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Structure-activity and *in vivo* evaluation of a novel lipoprotein lipase (LPL) activator

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ABSTRACT

Elevated triglycerides (TG) contribute towards increased risk for cardiovascular disease. Lipoprotein lipase (LPL) is an enzyme that is responsible for the metabolism of core triglycerides of very-low density lipoproteins (VLDL) and chylomicrons in the vasculature. In this study, we explored the structure-activity relationships of our lead compound (C10d) that we have previously identified as an LPL agonist. We found that the cyclopropyl moiety of C10d is not absolutely necessary for LPL activity. Several substitutions were found to result in loss of LPL activity. The compound C10d was also tested *in vivo* for its lipid lowering activity. Mice were fed a high-fat diet (HFD) for four months, and treated for one week at 10 mg/kg. At this dose, C10d exhibited *in vivo* biological activity as indicated by lower TG and cholesterol levels as well as reduced body fat content as determined by ECHO-MRI. Furthermore, C10d also reduced the HFD induced fat accumulation in the liver. Our study has provided insights into the structural and functional characteristics of this novel LPL activator.

Elevated triglycerides (TGs) are considered an independent risk factor for coronary heart disease (CHD). In many cases, the continuing residual CHD risk in spite of optimal low-density cholesterol (LDL-C) after statin monotherapy can be attributed to elevated apolipoprotein B (apo-B) and hypertriglyceridemia.[1–3] TGs associated with remnant lipoproteins formed as a result of partial hydrolysis by LPL, are atherogenic as well as subject to endothelial accumulation and uptake by macrophages to form foam cells.[4,5] Similarly type 2 diabetes mellitus (DM) and metabolic syndrome patients commonly present with combined dyslipidemia, which is characterized with fasting and postprandial hypertriglyceridemia and low HDL-C. These patients are at risk for CHD even if LDL-C levels are optimal. Hence, there is a significant need for novel approaches to control plasma TG levels.

Extracellular lipases have been shown to tightly regulate the plasma TG and /or HDL-cholesterol levels. The lipoprotein lipase enzyme (LPL) that is found lining the capillary endothelium is a critical regulatory protein involved in TG metabolism.[6,7] TG-rich lipoproteins such as VLDL and chylomicrons are catabolized by the action of LPL into free fatty acids and remnant lipoproteins. Such activity is crucial for effective utilization of TGs and for the resultant uptake of fatty acids by tissues. Furthermore, intact LPL activity is crucial to guard against fluctuations resulting from intake of high fat meals or production of TG rich lipoproteins from the liver.

As such, a reduction in the expression and activity of LPL is associated with hypertriglyceridemia. Both gain-of-function and loss-of-function genetic mutations of LPL, resulting in dysregulated plasma TG levels in patients, have been reported.[7,8] Glybera (alipogene tiparvovec), a gene therapy product that replaces the LPL gene is available in Europe from uniQure (www.uniqure.com) for the treatment of patients diagnosed with a genetic deficiency in familial lipoprotein lipase (LPLD). A large gap in the LPL field is the lack of clinically used small molecule drugs to modulate LPL activity. Previous attempts to modulate LPL activity using small molecules produced highly significant effects in several animal models of hyperlipidemia. Developed by Otsuka Pharmaceutical Factory (Japan), NO-1886 (generic name: Ibrolipim) remains the best characterized specific LPL activator. NO-1886 was discovered in early

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1990s and significantly stimulated LPL activity, lowered plasma triglycerides, as well as elevated the levels of HDL-C.[9,10] In addition to affecting LPL activity, NO-1886 also increases LPL mRNA thereby increasing post-heparin LPL mass.[9] In streptozotocin (STZ) treated diabetic rats, NO-1886 increased LPL activity 59% over the control.[11] Finally, long term NO-1886 administration to rats with experimental atherosclerosis caused by high-cholesterol feeding significantly inhibited the development of atherosclerotic lesions in coronary arteries.[9] Similar results were observed in rabbit model of atherosclerosis.[12]

Our group previously reported the identification of a novel small organic compound with LPL activation property.[13] This lead compound named C10d which was identified from an *in vitro* high-throughput (HTS) screening assay exhibited potent LPL activation twofold as compared to NO-1886. In the present study, we have carried out additional structure-activity relationship studies to identify the key structural features of the C10d molecule that is responsible for its LPL activation. Moreover, we present the first *in vivo* studies to show lipid lowering properties of C10d in a high fat diet model of hyperlipidemia.

