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Identification of a small molecule that stabilizes lipoprotein lipase *in vitro* and lowers triglycerides *in vivo*

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ABSTRACT

Patients at increased cardiovascular risk commonly display high levels of plasma triglycerides (TGs), elevated LDL cholesterol, small dense LDL particles and low levels of HDL-cholesterol. Many remain at high risk even after successful statin therapy, presumably because TG levels remain high. Lipoprotein lipase (LPL) maintains TG homeostasis in blood by hydrolysis of TG-rich lipoproteins. Efficient clearance of TGs is accompanied by increased levels of HDL-cholesterol and decreased levels of small dense LDL. Given the central role of LPL in lipid metabolism we sought to find small molecules that could increase LPL activity and serve as starting points for drug development efforts against cardiovascular disease. Using a small molecule screening approach we have identified small molecules that can protect LPL from inactivation by the controller protein angiopoietin-like protein 4 during incubations *in vitro*. One of the selected compounds, 50F10, was directly shown to preserve the active homodimer structure of LPL, as demonstrated by heparin-Sepharose chromatography. On injection to hypertriglyceridemic apolipoprotein A-V deficient mice the compound ameliorated the postprandial response after an olive oil gavage. This is a potential lead compound for the development of drugs that could reduce the residual risk associated with elevated plasma TGs in dyslipidemia.

1. Introduction

Dyslipidemia characterized by high levels of triglycerides (TGs), increased levels of LDL cholesterol, small dense LDL and low levels of HDL-cholesterol increases the risk for cardiovascular disease (CVD) and is often accompanied with lifestyle-related diseases such as obesity and type 2 diabetes mellitus [1]. Recent studies suggest that plasma TGs independently increase risk for coronary artery disease [2]. Statin therapy effectively reduces CVD events in patients with elevated LDL-cholesterol. However, certain patients remain at risk of experiencing a clinical event partly due to elevated plasma TGs [3]. The alternatives for add-on therapy to statins are usually restricted to fibrates, niacin or n-3 polyunsaturated fatty acids [4].

Lipoprotein lipase (LPL), an enzyme bound to the capillary endothelium, hydrolyzes TGs in TG-rich plasma lipoproteins (TRLs) [5]. LPL also plays a role in HDL maturation by providing surface material from chylomicrons and VLDL during TG hydrolysis [6]. LPL S447X, a common gain-of-function mutation associated with low levels of TGs and high levels of HDL, is more frequent among healthy subjects than patients with incident coronary heart disease [7]. Multiple genetic variants are likely to act in concert to cause hypertriglyceridemia that predispose individuals to develop CVD [reviewed [8]].

LPL is synthesized from essentially stable mRNA levels, while other gene products, with shorter half-lives for mRNA and protein, act as controllers of LPL activity [9]. During the last decade candidate proteins for this function have emerged in the form of angiopoietin-like proteins (ANGPTLs) that have the ability to inactivate

Abbreviations: ANGPTL4, angiopoietin-like protein 4; apo, apolipoprotein; BSA, bovine serum albumin; BW, body weight; CVD, cardiovascular disease; FA, fatty acid; HDL, high-density lipoprotein; i.p., intraperitoneal; i.v., intravenous; LDL, low-density lipoprotein; LPL, lipoprotein lipase; SPR, surface plasmon resonance; S/B, signal-to-background ratio; S/N, signal-to-noise ratio; TG, triglyceride; TRLs, triglyceride-rich lipoproteins; VLDL, very low-density lipoprotein; *Z′*, *Z*-factor; %CV, coefficient of variance.

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LPL [10]. ANGPTL4 has been extensively studied and is considered to cause down-regulation of LPL activity in adipose tissue during fasting [11,12]. LPL activity is higher in fed ANGPTL4-deficient mice compared to fed wild-type mice [12,13], indicating that LPL may be more or less inhibited by control proteins also in the fed state.

LPL is an interesting target for drug discovery because of its central role in TG metabolism. Increased LPL activity may compensate for deleterious alleles that increase CVD risk due to hypertriglyceridemia. Recently a study reported compounds with effects on LPL stability in the presence of ANGPTL4 [14]. We have identified other small molecules that protect LPL from inactivation by ANGPTL4 *in vitro*. In addition, one selected compound showed promising TG-lowering effects *in vivo*.

