Inhibitor discovery of phospholipases and N-acyltransferases

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Development and application of an ABPP assay to identify PLAAT3 inhibitors

2.1 Introduction

Phospholipase A acyltransferase 3 (PLAAT3, or PLA2G16), was first isolated from murine fibroblasts as a product of the HRASLS-gene (H-RAS-like suppressor) family, which also includes the phospholipase/acyltransferases, namely phospholipid-metabolizing enzyme A-C1 (A-C1, or PLAAT1), HRAS-like suppressor 2 (HRASLS2, or PLAAT2), retinoid acid receptor responder protein 3 (RARRES3, or PLAAT4) and Ca^{2+} -independent *N*-acyltransferase (iNAT, or PLAAT5).[1-3] PLAAT3 is an intercellular, single-pass transmembrane cysteine hydrolase with a molecular weight of 18 kDa with as major activity the hydrolysis of the sn-2 fatty acyl chain of phosphatidylcholine.[4, 5] PLAAT3 has a papain-fold motive consisting of three α-helices and five antiparallel β-sheets organized in a circular permutation and a conserved catalytic triad consisting of Cys113, His23 and His35 as determined by X-ray crystallography (PDB code: 4DOT) and site-directed mutagenesis studies.[6-9]

PLAAT3 is found in various cell lines (e.g. HepG2)[10, 11] and adipose tissue.[12, 13] Its expression is induced during adipocyte differentiation.[14, 15] In mouse models, PLAAT3 has been shown to play an important role in the development of obesity.[16] The nuclear receptor PPARγ (peroxisome proliferator-activated receptor) regulates the function and formation of adipose tissue and PLAAT3 was identified as the downstream target of PPARγ with a role in adipogenesis.[12] PLAAT3 (also known as HRSL3 or H-REV107-1) is also a member of a class II tumor suppressor gene family and it functions as tumor suppressor by regulating the activity of proto-oncogenes.[1, 17, 18] In tumor cell lines and experimental tumors, the growth-inhibitory activity of tumor and downregulation of PLAAT3 were observed[19] and PLAAT3 was shown to be involved in the oncogenic network which mediate the growth and survival of ovarian cancer cells.[17] PLAAT3 is also involved in clearance of the virus in host cells.[18, 20] The authors first created a Haplobank from haploid mouse embryonic stem cells lines and the subsequent reverse genetic screening led to the identification of PLAAT3 as a host factor which is required for cytotoxicity by rhinoviruses.[18] In another genome-wide screening, PLAAT3 was identified as picornavirus host factor because it can facilitate the viral genome translocation and prevent clearance.[21] Taken together, these studies highlight the therapeutic potential of PLAAT3. However, to date, there are no PLAAT3 inhibitors reported that can be used as pharmacological tools to validate PLAAT3 as a therapeutic target.

Currently, no ABPs have been reported for PLAAT3 that could enable inhibitor discovery. In this chapter, it is shown that MB064 (Figure 1A) labels PLAAT3 in an activity-dependent manner and is able to visualize endogenous PLAAT3 in adipose tissue. Screening of a focused lipase inhibitor library using ABPP resulted in the identification of an α-ketoamide (compound **1**, Figure 1B) as a selective PLAAT3 inhibitor.

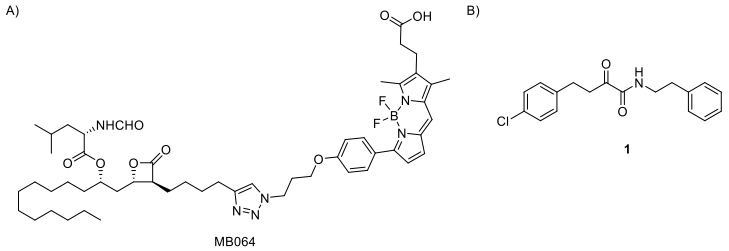

Figure 1. Chemical structures of probe MB064 and compound **1**.

2.2 Results and discussion

To test whether MB064 could also label PLAAT3, proteomes of transiently overexpressed human PLAAT3-FLAG in human embryonic kidney 293 T (HEK293T) cells was incubated with MB064, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence scanning. A fluorescent band was observed at the expected MW of 18 kDa, which overlapped with a band visualized by the FLAG-tag antibody and was absent in mock-transfected cells (Figure 2A). The labeling was probe- and protein-concentration-dependent (Figure 2B) and was optimal at pH 8, which is consistent with previously reported pH optimum of PLAAT3 activity.[14, 22] Site-directed mutagenesis of the catalytic Cys113 into alanine or serine abolished or significantly reduced labeling, respectively, whereas its expression was not substantially altered as witnessed by FLAG-tag antibodies (Figure 2C). Of note, MB064 was also able to cross-react with PLAAT2, PLAAT3 and PLAAT5 (Figure 2D). Finally, it was tested whether MB064 could also label endogenously expressed PLAAT3. To this end, MB064 was incubated with freshly isolated mouse white adipose tissue (WAT) and brown adipose tissue (BAT) extracts. A fluorescent band was observed at the expected MW, which also overlapped with a band visualized by PLAAT3 antibodies (Figure 2E). Taken together, these results demonstrate that MB064 reacts with active human PLAAT3 to form a covalent bond with Cys113 and visualizes native mouse PLAAT3 in brown and white adipose tissue.

