Inhibitor discovery of phospholipases and N-acyltransferases

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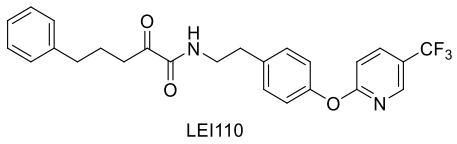
4

Biochemical and cellular profiling of LEI110

4.1 Introduction

The previous two chapters together outline the pathway to the discovery of a selective PLAAT3 inhibitor. PLAATs play an important role in lipid metabolism and biosynthesis of NAE phospholipids and studies on their individual contribution to these physiological processes are required, both for fundamental understanding and for unveiling their potential as therapeutic targets in obesity and/or the common cold. Recent immunoprecipitation studies have shown that PLAAT3 binds to peroxin-19 (Pex19), a protein known to function as a chaperone in the transport of peroxisomal membrane proteins.[1] Overexpression of PLAAT3 causes almost complete disappearance of peroxisomes in mammalian cells within 24 h, which further supports the regulatory role of PLAAT3 in the oxidation of fatty acids. Interestingly, the catalytically inactive point mutant of PLAAT3 (C113S) was neither able to disrupt the binding of Pex19 to other peroxisomal membrane proteins nor the peroxisomal function itself, while retaining the ability to bind Pex19. Therefore it has been suggested that PLAAT3 could exert its inhibitory effect through hydrolysis of peroxisomal membrane lipids, although more data is necessary to confirm this hypothesis. The dysregulation of peroxisomal function by PLAAT3 is in accordance with the observed phenotype of PLAAT3 deficient mice as elimination of adipocyte peroxisomes by ablation of a peroxin essential to peroxisome biogenesis, Pex5, in mice was reported to result in increased fat mass and a reduced rate of lipolysis in white adipose tissue (WAT).[2]

LEI 110 (Figure 1B), discovered by research in the previous **chapter 3** provides the potential means to do, at least for its preferred target, PLAAT3. For this potential to bear out, its behavior in live cells needs to be mapped. This chapter details studies performed to this end and entailing biochemical and cellular profiling of LEI110.



LEI110

Figure 1. Chemical structure of LEI110.

4.2 Results and discussion

In **chapter 3**, 2-oxo-5-phenyl-N-(4-((5-(trifluoromethyl)pyridin-2-yl)oxy)-phenethyl)-pentanamide (LEI110) was discovered as a PLAAT3 inhibitor with a pIC_{50} = 7.0 ± 0.1 in the ABPP-assay. In order to gain insight into the selectivity over other members of this protein family, LEI110 was tested against other PLAATs. The results showed that LEI110 was also active on PLAAT2 (pIC_{50}: 6.8), PLAAT4 (pIC_{50}: 6.8) and PLAAT5 (pIC_{50}: 7.6), (Figure 2A, Table 1). LEI110 was tested in a fluorescent substrate assay. In this assay, the

cytosolic protein fraction of HEK293T cells transiently overexpressing PLAAT3 was diluted to 0.6 mg/mL in assay buffer (50 mM Tris-HCl pH 8, 1 mM $CaCl_2$, 100 mM NaCl). 50 µL of protein dilution was added in a dark flat-bottom Greiner 96-well plate and incubated with the LEI110 at different concentrations. 50 µL of the substrate liposome solution, which was prepared by slowly adding a concentrated solution of the substrate Red/Green Bodipy PC-A2 (2.5 mM in DMSO), DOPC (10 mM in ethanol, Avanti Polar Lipids, Alabaster, AL) and DOPG (10 mM in ethanol, Avanti Polar Lipids, Alabaster, AL) into the assay buffer, was added (Bodipy PC-A2, final concentration: 2.5 µM) and the fluorescence measurement was started immediately on a TECAN infinite M1000 pro. A standard curve of Bodipy FL C5 (D3834, Invitrogen) was used for evaluating the amount of substrate conversion. LEI110 demonstrated a K_i of 20 nM (95% CI: 17-24 nM) (Figure 2B). The selectivity of LEI110 was profiled in mouse brain membrane and cytosol proteome with probe MB064 and FP-TAMRA, respectively. The results showed that LEI110 was selective over brain serine hydrolases as determined with a gel-based ABPP assay (Figure 2C).