2. Materials and methods

2.1. Materials

See Supplementary Material and Methods.

2.2. Screening compounds

The compound collection screened consisted of 17,340 small molecules originating from ChemBridge (San Diego, CA). The compound collection was selected based on chemical diversity and drug likeness. Statistical parameters were calculated as described [15]. See Supplementary Material and Methods for details.

2.2.1. Primary screen

The screening was performed at Laboratories for Chemical Biology Umeå (LCBU), Umeå University. In brief, compound or DMSO control was added to wells containing ANGPTL4 followed by the addition of LPL and the fluorogenic substrate pivaloyl–umbelliferone. Plate readouts were performed immediately after substrate addition (t_0) and after 5 h (t_5). See Supplementary Table S1 for details.

2.2.1.1. Selection criteria.

- Compounds with fluorescence intensities above 50% of the mean plate average at zero minutes (t_0) and values not measurable due to instrumental limitations after 5 h (t_5) were excluded.
- Raw data from each well were normalized on a per-plate basis. Compounds ameliorating more than 50% of the inactivation of LPL by ANGPTL4 were selected by means of normalized percent inhibition using the following equation:

$$\%\ \text{Effect} = 100 \times \frac{W_{i(t_5-t_0)} - \bar{X}_{\text{neg}(t_5-t_0)}}{\bar{X}_{\text{pos}(t_5-t_0)} - \bar{X}_{\text{neg}(t_5-t_0)}}$$

For all wells (W_i) the fluorescent intensity at start (t_0) was subtracted from that observed after 5 h (t_5). $\bar{X}_{\text{neg}}$ and $\bar{X}_{\text{pos}}$ are the means of measurements of the negative (DMSO + LPL + ANGPTL4) and positive (DMSO + LPL only) controls, respectively.

2.2.2. Confirmation screen

Selected compounds were further evaluated using identical conditions as in the primary screen, but in duplicate wells. To examine possible effects of the compounds on the substrate, they were also incubated with substrate only.

2.2.3. Secondary screen

In brief, LPL was pre-incubated in the presence of ANGPTL4 or compound/DMSO. After 30 min TRLs from human plasma were added to assess remaining LPL activity by means of released fatty acids (FAs). See Supplementary Table S2 for details.

2.2.3.1. Selection criteria.

- Compounds ameliorating more than 50% of the inactivation of LPL by ANGPTL4 were selected by means of normalized percent inhibition using the following equation:

$$\%\ \text{Effect} = 100 \times \frac{W_i - \bar{X}_{\text{neg}}}{\bar{X}_{\text{pos}} - \bar{X}_{\text{neg}}}$$

where W_i is the measured FA concentration of the ith well. $\bar{X}_{\text{neg}}$ and $\bar{X}_{\text{pos}}$ are the means of measurements of the negative (DMSO + LPL + ANGPTL4) and positive (DMSO + LPL only) controls, respectively.

2.3. Conditions for studies of effects on LPL activity

For follow-up studies on LPL activity we used revised versions of our secondary screen with different concentrations of compounds solubilized in DMSO (the same volume of DMSO was added to all wells). LPL was inactivated by ANGPTL4 during pre-incubation for 10 min at room temperature on an orbital shaker (600 rpm). Alternatively, LPL was subjected to mild heat inactivation at 37° on a SpectraMax 340 microplate reader with 5 s of shaking every 30 s for a total time of 10 min. The remaining LPL activity was then quantified by addition of 90 µl substrate containing the synthetic lipid emulsion (Intralipid) corresponding to 3.33 mg TG/ml, 8.33% (v/v), heat-inactivated rat serum as source of the LPL-activator apolipoprotein C-II, 10% bovine serum albumin (BSA), 27.75 U heparin/ml, 0.167 M NaCl, and 0.25 M Tris–HCl, pH 8.5. After 45 min the lipolysis was stopped by addition of 50 µl 10% (v/v) Triton X-100. The released fatty acids were quantified using the kit NEFA HR2 (Wako Chemicals).

