/L	105 ± 4	149 ± 6*	145 ± 6*
ApoB, nmol/L	431 ± 21	465 ± 18	509 ± 40

Compound A (20 or 60 mg/kg), or vehicle control (0.5% methyl cellulose) was administered via oral gavage daily into high fat diet–fed male Syrian golden hamsters (n = 8 per group) for 14 days. Hamsters were then sacrificed for various analyses. Plasma TC and FC were measured by Wako kits, with CE then derived by subtraction. Plasma TG, PC, and LysoPC were analyzed via LC/MS profiling of each species in each lipid class with the sum then derived to represent the class. HDLc, LDLc, and VLDLc were analyzed by FPLC. Plasma apoA-I and apoB levels were measured via internally developed LC/MS method.

* $P < .05$ compared with vehicle.

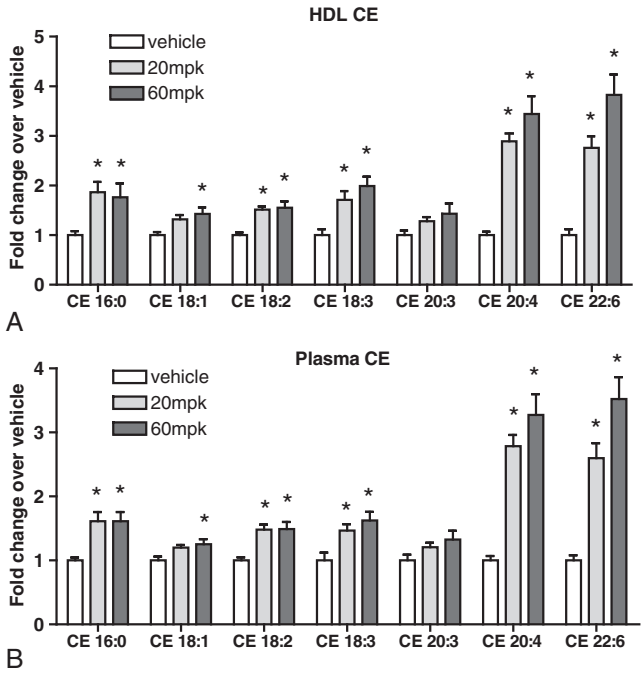

Fig. 6 – Chronic dosing resulted in robust increases in HDL CE and plasma CE. All CE species in the HDL particles isolated from polyacrylamide gel electrophoresis or in unfractionated plasma samples were quantitated via LC/MS and then expressed as fold change over vehicle control, with vehicle control defined as 1-fold. A, CE increase in HDL. B, CE increase in plasma. *P < .05 compared with vehicle.

2-fold increase in bile acids content compared with the vehicle control (Fig. 8).

4. Discussion

In models of genetic or somatic LCAT overexpression, including those in higher animal species (rabbit, hamster, and nonhuman primate), LCAT gain of function is associated with changes in lipoprotein metabolism that are consistent with an antiatherosclerotic benefit (eg, increased HDLc) [19-21]. Although studies describing beneficial effects of

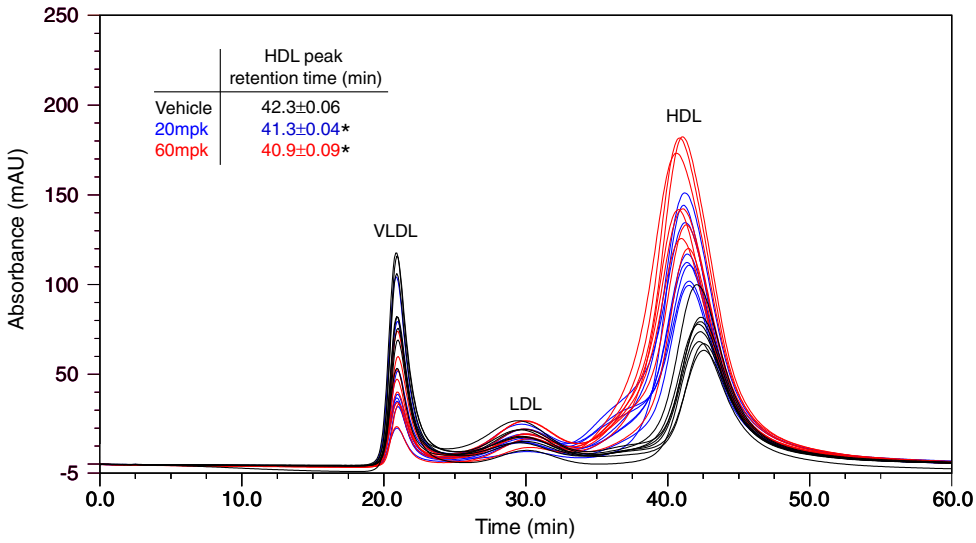

Fig. 5 – FPLC analysis of the lipoproteins in the hamsters after chronic dosing. All plasma samples post chronic dosing were subjected to lipoprotein fractionation by FPLC. Each line represents one animal in the corresponding dosing group. *P < .05 compared with vehicle.