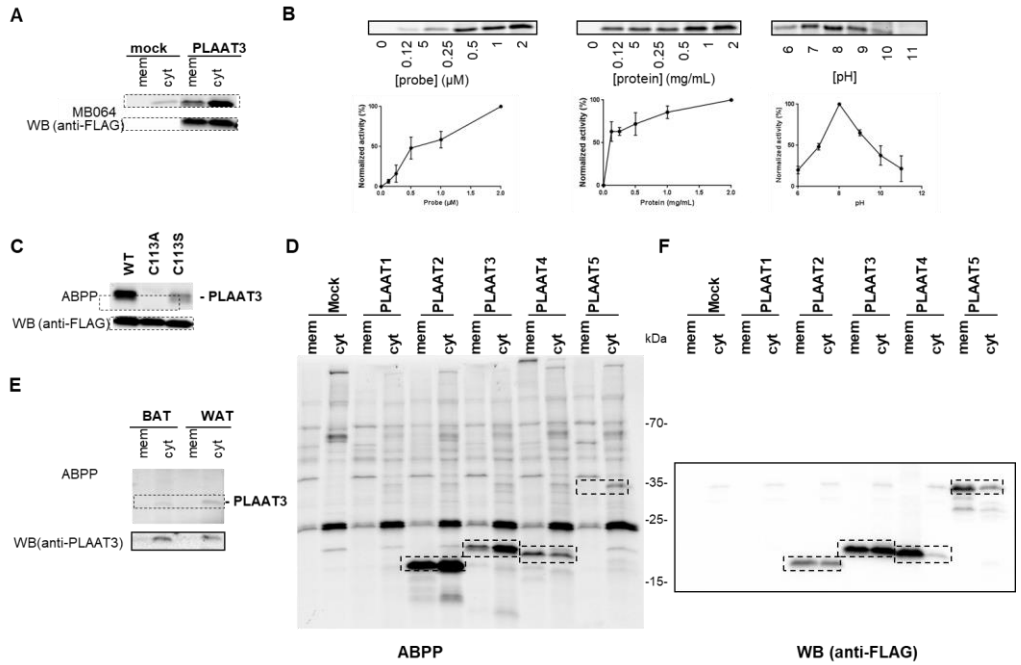

Figure 2. Characterization of MB064 as an ABP for PLAAT3. (A) ABPP using MB064 with PLAAT3 membrane (mem) or cytosol (cyt) proteome (1 mg/mL) transiently overexpressed in HEK293T cells and Western blot of the ABPP gel using an anti-FLAG antibody. (B) ABPP condition optimization for human PLAAT3 cytosol proteome using MB064. For the probe concentration test, 0.5 mg/mL protein lysate was used. For the protein concentration test, probe of 500 nM was used. (C) ABPP using MB064 with different human PLAAT3 constructs, and Western blot of the ABPP gel using an anti-FLAG antibody. (D) ABPP using probe MB064 on HRASLS protein family members (transiently overexpressed in HEK293T cells). (E) Labelling of endogenous PLAAT3 in WAT and BAT cytosol proteome by MB064, and Western blot of the ABPP gel using an anti-PLAAT3 antibody. (F) Western blot of the ABPP gel using an anti-FLAG antibody. All HRASLS family members except PLAAT1 were successfully overexpressed in HEK293T cells and the overexpressed proteins could be labelled by probe MB064.

Having identified MB064 as a suitable chemical probe targeting PLAAT3, it was decided to screen a small focused library of 50 lipase inhibitors representatively selected from a compound library as in our previous study[23] at 10 μM in a competitive ABPP format. This led to discovery of 4-(4-chlorophenyl)-2-oxo-N-phenethylbutanamide, an α-ketoamide (compound **1**) as a hit that almost completely abolished PLAAT3 labeling at 10 μM (Figure 3A).