2.4. Surface plasmon resonance (SPR) experiments

For investigation of binding of small molecules to LPL or ANGPTL4 we used SPR. LPL or ANGPTL4 were immobilized to the sensor chip surface. To assess binding kinetics, increasing concentrations of compounds were injected to the flow cells in 20 mM Hepes, 0.15 M NaCl, pH 7.4 containing 0.1% Triton X-100 (running buffer) at 25 °C.

2.5. Heparin-Sepharose chromatography

For separation of active LPL dimers from inactive, monomeric forms of LPL we used heparin-Sepharose chromatography as previously described [16,17]. Samples containing ^{125}I-labeled LPL, with or without ANGPTL4, were incubated at 37 °C or room temperature in Eppendorf tubes in the presence or absence of 50 µM 50F10. After the incubation, 30 µl of 10% (w/v) deoxycholic acid was added to stabilize LPL against further inactivation by temperature and/or ANGPTL4. All samples were put on ice for 2 min prior to dilution by 700 µl 20 mM Tris–HCl, 0.1 M NaCl, pH 7.4 containing 1 mg/ml BSA, 20% glycerol and 0.01% TX-100. The samples were briefly centrifuged and 500 µl were loaded on HisTrap columns. A gradient from 0.1 M NaCl to 1.5 M NaCl was applied (in 20 mM Tris–HCl, 1 mg/ml BSA, 20% glycerol, 0.01% TX-100, pH 7.4) to elute the LPL protein.

2.6. In vivo experiments

All animals were between 10 and 12 weeks old. For experiments with compound all injections of animals were made with PBS containing 3% DMSO (v/v) and 0.2% Triton X-100 (v/v), with or without (vehicle only) 1 mM compound 50F10. ApoA-V deficient mice were treated once daily in the morning for 4 days with intraperitoneal injections (i.p.) corresponding to 10 µg of compound/kg BW, or vehicle. Food was removed 15 h before the

last injections which commenced at 08:00 h. An oral gavage of 100 μl olive oil was given 1 h after the final i.p. injection. Blood was collected after 3 h from the tail vein in EDTA-coated capillaries. Plasma lipids were analyzed with Trig/GB kit (Roche/Hitachi) scaled to microtiter plates.

For chylomicron clearance experiments normal C57BL/6 mice, sedated with hypnorm/dormicum, were injected with 1 μg of radiolabeled chylomicron TG in the right jugular vein. Blood was collected from the left jugular vein at the indicated time points. The blood was immediately mixed with a lipid extraction solution and analyzed as described [18].

Lipoprotein production was investigated in fasted C57BL/6 by i.v. administration of Triton WR-1339 (0.5 mg/g BW, 10% (w/v) in PBS) to overnight fasted C57BL/6 mice [19]. For studies of VLDL production, blood was collected over 3 h after the injections. For studies of chylomicron production mice were given an oral lipid gavage containing ^{14}C-labeled triolein (40 μCi).

ApoA-V mice deficient mice were from the same breed as described in [19]. Normal C57BL/6 mice were from Taconic (Denmark). The mice were kept at room temperature with light on between 06.00 h a.m. and 06.00 h p.m., with free access to chow and tap water.

All animal experiments were approved (No.: A54/11) by the regional Ethical Committee for Animal Experiments at Umea University.

3. Results

3.1. Small molecule screening

A small molecule screening was employed to identify compounds that could protect LPL from inactivation by ANGPTL4. Based on our selection criteria, 2064 compounds were excluded due to autofluorescence or effects on the substrate. Of the remaining compounds, 84 protected LPL so that more than 50% of the enzyme activity remained after incubation with ANGPTL4. Confirmation screens showed that 48 of these initial hits were due to effects on the substrate by the compounds themselves. The remaining compounds were then re-screened, but with TRLs as substrate for LPL. This resulted in identification of 8 compounds that prevented inactivation of LPL by 50% or more (Table 1).