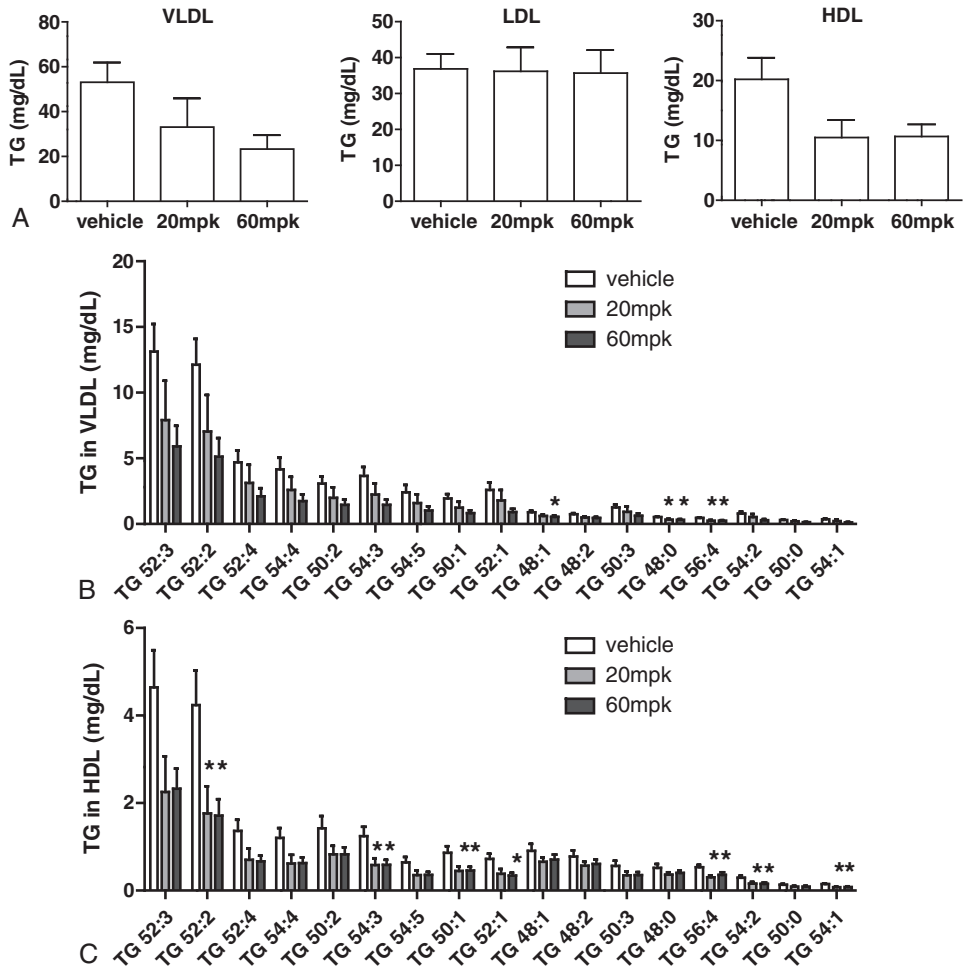

Fig. 7 – Triglycerides in the lipoprotein particles. A, Triglyceride content in the lipoprotein particles. Very low-density lipoprotein, LDL, and HDL were isolated from polyacrylamide gel electrophoresis; and their TG species were quantitated via LC/MS. Shown is total TG in each lipoprotein fraction. B, The content of each TG species in VLDL. C, The content of each TG species in HDL. *P < .05 compared with vehicle.

LCAT overexpression highlight LCAT as a possible target for therapeutic intervention, the notion that small molecule activation of LCAT might provide a similar benefit has been largely untested in the literature. A class of putative small molecular LCAT activators (eg, compound A) was recently described [24,25]; however, detailed pharmacological evaluation has not been reported. The purpose of the present study was to characterize compound A both in vitro and in vivo to assess the therapeutic potential of small molecule activation of LCAT.