Compound **1** was resynthesized using previously reported procedures and tested in a concentration-response ABPP assay. Compound **1** displayed a half-maximum inhibitory concentration (pIC_{50} ± SEM) of 6.0 ± 0.1 (n=3). Furthermore, it demonstrated similar activity on the other proteins of the *HRASLS*-gene family (PLAAT2, PLAAT3and PLAAT5) with a pIC_{50} in the range of 6.0-6.2 (Figure 3A and Table 1). Next, the inhibitory activity

of compound **1** was confirmed in a previously reported orthogonal biochemical fluorescence assay that uses the Green/Red Bodipy PC-A2 as a surrogate substrate (with a K_M of 7.8 ± 2.2 µM) and cytosol PLAAT3 fraction of HEK293T cells overexpressing human PLAAT3.[8] Compound **1** displayed a K_i of 80 nM (95% confidence interval CI: 72-96 nM) (Figure 3B). α-Ketoamides have previously been reported to inhibit serine hydrolases expressed in the brain.[24-27] To determine the selectivity of compound **1** on endogenously expressed serine hydrolases, a competitive ABPP experiment was performed in mouse brain proteomes using the broad-spectrum serine hydrolase ABPs, fluorophosphonate (FP)-TAMRA, and MB064. Compound **1** (10 µM) did not reduce the labeling of any proteins in mouse brain targeted by FP-TAMRA or MB064 (Figure 3C). Taken together, these results indicate that α-ketoamide **1** is a selective inhibitor of PLAAT3 and its family members.

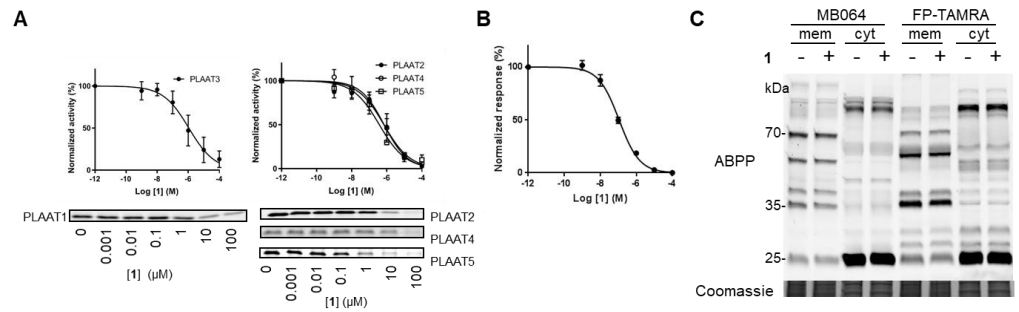

Figure 3. Discovery and biochemical characterization of compound **1**. (A) Dose-response curves for **1** on PLAAT3 (left) and other HRASLS family members, PLAAT2, PLAAT4 and PLAAT5 (right) measured by competitive ABPP using cytosol proteome prepared from transfected HEK293T cells with probe MB064. Under the curves are the corresponding ABPP gels: concentration-dependent inhibition of **1** against different proteins (n=3). (B) Dose-response curve of **1** for PLAAT3 (cytosol proteome prepared from PLAAT3 overexpressing HEK293T cells) with the PC-A2 fluorescent substrate assay (n = 3). (C) Selectivity of **1** against MB064 and FP-TAMRA in mouse brain membrane (mem) and cytosol (cyt) proteome. Coomassie was used as a protein loading control. Minus sign (-) indicates control (with DMSO), plus sign (+) indicates with compound **1** at 10 µM.

Table 1. pIC_{50} ± SEM (n = 3) of compound **1** 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

2.3 Conclusions

In conclusion, in this chapter, an activity-based protein profiling assay has been developed to label PLAAT3 in vitro, using MB064. Thereafter, this method was applied to screen inhibitors for PLAAT3. α-Ketoamides were discovered as the first selective PLAAT3 inhibitors.

2.4 Experimental procedures

Plasmids. Full-length human cDNA of PLAAT3, PLAAT1, 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 and site-directed mutagenesis was used to remove restriction by silent point mutations. Two step PCR mutagenesis was performed to substitute the active site cysteine for an alanine (A) or serine (S) in the hPLAAT3-FLAG, hPLAAT2, PLAAT4 and PLAAT5, respectively to obtain the corresponding mutants. Plasmids were isolated from transformed XL-10 Z-competent cells (Maxi Prep kit: Qiagen) and sequenced at the Leiden Genome Technology Center. Sequences were analyzed and verified (CLC Main Workbench).

Cell culture

General. HEK293T 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.[28] In brief, HEK293T cells were seeded in 15cm 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.