3.2. Effects of compounds

One of our identified hits had a similar molecular structure as another compound that did not fulfil the selection criteria in our secondary screen. For the possibility that there may exist a structure–activity relationship for these two compounds we decided to evaluate them further (Table 2). We noted that these compounds gave different results depending on which assay was employed. Compound 61A2 showed a higher ability to prevent LPL inactivation with the fluorogenic assay than with TRLs as substrate, whereas compound 50F10 showed similar effects on LPL activity independent of assay conditions. Next, we performed dose–response curves for these compounds using identical conditions as in our secondary screen (Fig 1A). Both compounds dose-dependently protected LPL from inactivation by ANGPTL4, with 50F10 being almost four times more potent than 61A2 when TRLs were used as substrate. Based on this we decided to study 50F10 in more detail. To investigate if 50F10 had effects directly on LPL we incubated the enzyme at 37 °C in the absence of ANGPTL4 and found that 50F10 stabilized LPL with an EC50 similar to that when ANGPTL4 was used for inactivation of the enzyme at room temperature (Fig. 1B).

3.3. Binding studies using SPR

To verify binding to LPL and examine if 50F10 bound to ANGPTL4 we used SPR. Detectable binding of 50F10 to both proteins was observed (Fig. 2A and B). In the case of LPL, the SPR binding curves could only be fitted to a two binding site model according to which 50F10 binds at least to two distinct binding sites on LPL. Corresponding kinetic parameters were as follows: $k_{ass1} = 58$ $M^{-1}s^{-1}$, $k_{diss1} = 1.9 \times 10^{-5}$ s^{-1}, $K_{d1} = 0.32$ μM and $k_{ass2} = 120$ $M^{-1}s^{-1}$, $k_{diss2} = 0.02$ s^{-1}, $K_{d2} = 0.16$ mM. Binding of 50F10 to ANGPTL4 followed a simple 1:1 binding model with kinetic parameters: $k_{ass} = 1.5 \times 10^{3}$ $M^{-1}s^{-1}$, $k_{diss} = 1.5 \times 10^{-3}$ s^{-1}, $K_d = 0.95$ μM.

3.4. Heparin-Sepharose chromatography

To investigate if 50F10 stabilized the dimeric form of the enzyme, we used heparin-Sepharose chromatography and ^{125}I-labeled LPL to follow the protein. In the absence of preincubation or added compound, LPL eluted at about 1 M NaCl (Fig. 3A), as expected for active LPL dimers [17]. Preincubation at room temperature for 30 min in the absence of compound resulted in one additional peak of radioactivity eluting already at 0.5 M NaCl, characteristic for LPL monomers [17]. Preincubation with 50F10 prevented conversion of LPL from the form with high affinity to heparin to that with lower affinity (Fig. 3A). Next, we inactivated LPL by pre-incubation at 37 °C or in the presence of ANGPTL4 at room temperature. In both cases this resulted in almost complete conversion from the form with high affinity to heparin to presumably monomeric forms of LPL. In both cases, addition of 50F10 to the pre-incubation mixtures prevented this conversion (Fig. 3B and C).

3.5. In vivo experiments

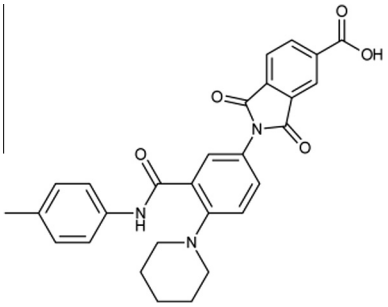
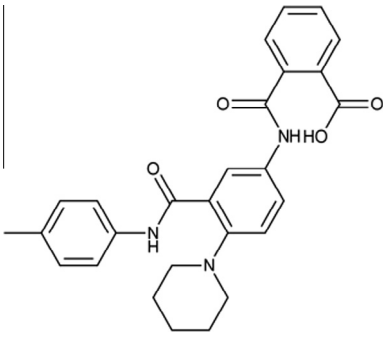
To test if 50F10 had effects *in vivo* we turned to a mouse model with hypertriglyceridemia (the apoA-V knockout mice). Treatment with i.p. injections of 50F10 resulted in significant reductions in the postprandial response to an oral lipid load (100 μl olive oil) compared to vehicle-treated controls (Fig. 4A). To further characterize the lipid clearance we injected radiolabeled rat chylomicrons in C57BL/6 wild-type mice. Mice treated for 4 days by i.p. injections of 50F10 showed a significantly more rapid clearance compared to animals that had received only vehicle (Fig 4B). When

Table 1
Summary of assay results.