To explore the structure-activity around C10d, we synthesized benzoic acid derivatives of C10d through a condensation of 1-(3-aminopropyl) imidazole with the appropriate aromatic moiety. The carboxylic group of the aromatic side chain was first activated using 1′-carbonyldiimidazole (CDI) by stirring at room temperature for 24 h in tetrahydrofuran (THF), after which the imidazole was added (Scheme 1). Analytical data of the compounds given in the Supplementary data.

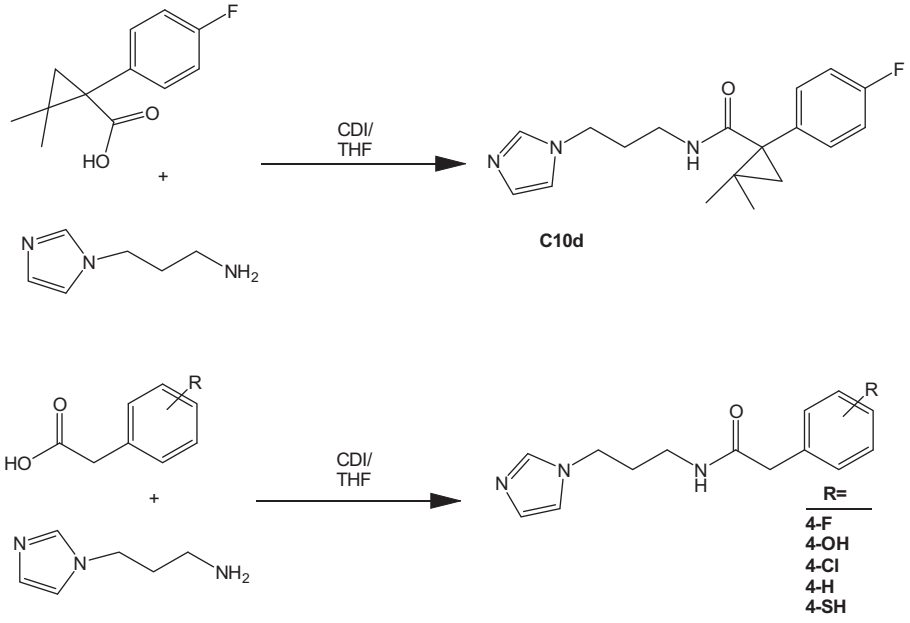
In this study, we evaluated the structure-activity relationship surrounding the imidazole moiety of C10d. Compounds 2A–5E were tested in comparison with C10, our originally identified hit compound and C10d, the more potent activator identified in subsequent studies (Figs. 1 and 2). Compound 2A improved LPL activity, although still much less than C10d (Fig. 2). Similar trend was observed for the benzoic acid derivatives of C10d, including C10d-Cl, C10d-H, and C10d-F as well. Compounds that acted as substantial inhibitors of LPL enzyme activity were 2E, 2F, 4E, C10d-SH, and C10d-OH.

To further explore the activity of C10d, we synthesized several benzoic acid derivatives lacking the cyclopropyl group present in C10d. We found that C10d-H and C10d-Cl have similar activity to the control compound C10, but less than C10d (Fig. 2). Based on previous docking studies, we expect that the aromatic ring of C10d is oriented towards a pocket ideal for aromatic or hydrophobic interactions. The lack of the cyclopropyl group allows for more flexibility and can potentially lead to decreased occupancy in this pocket, explaining in part the loss of activity compared to C10d. However, since the compounds lacking the cyclopropyl group also show agonist activity, it suggests that the cyclopropyl moiety of C10d is not essential for activity on LPL and can be removed for alternative scaffold hopping efforts.

To understand how C10d acts as activator of LPL, we docked C10d onto the homology model of LPL we had previously published (Fig. 3).[6,13] We found that upon docking of C10d to LPL, the catalytic amino acids are pushed closer in space suggesting that the induced docking of C10d may lead to more efficient enzyme kinetics via a stabilization of the catalytic unit resulting in lowering of the activation energy of TG catalysis. In comparison with C10d, the compounds that act as inhibitors, for example 2E, seem to bind to LPL but prevent the induced fit suggested from the C10d experiments. As can be seen from our docking studies, when C10d binds to LPL, hydrogen bonds are formed with Ser216 and an aromatic interaction with Pro217 occurs. The induced fit docking also indicated that the catalytic residues are moved slightly towards the catalytic site via the amino acids Ile272-Phe275. In the case of the antagonists such as 2E, an extra hydrogen bond is formed with Lys294, effectively preventing the inductive effect seen from the C10d binding.