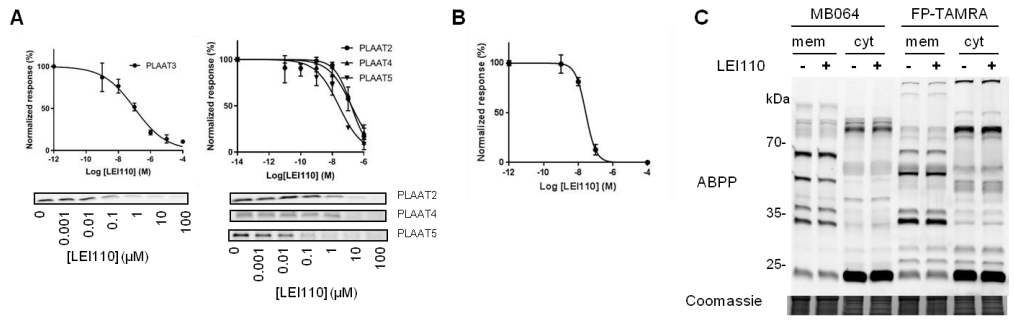


Figure 2. Biochemical characterization of LEI110. (A) Dose-response curves of LEI110 against PLAAT3 and other PLAAT family members with probe MB064. (B) Dose-response curve of LEI110 for PLA2G16 with the PC-A2 fluorescent substrate assay. (C) Selectivity of LEI110 against MB064 (2 µM) and FP-TAMRA in mouse brain membrane (mem) and cytosol (cyt) proteome. Coomassie staining was used as a protein loading control. – indicates control (with DMSO), + indicates with LEI110 at 10 µM.

Table 1. pIC_{50} ± SEM (n = 3) of compound **1** and LEI110 against HRASLS protein family members from the ABPP assay.

Compound	PLAAT3	PLAAT2	PLAAT4	PLAAT5
1	6.0 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.4 ± 0.1
LEI110	7.0 ± 0.1	6.8 ± 0.1	6.8 ± 0.1	7.6 ± 0.1

PLAAT3 is endogenously expressed in brown and white adipose tissue and its activity could be visualized by MB064 (Figure 3A). Therefore it was decided to test whether compound **1** and LEI110 were able to block PLAAT3 activity in adipose tissue. Indeed,

both compounds completely abolished labeling of native PLAAT3 by MB064, whereas the labeling of other proteins in brown and white adipose tissue was not affected (Figure 3A). The selectivity of LEI110 in adipose tissue was confirmed in a chemical proteomics assay using MB108 (THL-biotin, Figure 3B) and FP-biotin (Figure 3C), respectively (Figure 3D and 3E, respectively). Based on its activity and selectivity profile, it was decided to test LEI110 in a cellular assay to investigate how the PLAAT3 product formation was affected by this inhibitor. To this end, human PLAAT3 was transfected in U2OS cells which were then incubated with vehicle (DMSO) or LEI110 (10 µM) in serum-free conditions. This led to a time-dependent increase in arachidonic acid, a product of PLAAT3, as determined by targeted lipidomics, which could be almost completely abolished by LEI110 (Figure 3F). Taken together, the results indicate that LEI110 is a potent, selective and cell-permeable PLAAT3 inhibitor.