Screen type	Number of compounds	Selection criteria	Number of selected compounds	Assay statistics			
				Z′[a]	S/B[a]	S/N[a]	%CV[a]
Primary screen	17,340	%Effect > 50%[b]	84	0.74 ± 0.09	2.74 ± 0.16	16.50 ± 5.56	3.92 ± 1.64
Confirmation screen	84	%Effect > 50%	48	0.77 ± 0.12	3.09 ± 0.34	20.14 ± 6.74	4.51 ± 2.50
Secondary screen	48	%Effect > 50%	8	0.57 ± 0.11	2.57 ± 0.03	8.90 ± 2.21	5.75 ± 1.64

[a] Data are represented as mean values ± SD.
[b] Additional criteria include autofluorescence and substrate effects as described in the method section.

Table 2
Two compounds with similar structure and their effects in different assays.

Structure	% Effect		
	Primary screen	Confirmation screen [a]	Secondary screen [a]
50F10 	67.23	77.23 ± 0.02	68.17 ± 0.02
61A2 	91.78	92.88 ± 0.02	15.32 ± 0.04

[a] Data are represented as mean values of duplicate samples ± SD.

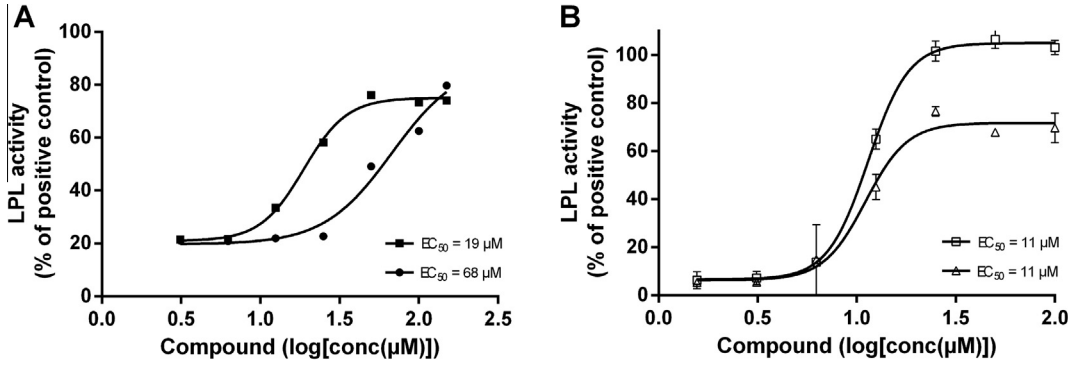


Fig. 1. LPL activity in response to compounds under inactivating conditions. (A) LPL (15 nM) and ANGPTL4 (75 nM) was pre-incubated with 50F10 (■) or 61A2 (●) at room temperature for 10 min and TRLs were then added. LPL activity was measured and divided with the corresponding mean value for LPL without ANGPTL4 (positive control). Data points represent values of single samples. (B) LPL (15 nM) and 50F10 were pre-incubated for 10 min at 37 °C (□) or with 75 nM ANGPTL4 (Δ) at room temperature. Intralipid was then added and LPL activity was measured and divided with the corresponding mean values for LPL incubated on ice or without ANGPTL4, respectively (positive controls). Data points represent mean values of triplicate samples ± SD, EC_{50} values were calculated using Graphpad Prism 6.

we analyzed the tissue distribution of radioactivity we found no significant differences, indicating that plasma TGs had been cleared to the same tissues in both groups (data not shown). Finally, we examined if there were any effects of 50F10 on lipoprotein production. To assess VLDL and chylomicron production rates, Triton WR-1339, an efficient inhibitor of LPL *in vivo* [20], was administrated i.v. to wild-type mice, either fasted or given an olive oil bolus that included a ^{14}C-labeled triolein tracer. In neither nutrition group did we observe any significant differences between mice given compound and those receiving vehicle. This indicated that the effect of 50F10 on plasma lipids was primarily due to an increased rate of TG clearance (Fig. 4C and D).