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 membrane and cytosolic fractions of the cell lysate were separated by ultracentrifugation (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 QubitTM 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 further 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 x g, 45 min, 4°C) the supernatant was collected as the cytosol proteome, flash frozen in liquid nitrogen and stored at -80 °C until 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.[28] 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 PLAAT2, PLAAT3 and PLAAT5 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 were 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.[29] After the ABPP assay, the proteins on the SDS-PAGE gel were transferred to a membrane using a Trans-Blot Turbo™ Transfer system (Bio-Rad). For anti-FLAG antibody, Membranes were washed with TBS (50 mM Tris, 150 mMNaCl) and blocked with 5% milk in TBST (50 mM Tris, 150 mMNaCl, 0.05% Tween 20) for 1 hat 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 XRS (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 was used for the total RNA isolation, the transferase (Thermo Scientific Maxima Reverse Transcriptase) was used for cDNA synthesis and the 2x SYBR Green qPCR Master (bimake.com) was used for qPCR

following the manufactures 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.[8] 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 mMNaCl).

Relevant concentrations of compounds were 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, scanning, 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 µM and then evaluated using GraphPad Prism® 7.

2.5 References

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2.6 Supplementary Information

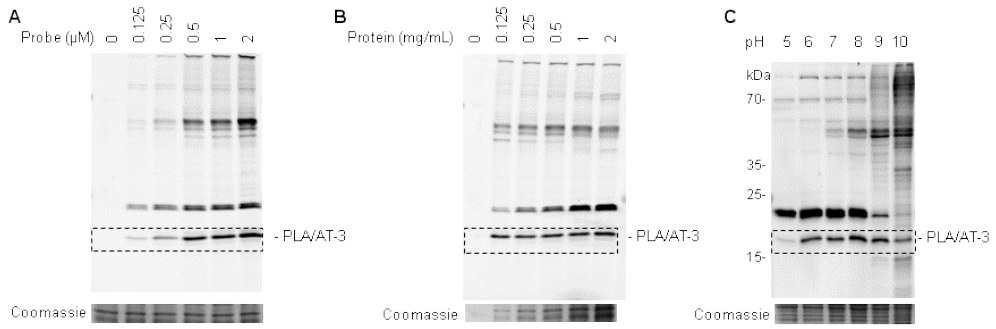

Figure S1. ABPP condition optimization for human PLAAT3 cytosol proteome using MB064. For the probe concentration test, 0.5 mg/mL protein lysate was used. For the protein concentration test, probe of 500 nM was used. Coomassie staining was used as a protein loading control. The lysis buffer with a pH 8 gives the optimal fluorescence signal.

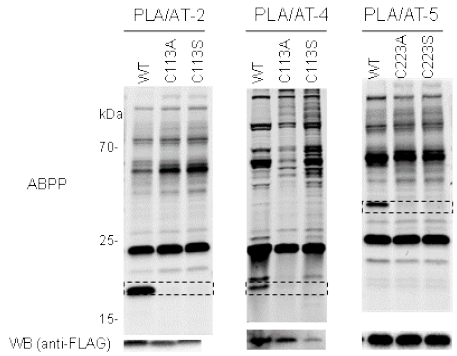

Figure S2. ABPP using MB064 (500 nM) with different enzyme constructs (cytosol fraction from transiently transfected in HEK293T cells) at the same protein concentration, respectively;and western blot of the ABPP gel using an anti-FLAG antibody.

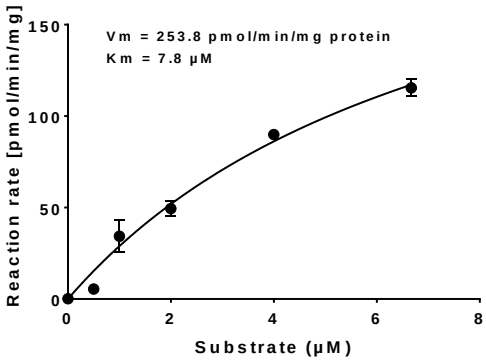

Figure S3. Phospholipase A2 activity of hPLAAT3. Red/Green BODIPY PC-A2 was used to measure the rate of reaction as a function of substrate concentration. The enzyme kinetic data V_{max} and K_m were calculated through sum-of-squares non-linear regression.

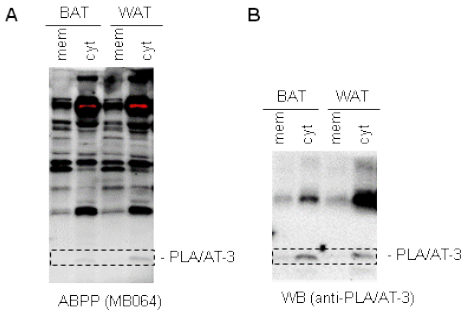

Figure S4. ABPP (A) using MB064 (2 µM) with BAT or WAT membrane (mem) or cytosol (cyt) proteome at the same protein concentration, and western blot (B) of the ABPP gel using an anti-PLAAT3 antibody.