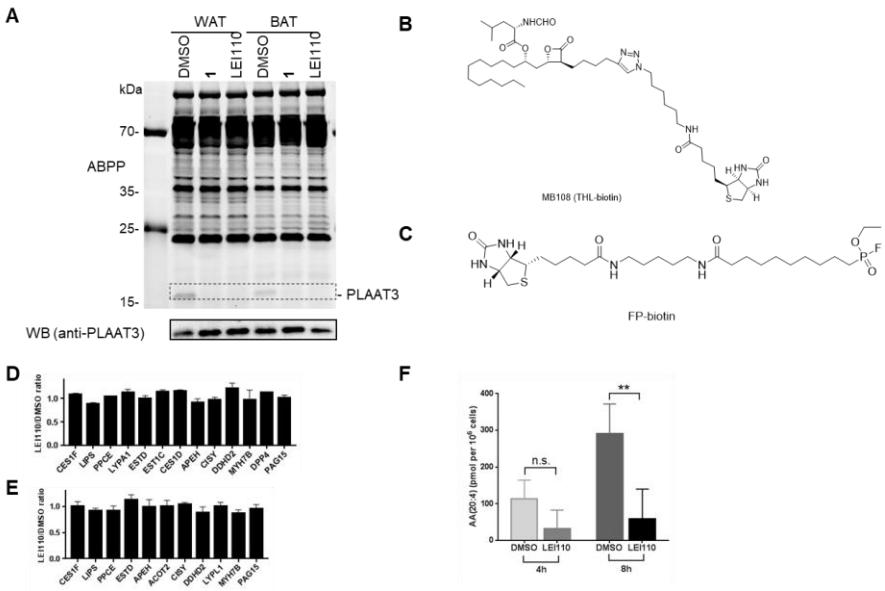


Figure 3. (A) Competitive ABPP of compound **1** and LEI110 against endogenous PLAAT3 using MB064 in the cytosol proteome of mouse WAT and BAT and Western blot of the ABPP gel using an anti-PLAAT3 antibody. Both inhibitors block the activity of PLAAT3 at 10 µM. (B) Chemical structure of MB108 (THL-biotin). (C) Chemical structure of FP-biotin. (D) MB108 and FP-biotin based chemoproteomic analysis of serine hydrolase activities in the mouse WAT cytosol proteome treated with LEI110 (10 µM). (E) MB108 and FP-biotin based chemoproteomic analysis of serine hydrolase activities in the mouse BAT cytosol proteome treated with LEI110 (10 µM). (F) *In situ* treatment of U2OS cells overexpressing PLAAT3 with LEI110 (10 µM, 4 h or 8 h) reduced arachidonic acid (AA) levels that were induced by PLAAT3.

Genetic studies have previously demonstrated that PLAAT3 regulates lipolysis;[3, 4] therefore it was decided to test LEI110 phenotypic assay with human hepatocytes.

Stimulation of HepG2 cells with oleic acid induces steatosis that eventually results in lipid droplet formation, as visualized by Adipored, which is a widely used *in vitro* model to study fatty liver disease.[5, 6] Since PLAAT3 activity could not be visualized in HepG2 cells using the ABPP method which was described in **chapter 2** and this may be probably due to its low abundance, a quantitative RT-PCR method was used to confirm PLAAT3 mRNA expression in HepG2 cells (Table 2). Of note, relatively high level of mRNA for PLAAT3 (Ct value: 24.1) was found when compared to PLAAT1 (Ct value: 34.0), PLAAT2 (Ct value: 32.3) and PLAAT4 (Ct value: 30.8). In addition, the selectivity of LEI110 in HepG2 cells was confirmed in a chemical proteomics assay with MB108 and FP-biotin (Figure 4A). Next, HepG2 cells were incubated with LEI110 (10 μM) before oleic acid treatment and a reduction in lipid droplet formation was observed, which indicates that LEI110 modulates lipolysis (Figure 4B). This is in line with previous reports showing that PLAAT3 modulates lipid metabolism in HepG2 cells or adipocytes.[3, 7]

Table 2. Summary table of the Ct values in qPCR analysis for different *HRASLS*-gene detection in HepG2 cells (*GAPDH* as the house-keeping gene)

Gene name	***GAPDH***	***PLAAT1***	***PLAAT2***	***PLAAT3***	***PLAAT4***	***PLAAT5***
Ct values	15.0 ± 0.3	34.0 ± 0.3	32.2 ± 0.5	24.1 ± 0.4	30.8 ± 0.4	n.d.*