4. Discussion

We have demonstrated that a small molecular compound, 50F10, identified by our screening campaign, caused a dramatic stabilization of LPL activity *in vitro*. In addition, we show that 50F10 has effects on plasma TGs in mice.

Catalytically active LPL is a non-covalent, unstable homodimer [21]. Dissociation of LPL dimers to monomers leads to loss of activity and is practically irreversible [22]. Inactivation of LPL by ANGPTL4 was previously shown to be due to dissociation of active LPL dimers to inactive monomers [11]. In our primary screen we used the fluorogenic ester pivaloyl–umbelliferon as substrate

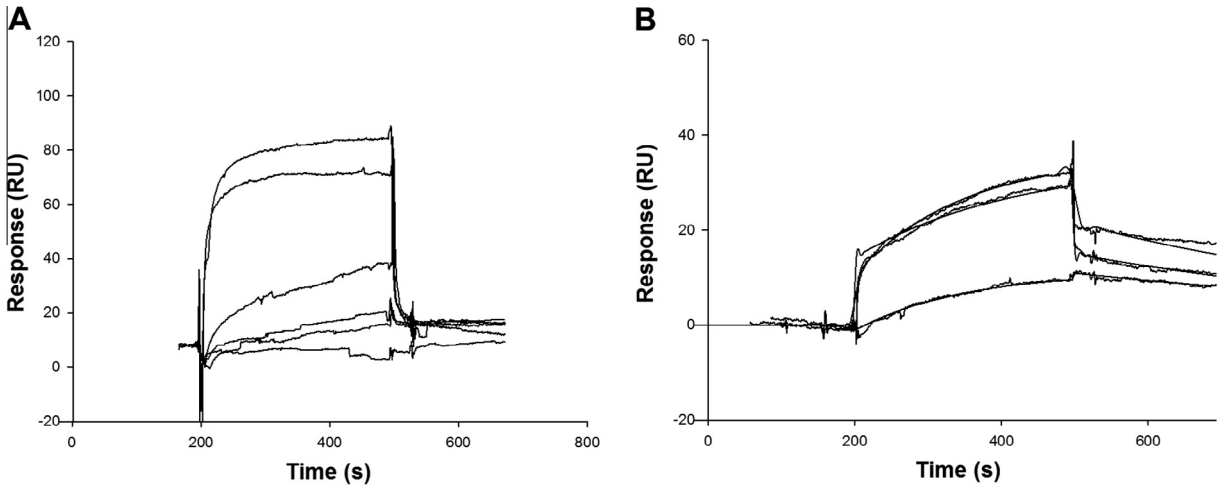


Fig. 2. Binding of 50F10 to LPL and ANGPTL4 on SPR. (A) Binding of 50F10 to immobilized LPL (8.3 ng/mm^2) at concentrations: 1.2 μM, 4.6 μM, 7.6 μM, 11.2 μM, 23 μM, 37 μM. (B) Binding of 50F10 to immobilized ANGPTL4 (5.5 ng/mm^2) at concentrations: 3.3 μM, 12 μM and 22 μM.