We tested the enzyme kinetics of C10d and found that the K_m for the enzymatic reaction is significantly lowered (K_m = 648 μM in presence of C10d versus K_m = 8.15 mM in presence of DMSO) by using C10d, suggesting enhanced affinity of LPL for its substrate in the presence of C10d (Fig. 4).

We furthermore explored the *in vivo* efficacy of C10d in its ability to lower serum triglycerides. Mice were fed a high fat diet (60% kcal from fat) for four months. In the last week of the study, we



Scheme 1. Synthesis of the C10d and its benzoic acid derivatives which are devoid of the cyclopropyl moiety. The carboxylic group of the aromatic side chain is activated with CDI in THF for 24 h, after which the 1-(3-aminopropyl) imidazole is added and stirred for 48 h until workup.

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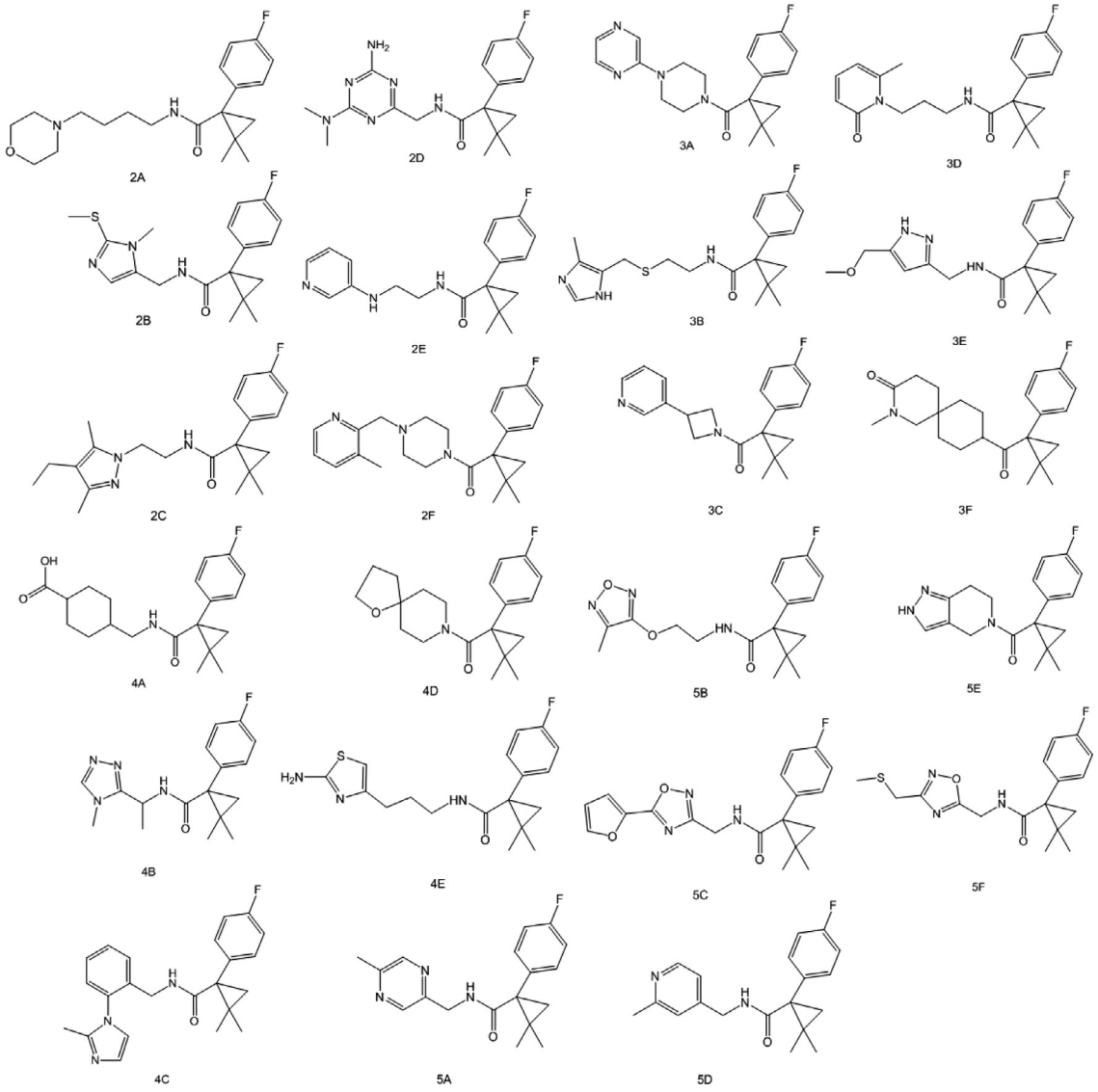