* n.d. indicates not determined

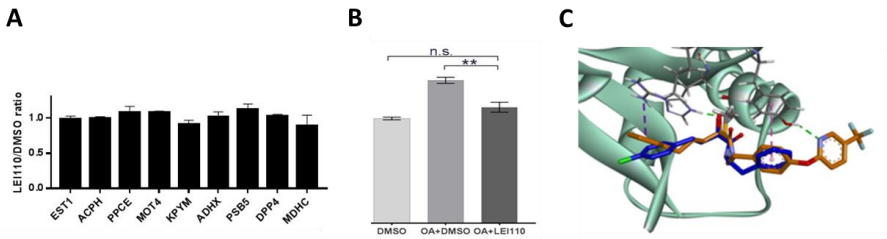


Figure 4. Biochemical characterization of compound LEI110. (A) MB108 and FP-biotin based chemoproteomic analysis of serine hydrolase activities in HepG2 cell lysate proteome treated with LEI110 (10 μM). (B) *In situ* treatment of HepG2 cells with LEI110 (10 μM, 24 h) reversed the lipid accumulation in the cells induced by oleic acid (OA, 100 μM, 24 h). (C) Structure-guided modelling of **1** and LEI110. Compounds **1** (blue) and LEI110 (orange) in complex with PLAAT3, covalently bound to Cys113. Green dotted lines represent a hydrogen bond, pink and purple represent π-interactions. Data represent mean values ± SEM for at least 3 replicates. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ using Student's t-test.

4.3 Conclusions

In summary, here it was shown that LEI110, a potent PLAAT3 inhibitor (K_i = 20 nM), reduces cellular arachidonic acid levels in PLAAT3-overexpressing U2OS cells and oleic acid-induced steatosis in human HepG2 cells. Gel-based ABPP and chemical proteomics showed that LEI110 is a selective pan-inhibitor of the HRASLS-family of thiol hydrolases (i.e. PLAAT2, PLAAT3 and PLAAT5). α-Ketoamides have previously been employed as pharmacophore for the inhibition of hydrolases[8-10] and are incorporated in marketed drugs for the treatment of the viral infection with hepatitis C (e.g., boceprevir);[11, 12] therefore, it is anticipated that LEI110 constitutes an excellent starting point for the structure-based drug development of novel molecular therapies for obesity and/or common cold.

4.4 Experimental procedures

Plasmids. Full-length human cDNA of PLAAT3, A-C1, PLAAT2, PLAAT4 and PLAAT5 (from Prof. N. Ueda's lab) were cloned into mammalian expression vector pcDNA3.1, containing genes for ampicillin and neomycin resistance. The inserts were cloned in frame with a C-terminal FLAG-tag. Two-step PCR mutagenesis was performed to substitute the active site cysteine for an alanine (A) or serine (S) in the PLAAT3-FLAG, PLAAT2, PLAAT4 and PLAAT5, respectively to obtain the corresponding mutants. Plasmids were isolated from transformed XL-10 Goldcompetent cells (Maxi Prep kit: Qiagen) and sequenced at Macrogen. Sequences were analyzed and verified (CLC Main Workbench).

Cell culture

General. HEK293T, U2OS and HepG2 cells were kept in culture at 37 °C under 7% CO_2 in DMEM containing phenol red, stable glutamine, 10% (v/v) New Born Calf Serum (Thermo Fisher), and penicillin and streptomycin (200 µg/mL each; Duchefa). Medium was refreshed every 2-3 days and cells were passaged twice a week at 80-90% confluence. Cells lines were purchased from ATCC and were regularly tested for mycoplasma contamination.

Transient transfection. Transient transfection was performed as previously described.[13] In brief, HEK293T cells were seeded in 15-cm petri dishes one day prior to transfection. Prior to transfection, culture medium was aspirated and a minimal amount of medium was added. A 3:1 (m/m) mixture of polyethyleneimine (PEI, 1 mg/mL) (60 µg/15-cm dish) and plasmid DNA (20 µg/dish) was prepared in serum free culture medium and incubated for 15 min at RT. Transfection was performed by dropwise addition of the PEI/DNA mixture to the cells. Transfection with the empty pcDNA3.1 vector was used to generate control samples (mock groups). After 24 h, medium was refreshed. Medium was aspirated 72 h post-transfection and cells were harvested by resuspension in PBS. Cells were pelleted by centrifugation (5 min, 1,000 g) and the pellet was washed with PBS. Supernatant was discarded and cell pellets were snap-frozen in liquid nitrogen and stored at -80 °C until sample preparation.