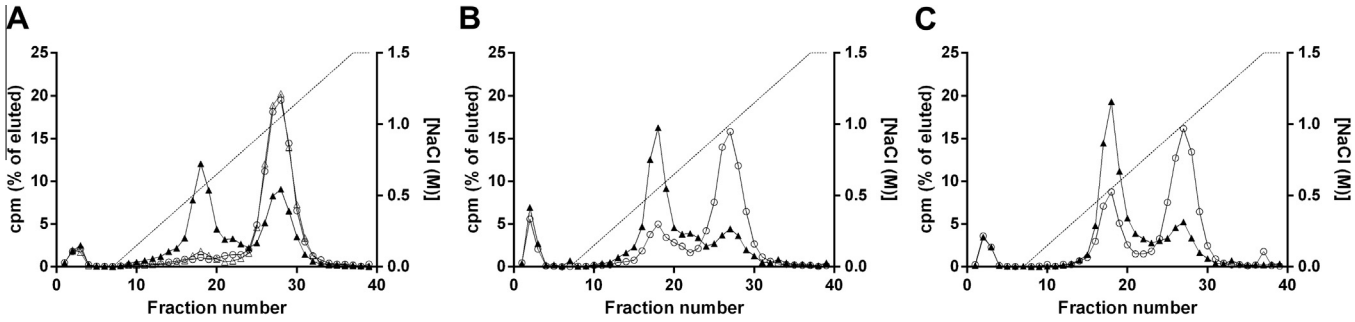


Fig. 3. Effects of 50F10 on the LPL dimer as analyzed by heparin-Sepharose affinity chromatography. ^{125}I-labeled LPL (15 nM) was subjected to different conditions, both in the absence and presence of 50 μM 50F10, and then applied to a heparin-Sepharose column, later eluted by a NaCl gradient to separate different forms of LPL. (A) LPL was applied directly to the column (Δ) or incubated for 30 min at room temperature with (○) or without (▲) compound. (B) LPL was incubated at 37 °C for 10 min with (○) or without (▲) compound. (C) LPL and ANGPTL4 (75 nM) was incubated for 10 min at room temperature with (○) or without (▲) compound.

because it was reported to be water-soluble and have low non-specific reactivity in aqueous solutions [23]. Studies of LPL had shown a dissociation between the more rapid loss of activity against emulsions of TGs with long acyl chains compared to the loss of activity against the short-chain TG tributyrin [24]. Therefore it was important to demonstrate that hits from the primary screen did in fact cause retention of enzymatic activity also against TRLs, the natural substrate for LPL. One draw-back with TRLs from a drug screening perspective is that LPL becomes stabilized by the lipoproteins and cannot easily be inactivated by ANGPTL4 [25]. We circumvented this problem by using a pre-incubation procedure in which ANGPTL4 was allowed to interact with LPL in the presence of compounds prior to addition of the lipid substrate. For practical reasons, TRLs were later substituted with the commercial lipid emulsion Intralipid, yielding similar results (Fig. 1A and B).

LPL is unstable *in vitro* and activity decreases rapidly at 37 °C unless stabilizing agents are present [11,26]. Compound 50F10 preserved the activity of LPL at 37 °C with similar EC50 as when ANGPTL4 was used to inactivate the enzyme at room temperature. By comparing elution profiles from heparin-Sepharose columns for LPL incubated with or without 50F10 we could conclude that the compound preserved the active, dimeric form of LPL. SPR experiments revealed binding of 50F10 to both LPL and ANGPTL4. It was previously shown that FAs protect LPL from inactivation by ANGPTL4 and that FAs bind to both LPL and ANGPTL4 [27]. It remains to be determined if there are any similarities in the mechanisms behind the *in vitro* observations.

We found discrepancies in the effectiveness of compounds 50F10 and 61A2 depending on what assay was employed to measure LPL activity. It is possible that 61A2 is as good, or even better, in preserving the active LPL dimer as 50F10, while the latter compound may better preserve the lipid-binding properties that are known to reside mostly in the C-terminal part of LPL [28]. Possibly the same is true for the recently described LPL agonist [14]. This compound failed to retain LPL activity in our Intralipid-based assay at concentrations up to 400 μM (Fig. S1).

We challenged our candidate compound by injecting it to hypertriglyceridemic apoA-V deficient mice, as they were previously reported to reach normal TG levels when crossbred with mice overexpressing LPL [19]. Interestingly, 50F10 significantly decreased the plasma TG levels of apoA-V deficient mice in the postprandial state. By i.v. injection of radiolabeled chylomicrons we demonstrated an accelerated plasma clearance of TGs in treated wild-type animals (Fig. 4B).

Reductions in postprandial plasma TG levels could be due to decreased intestinal lipid absorption or impaired assembly and secretion of chylomicrons and/or VLDL. Administration of the LPL inhibitor Triton WR-1339 to wild-type mice resulted in continuous increase in plasma TG levels in fasted or lipid-fed animals. There were no significant differences in the TG accumulation in mice treated with 50F10 compared to untreated animals, indicating that our compound did not lower plasma TGs primarily by affecting factors involved in lipoprotein assembly or secretion.