Fig. 1. Structures of C10d-derivatives tested in the LPL assay.

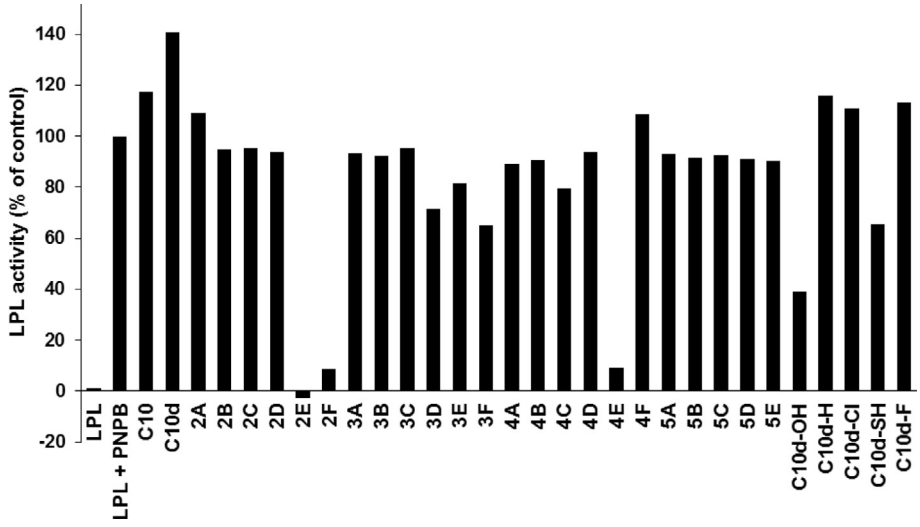

Fig. 2. Activity profile of the hit compound C10, the lead compound C10d and structural derivatives using an *in vitro* recombinant lipoprotein lipase (LPL) activity high-throughput (HTS) screening assay. All compounds were screened at 100 μM, in an assay set up in a 384 well plate. p-Nitrophenyl butyrate was used as a substrate in the assay and absorbance detection at 405 nm was performed to detect product of cleavage, butyric acid. The fold change in absorbance as a measure of LPL activation is plotted. Bars represent average ± S.D. with the error bars contained within the size of the bar where N = 2.

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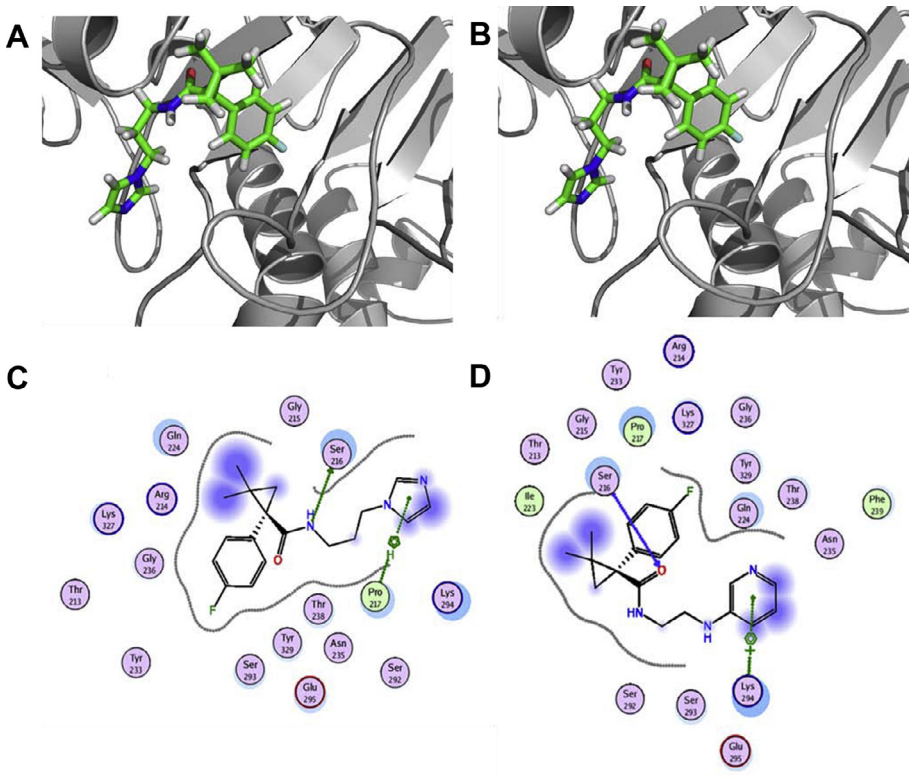