For Figure 3F, a slightly altered procedure and a different cell line was used: one day prior to transfection, U2OS cells were seeded in 6-cm dishes. After 24 h, a mixture of polyethyleneimine (PEI, 1 mg/mL) (8 µg/6-cm dish) and plasmid DNA (2.7 µg/dish) were incubated in serum free culture medium (15 min, RT), and then added dropwise to the cells. After 24 h, medium was aspirated and cells were washed once with serum free medium. New serum free medium supplemented with DMSO (final concentration 0.1%,

v/v) or LEI110 (final concentration: 10 µM) were added in the dishes. After 4 or 8 h incubation at 37 °C under 7% CO_2, cells were harvested for lipidomics analysis.

In situ treatment on HepG2 cells. For Figure 3E, a liver hepatocellular carcinoma cell line HepG2 cells was used. One day prior to treatment, HepG2 cells were seeded in a 48-well plate. After 24 h, the medium was aspirated and cells were treated with 100 µM oleic acid (OA) or DMSO in the absence or presence of LEI110 (final concentration: 10 µM). After 24 h, medium was aspirated and cells were washed once with PBS, then AdipoRed Assay Reagent (3% in PBS, PT-7009, Lonza) was added. After 10 min, place the plate in the fluorimeter (Tecan infinite M1000 pro), and measure the fluorescence in a 3 x 3 grid with excitation at 485 nm and emission at 572 nm.

Sample preparation

Cell membrane and cytosol proteome preparation. Cell pellets were thawed on ice, resuspended in cold lysis buffer (50 mM Tris-HCl pH 8, 2 mM DTT, 1 mM $MgCl_2$, 2.5 U/mL benzonase) and incubated on ice (30 min). The cell lysate was collected and centrifuged (100.000 *g*, 45 min, 4 °C, Beckman Coulter, Ti 70.1 rotor). The supernatant was collected (cytosolic fraction) and the membrane pellet was resuspended in cold storage buffer (50 mM Tris HCl, pH 8, 2 mM DTT) by thorough pipetting and passage through an insulin needle (29G). Protein concentrations were determined by a Quick Start™ Bradford Protein Assay or Qubit™ protein assay (Invitrogen). Samples were flash frozen in liquid nitrogen and stored at -80 °C until further use.

Mouse tissue proteome preparation. Mouse brains (C57Bl6) were isolated according to guidelines approved by the ethical committee of Leiden University (DEC#13191), frozen in liquid nitrogen, and stored at -80 °C until use. Tissues were thawed on ice, dounce homogenized in cold lysis buffer (50 mM Tris HCl, pH 8, 2 mM DTT, 1 mM $MgCl_2$, 2.5 U/mL benzonase) and incubated on ice (15 min), followed by low-speed centrifugation (2500 g, 3 min, 4°C) to remove debris. After high-speed centrifugation (100.000 g, 45 min, 4°C) the supernatant was collected as the cytosol proteome, flash frozen in liquid nitrogen and stored at -80 °C for further use.

Mouse white or brown adipose tissue (C57Bl6) were isolated according to guidelines approved by the ethical committee of Leiden University (DEC#13191) and were immediately dounce homogenized in cold lysis buffer, followed by low-speed spin (2500 g, 3 min, 4°C) to remove the debris. The membrane and cytosol proteome were prepared followed by the same procedure as for the mouse brains described above.