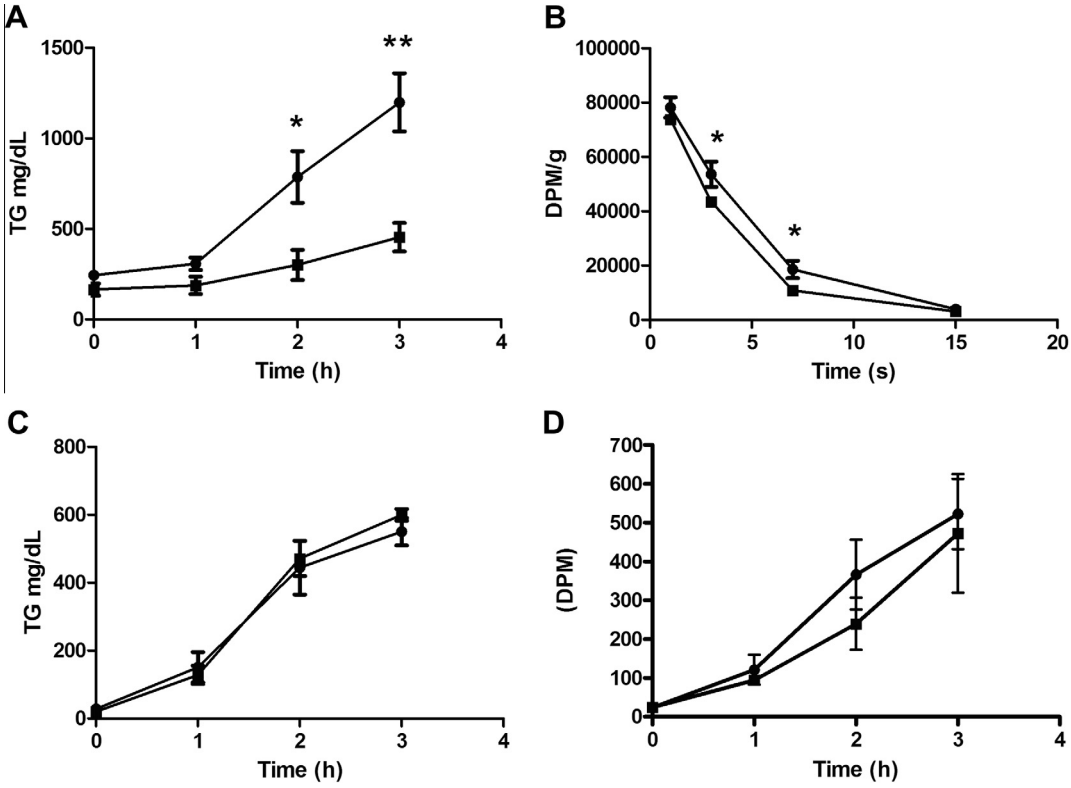


Fig. 4. Effects of 50F10 in mice. (A) ApoA-V deficient mice were injected i.p. with 50F10 (■) ($n = 4$) or vehicle (●) ($n = 6$) once daily for 4 days. One hour after the last injection mice were given a 100 µl olive oil oral gavage. Plasma TG levels were investigated at the indicated time points. (B) For studies of TG clearance, radiolabeled chylomicrons (1 µg TG) were injected intravenously to sedated wild-type mice ($n = 5$/group) pre-treated by daily i.p. injections with 50F10 (■) or vehicle (●) for 4 days. To study lipoprotien production rates, overnight fasted WT mice were pre-treated with 50F10 (■) or vehicle (●) once daily i.p. for 4 days and then given i.v. injections with Triton WR1339 (0.5 mg/g BW). (C) TG levels were investigated in fasted mice treated with 50F10 ($n = 4$) or vehicle ($n = 5$). (D) Mice treated with 50F10 or vehicle ($n = 5$/group) were given an oral lipid gavage containing ^{14}C-labeled triolein. Data for TG and blood radioactivity are mean values with standard error of mean. $^{*}p < 0.05$, $^{**}p < 0.01$.

There is a need for additional drugs in the prevention of CVD, especially for high-risk patients. Evidence is emerging around hypertriglyceridemia as a cause for CVD [2]. Here we present a potential lead compound that stabilizes LPL *in vitro* and lower plasma TGs *in vivo*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.114.

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