Fig. 3. Docking studies of the lead compound C10d (**A** and **C**) and compound 2E (**B** and **D**) in the homology model of LPL.[13] C10d shows agonist behavior and 2E shows antagonist behavior. Based on the docking studies, it appears that C10d allows for the amino acids in the catalytic site to be optimally place for a lower activation energy, while the tight binding of 2D inhibits the flexibility of the protein to allow for efficient catalysis of the TG.

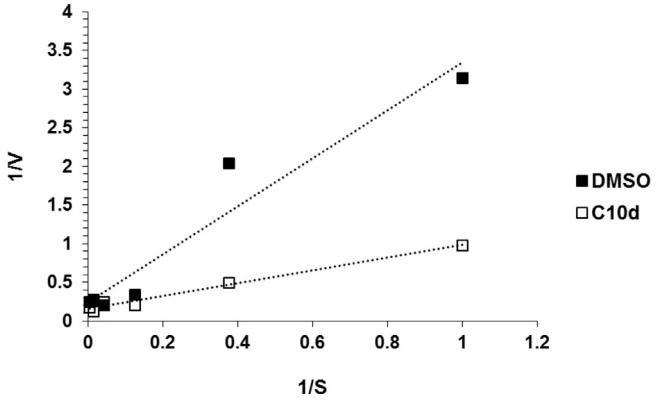

Fig. 4. Enzyme kinetics for LPL stimulated hydrolysis was determined in the presence or absence (DMSO vehicle control) of C10d. Using a dilution series for the substrate, velocity of the reaction was determined. A Lineweaver-Burk plot of 1/V vs. 1/S is shown. C10d significantly lowered the Km from 8.15 mM for the vehicle treated to 0.648 mM in the presence of C10d. Symbols represent average ± S.D. with the error bars contained within the size of the symbols where N = 3.

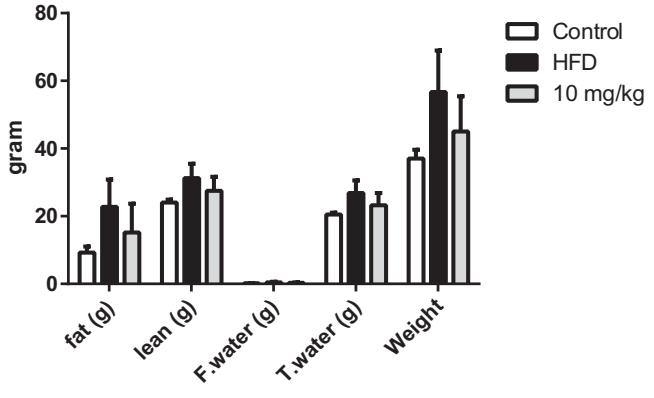

Fig. 5. ECHO-MRI analysis for different groups of animals in the high-fat diet (HFD) study. At the conclusion of the four months of high fat diet feeding, body weight, as well as total body fat and lean mass, were monitored by EchoMRI. Each bar represents mean ± S.D., where n = 4.

treated the mice with C10d at 10 mg/kg dose i.p. daily until the end of the study. The dose of C10d used for the *in vivo* study was selected based on a dose response study wherein a 10 mg/kg dose of C10d exhibited an increase in LPL activity whereas the doses of 1 mg/kg and 5 mg/kg did not alter the LPL activity (data not shown). Before sacrifice, ECHO-MRI was performed on the animals. C10d treated animals showed a small but not significant decrease in body weight as compared to untreated HFD controls as well as lower total body fat content (Fig. 5).