Activity based protein profiling on transiently transfected HEK293T cell lysate. Gel-based activity based protein profiling (ABPP) was performed with minor alterations of

the previously reported protocol.[13] For ABPP assays on HEK293T cells overexpressing PLAAT3, the cytosol proteome (0.5 mg/mL, 20 µL) was pre-incubated with vehicle (DMSO) or inhibitor (0.5 µL in DMSO, 30 min, RT) followed by an incubation with the activity based probe MB064 (final concentration: 250 nM, 20 min, RT). The incubation protocols for HRASLS2, RARRES3 and iNAT were similar except for the protein concentration (0.25 mg/mL, 1 mg/mL, 1 mg/mL, respectively) and probe concentration (250 nM, 500 nM, 500 nM). Final concentrations for the inhibitors are indicated in the main text and figure legends. Only cytosol proteome was used for the dose-response test in ABPP or substrate assay. Reactions were quenched with 7 µL of 4x Laemmli buffer (5 µL, 240 mM Tris (pH 6.8), 8% (w/v) SDS, 40% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.04% (v/v) bromophenol blue). 10 µl sample per reaction was resolved on a 10% or 15% acrylamide SDS-PAGE gel (180 V, 70 min). Gels were scanned using a ChemiDoc MP system with Cy3 and Cy5 multichannel settings (605/50 and 695/55, filters respectively) and stained with Coomassie after scanning. Experiments were done 3 times individually. Fluorescence was normalized to Coomassie staining and quantified with Image Lab (Bio-Rad). IC_{50} curves were fitted with Graphpad Prism® 7 (Graphpad Software Inc.).

Activity based protein profiling on mouse brain proteome. Mouse brain membrane or cytosol proteome (2 mg/mL, 20 µL) was incubated with vehicle (DMSO) or inhibitor (0.5 µL in DMSO, 30 min, RT) followed by an incubation with the activity based probe MB064 (final concentration: 500 nM, 20 min, RT) or FP-TAMRA (Thermo Fisher, 88318, final concentration: 500 nM, 20min, RT). The reaction was quenched with 7 µL of 4x Laemmli buffer and the proteins were resolved and visualized using the same procedures as for the transfected HEK293T cells.

Activity based protein profiling on mouse adipose tissue. Brown or white adipose tissue cytosol proteome (1 mg/mL, 20 µL) was incubated with vehicle (DMSO) or inhibitor (0.5 µL in DMSO, 30 min, RT) followed by an incubation with the activity based probe MB064 (final concentration: 2 µM, 20 min, RT). The reaction was quenched with 7 µL of 4x Laemmli buffer and the proteins were resolved and visualized using the same procedures as for the transfected HEK293T cells.

Western Blot. Western blots were performed as previously reported.[7] After the ABPP assay, the proteins on the SDS-PAGE gel were transferred to a PVDF membrane using a Trans-Blot Turbo™ Transfer system (Bio-Rad). For anti-FLAG antibody, membranes were washed with TBS (50 mM Tris, 150 mM NaCl) and blocked with 5% milk in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20) for 1 h at RT (anti-FLAG antibody) or overnight at 4 °C (for anti-PLAAT3 antibody). Membranes were then incubated with primary antibody in 5% milk TBST for 1 h at RT, washed with TBST, incubated with matching secondary antibody in 5% milk TBST for 1 h at RT and subsequently washed with TBST and TBS. The

blot was developed in the dark using an imaging solution (10 mL luminal solution, 100 µL ECL enhancer and 3 µL 30% H_2O_2). Chemiluminescence was visualized using a ChemiDoc MP (BioRad) with standard chemiluminescence settings. For anti-PLAAT3 antibody, the protocol was similar except that PBS or PBST were used as the washing buffer and blocking buffer instead of TBS or TBST, respectively.

Primary antibodies: monoclonal mouse-anti-PLAAT3 (1:200, Abnova, H00011145-M02), monoclonal mouse-anti-FLAG (1:5000, Sigma Aldrich, F3156). Secondary antibodies: HRP-coupled-goat-anti-mouse (1:5000, Santa Cruz, sc2005).

qPCR. For the qPCR experiments, NucleoSpin® RNA kit (Macherey-Nagel) was used for the total RNA isolation, the transcriptase (Thermo Scientific Maxima Reverse Transcriptase) was used for cDNA synthesis and the 2x SYBR Green qPCR Master (Bimake) was used for qPCR following the manufacturer's protocols. Primers used were listed in the supplementary figures. The experiment was carried out in duplicate and repeated twice.