In in vitro experiments in plasma isolated from multiple species, compound A enhanced plasma LCAT activity with micromolar potency (EC_{50} range of 1-10 μmol/L for all d6-CE products, Fig. 1B). It had been postulated that compound A increases LCAT activity by interacting with the free sulfhydryl group in cysteine (C)31 near its catalytic site [25]. Based on evaluation of LCAT sequence information in the publicly available domain (National Center for Biotechnology Information), C31 appears to be a conserved residue in LCAT from multiple species. Our observation of the similar activity of compound A across multiple species supports the notion that compound A is acting via a similar mechanism in these species. In the present study, the plasma LCAT activity assay used the same proteoliposome substrate format as described

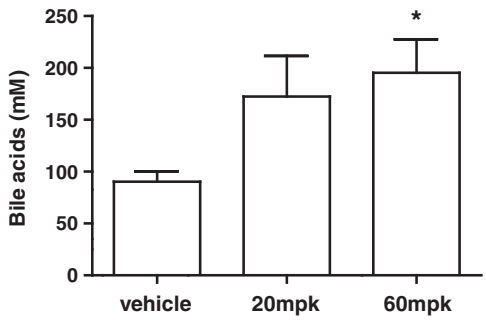

Fig. 8 – Gallbladder bile acids content. Gallbladder bile was taken by aspiration from the animals at study termination. Each bile acid species in the bile was quantitated by LC/MS. Shown is the total bile acids content in each treatment group. *P < .05 compared with vehicle.

by others [26] except that stable isotopes were used to allow LC/MS detection and determination of specific CE product species from the PC donor. It was observed that in the hamster, like the rhesus monkey and human, a larger window of activation was seen with CE 22:6 and CE 20:4 compared with CE 18:2 and CE 18:1, whereas in the mouse, the opposite relation was seen (Fig. 1B). It has been shown that LCAT from hamster and baboon has similar substrate and positional specificity as human LCAT, in contrast to the mouse [31]. The observation from the present study that the LCAT specificity is similar between rhesus, human, and hamster plasma but distinct from the mouse further supports this notion.

For in vivo characterization, an acute single-dose experiment was conducted in C57Bl/6 mice to establish a relationship between target engagement (activation of LCAT) and downstream plasma lipid profile (pharmacodynamics). After a single dose of compound A (20 mg/kg), the increase in ex vivo plasma LCAT activity within 1 to 5 hours following dosing correlated with changes in plasma HDLc, CE/FC ratio, and HDL particle size, suggesting target engagement and pharmacodynamic effects at this dose. The "rebound" of non-HDL at 24 to 48 hours suggests that chronic treatment with compound A in C57Bl/6 mice may result in increased non-HDL. It had been reported that LCAT transgenic mouse had increased non-HDL and exacerbated atherosclerosis, whereas further introduction of CETP transgene decreased non-HDL and reduced atherosclerosis in the LCAT transgenic background [17,18]. Our observation on non-HDL is thus in line with literature and suggests that CETP needs to be present to extrapolate effects of LCAT activation to human.

In C57Bl/6 mice, liver mRNA for Abcg5/g8 (encoding the major cholesterol exporters in liver) and Cyp7a1 (encoding the key enzyme in bile acids synthesis from cholesterol) increased in response to compound A treatment, with slightly delayed time course compared with LCAT activity and the increase in HDLc (Fig. 3F). This delay suggests that the increase in HDLc precedes cholesterol offloading to the liver, resulting in a compensatory increase in liver genes responsible for cholesterol elimination.

In the acute single-dose study in hamsters, ex vivo LCAT activity was increased with respect to formation of d6-CE 22:6 and d6-CE 20:4 but not d6-CE 18:2 and d6-CE 18:1. This is possibly due to the small activation window (1.5- to 1.8-fold) for d6-CE 18:2 and d6-CE 18:1 with compound A on hamster LCAT (Fig. 1). Analysis of downstream pharmacodynamic markers revealed a significant increase in HDLc throughout the time course and a significant reduction in non-HDLc and TG in the early time points, which tracked with the changes in LCAT activity increase and suggest that the effects on lipids were likely due to LCAT activation by compound A.