The lowering of total body fat content observed after C10d treatment of mice only in the final week of the four-month feeding suggests a potent *in vivo* efficacy of this LPL activator. A decrease in serum triglycerides and cholesterol was also observed (Fig. 6 A and B).

Additionally, mice treated with C01d were found to have livers which morphologically resembled that of the mice fed a normal

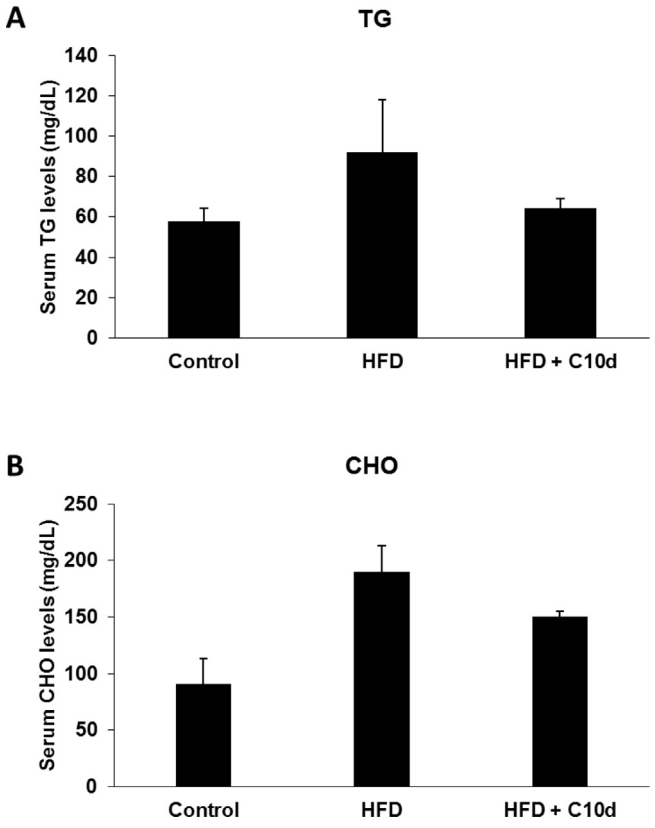

Fig. 6. Serum triglycerides (**A**; TG) and cholesterol (**B**; CHO) measurements in mice. High-fat diet (HFD) feeding was performed in mice for 4 months. HFD + C10d group received 10 mg/kg i.p. of C10d in the last week of the study. The mice were sacrificed and lipids were measured in the serum using commercially available assay kits (n = 3). Data show mean ± SE.

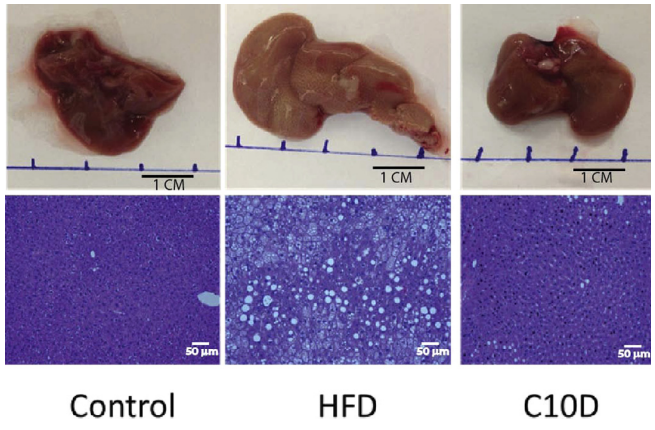

Fig. 7. Mice treated with C10d (10 mg/kg) show reduction in liver fat deposition from a high-fat diet (HFD) as compared to mice on a HFD alone. The livers of mice on HFD were enlarged as expected, and treatment with C10d showed a reduction in liver size (Top panel). Histochemical staining with hematoxylin and eosin (H and E) showed reduction in lipid accumulation in the livers treated with C10d (bottom panel; 20× magnification).