Fluorescent substrate assay. The fluorescent substrate assay was based on a previously reported method.[14] Liposomes were prepared by slowly injecting a concentrated solution of the substrate Red/Green Bodipy PC-A2 (2.5 mM, in DMSO, Invitrogen, A10072), DOPC (10 mM in ethanol, Avanti Polar Lipids, Alabaster, AL) and DOPG (10mM in ethanol, Avanti Polar Lipids, Alabaster, AL) into the assay buffer (50 mM Tris-HCl pH 8, 1 mM $CaCl_2$, 100 mM NaCl).

Relevant concentrations of compounds are prepared in DMSO. The cytosolic protein fraction of HEK293T cells transiently overexpressing PLAAT3 was diluted to 0.6 mg/mL in assay buffer. 50 µL of protein dilution was added in a dark flat-bottom Greiner 96-well plate and incubated with the compounds or vehicle for 30 min at RT. A sample with mock transfected cytosolic protein lysate incubated with DMSO was incorporated for background subtraction. 50 µL of the substrate liposome solution was added (Bodipy PC-A2, final concentration: 2.5 µM) and the fluorescence measurement was started immediately on a TECAN infinite M1000 pro (37 °C, continuous scanning for 1 h; excitation 488 nm, emission 530 nm). A standard curve of Bodipy FL C5 (D3834, Invitrogen) (the cleavage product of Bodipy PC-A2) was used for evaluating the amount of substrate conversion). The enzyme activity rates were calculated from the steady-state measured in the first 10 min of the reaction. The assay was carried out in duplicate and repeated twice. The data were corrected by the background of mock lysate, normalized by the residual protein activity at 100 % and then evaluated using GraphPad Prism® 7.

Targeted lipidomics

Targeted lipidomics was performed as described previously with minor modifications.[7, 15] In brief, lipids were extracted using liquid-liquid extraction method (MTBE: Methyl *tert*-butyl ether) from *in situ* treated U2OS cells (hPLAAT3-construct transiently expressed, 4 or 8 h, 10 µM LEI110 or vehicle (0.1% DMSO) treatment). The resulting samples are separated and quantified using LC-MS/MS platform.

Activity-based proteomics

Activity-based proteomics was performed based on previously described procedures.[16, 17] In summary, mouse adipose tissue cytosol proteome or HepG2 cell cytosol proteome (250 µL at 1.0 mg/mL) was incubated with vehicle (2% DMSO) or inhibitor (LEI110, 10 µM, 30 min, RT). The heat-inactivated proteome were used for background correction. The proteomes were then labeled with MB108 (THL-biotin) or FP-Biotin (sc-215056A, 10 µM, 20 min, RT), enriched using avidin (pulldown) and on-bead digestion. The resulting peptides are measured, identified and quantified by LC-MS/MS.

Statistical methods

All data are shown as the mean ± SEM where applicable. A Student's t-test (unpaired, two-tailed) was used to determine differences between two groups. All statistical analyses were conducted using Excel or GraphPad Prism version 7, and a p-value less than 0.05 was considered significant throughout unless indicated otherwise.

A sample size of n = 3 was sufficient to detect ≥ 50% inhibition of protein labeling with a 20% standard deviation and a power of 80% at a $p < 0.05$. Routinely, a protein is considered to be an off-target, if 50% inhibition of activity is reached at 10 µM.

4.5. References

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4.6 Supporting Information

Table S1. Summary table of the primers used for the qPCR analysis with their corresponding gene names.

Accession Number	Gene name	Forward(5′–3′)	Reverse (5′–3′)
XM_011544751	*HRASLS*	GTTCCGTCCTGGCTATCAGC	CTGCATTTTCACCAGGGCCT
XM_011545120	*HRASLS2*	GAGACTTGGAGACCTGATTGA	GCTTGTTATTGACCCTGTAG
XM_006718426	*PLA2G16*	AGCGAAATCGAGCCTGG	CCAGATGAACCACATATCCA
NM_004585	*RARRES3*	AGCTGATCCACAAACAAGAG	CCAGATGGATCACGTAGCCA
XM_011544751	*HRASLS5*	AATTGGCTATGAGCACTGG	AGACGACTGTATTTCACCAC