The above results establish a relationship between target engagement and pharmacodynamic markers under acute conditions and are consistent with the current understanding of the role of LCAT in HDL metabolism and RCT. Therefore, a chronic study in hamsters was pursued to characterize the effect of LCAT activation by compound A on lipid profiles in detail. A significant increase in HDLc, HDL particle size, and plasma apoA-I level was observed as well as dose-dependent decreases in VLDLc and multiple TG species. Low-density lipoprotein cholesterol remained the same, possibly because the baseline LDL level in this model is modest and thus gives a small window for reduction. Epidemiological studies have suggested that increased HDLc, decreased VLDLc, and decreased plasma TG are all associated with risk reduction for atherosclerosis [32,33]. In addition, large, lipid-rich HDL particle and increased plasma apoA-I level are also associated with reduced risk for coronary heart disease [32,34,35]. The global changes in the lipoprotein profile that we observed are thus potentially atheroprotective. Additional changes in plasma lipids that were observed in the hamster experiment included increased LysoPC. Because LysoPC is a direct product from the LCAT reaction, this observation is consistent with the notion that compound A treatment resulted in enhanced cholesterol esterification. PC, the donor for the fatty acyl chain for LCAT reaction, did not exhibit any decrease. This may be due to an adaptive increase in plasma PC production to accommodate increased HDL particle size, possibly through enhanced RCT, as its first step is the transfer of cellular FC and PC onto nascent HDL (lipid-poor apoA-I) [36,37]. The robust increase in gallbladder bile acids at the termination of this chronic study in hamster was in line with the large increase in Cyp7a1 gene expression in the C57Bl/6 mouse study and in aggregate supports the hypothesis that activation of LCAT via compound A treatment had resulted in enhanced RCT.

Detailed analysis of the lipoprotein particles from the chronic study revealed that the HDL particle had indeed become CE enriched (Fig. 6A), whereas VLDL and LDL particles had not (not shown). The increase in plasma CE mirrors that of HDL (Fig. 6B). The CE species that yielded the largest fold increase in HDL and in plasma were CE 20:4 and CE 22:6. Changes in endogenous CE after chronic treatment with compound A are thus consistent with the acute increase in the ex vivo LCAT activity on CE 20:4 and CE 22:6 (Fig. 4A). These observations suggest that, in this high fat diet–fed dyslipidemic hamster model, LCAT's major site of action remains the HDL particle and that LCAT accounts for the majority of circulating CE on plasma lipoproteins. Triglyceride analysis in the lipoprotein particles demonstrated that TG reduction resided in VLDL and HDL, but not LDL. In addition, HDL TG composition was strikingly similar to VLDL (Fig. 7). Given that key genes in liver TG synthesis and secretion in the C57Bl/6 study did not change (not shown) and that FLD patients tend to have hypertriglyceridemia, possibly due to defective chylomicron and VLDL clearance [14,38], it is possible that enhanced lipolysis has occurred upon LCAT activation and is one contributing factor for TG lowering. It also appears that TG reduction in HDL was primarily driven by TG reduction in VLDL and mediated by CETP in hamsters.

It is to be noted that compound A levels were undetectable in plasma during the acute window postdosing in the above studies or in independent single-dose pharmacokinetics studies (not shown). Additional analyses indicated that the plasma stability of compound A and liver microsomal stability were poor (not shown), which may account for the short duration of LCAT activation observed ex vivo. Interpretations of our findings thus have a limitation. Nonetheless, the correlation between pharmacodynamic markers and LCAT activity strongly supports the conclusion that compound A elicited global changes in lipoprotein metabolism via activation of LCAT.

In summary, the results from the current study demonstrate that activation of LCAT via compound A produces concomitant changes in the lipoprotein profile in mice and hamsters and raise the possibility for small molecule therapeutic intervention of dyslipidemia and atherosclerosis via modulating LCAT activity. The findings of enhanced HDLc and decreased TG are consistent with previous LCAT overexpression studies in higher animal species and are in line with observations from humans with LCAT deficiency. This similarity between preclinical findings and the LCAT-deficient human phenotype offers the possibility that the changes induced by a small molecule activator of LCAT might translate into humans. The finding of decreased VLDL may translate into decreased LDL in human, as LDL is the predominant non-HDL lipoprotein particles in human. Beneficial effect of LCAT activation may also exhibit additivity to statin, which has an independent mechanism of action (suppression of liver cholesterol synthesis). The detailed characterization from the current study of lipoprotein particle composition also sheds new light on LCAT's mechanism of action in lipoprotein metabolism.

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Conflict of Interest

All authors are (or were) employees of Merck Sharp & Dohme and potentially own stock and/or hold stock options in the company.

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