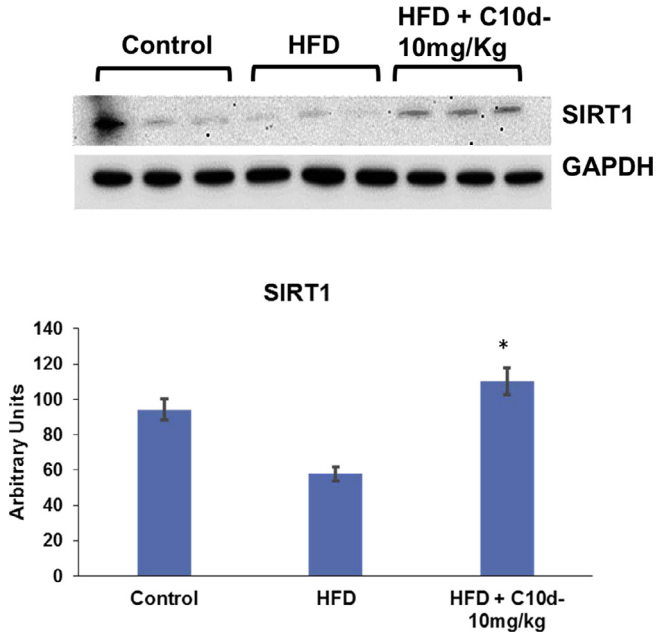

Fig. 8. Mice treated with C10d show reduction in liver injury from a high-fat diet (HFD). Mice were fed a HFD for four months, and treated with C10d for seven days at 10 mg/kg. The protein levels of SIRT1 was increased in mice treated with C10d. *Statistical significance P < 0.05. n = 5 mice.

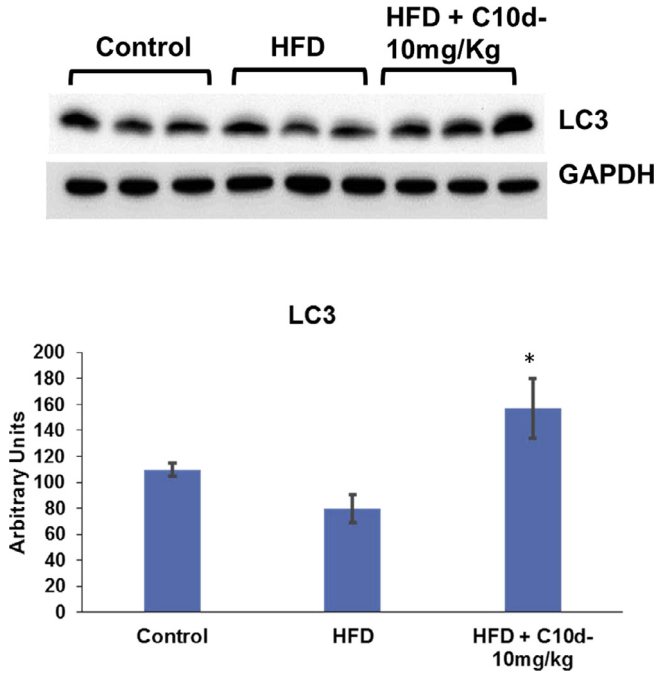

Fig. 9. Mice treated with C10D show reduction in liver injury from a high-fat diet (HFD). Mice were fed a HFD for four months, and treated with C10D for seven days at 10 mg/kg. The protein level of LC3 was increased in mice treated with C10D. *Statistical significance P < 0.05. n = 5 mice.

diet (Fig. 7). In contrast, the livers of mice fed a high fat diet (HFD) only, appeared enlarged and steatotic. Histochemical H & E staining indicated that the livers of mice treated with C10d showed less lipid accumulation as compared to the HFD-only group.

Based on our morphological findings of the livers, we evaluated the protein levels of two markers that are associated with liver protection, the NAD dependent deacetylase – sirtuin 1 (SIRT1) and the autophagy marker – microtubule associated protein 1 light chain 3 alpha (LC3). These markers were increased in mice treated with C10d (Figs. 8 and 9), suggesting a likely mechanism of action of C10d in the liver might be via stimulation of liver protection pathways.

In conclusion, we explored the SAR for our lead compound C10d as LPL agonist and found that the imidazole moiety plays an

important role in determining agonist/antagonist activity. Additionally we tested the compound C10d in a high fat diet study to evaluate its effect on lipid lowering activity. We found the compound can reduce whole body fat, serum triglyceride levels, and hepatic fat accumulation in mice fed high fat diet. Taken together our data shows the feasibility of using LPL agonists in lowering triglycerides. Lastly, the novel scaffolds here may lead to identification of new classes of lipid lowering drugs which has therapeutic potential in a variety of metabolic diseases such as dyslipidemia, obesity, diabetes and fatty liver disease.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.11.053.

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