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Development of a Selective Activity-Based Probe for Glycosylated LIPA

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ABSTRACT: Loss of LIPA activity leads to diseases such as Wolman's Disease and Cholesterol Ester Storage Disease. While it is possible to measure defects in LIPA protein levels, it is difficult to directly measure LIPA activity in cells. In order to measure LIPA activity directly we developed a LIPA specific activity based probe. LIPA is heavily glycosylated although it is unclear how glycosylation affects LIPA activity or function. Our probe is specific for a glycosylated form of LIPA in cells, although it labels purified LIPA regardless of glycosylation.

Lysosomal Acid Lipase (LIPA also known as LAL) is a lysosomal serine hydrolase responsible for the hydrolysis of cholesterol esters and triglycerides delivered to the lysosome[1]. Loss of LIPA activity leads to disease. Complete loss of function mutations are associated with Wolman's disease characterized by accumulation of triglycerides and cholesteryl esters in all tissues of the body and death within the first year of life[2]. Partial loss of activity in the enzyme results in Cholesterol Ester Storage Disease (CESD). CESD is a more benign disease characterized by hypercholesterolemia and premature atherosclerosis[3]. One mutation found to be associated with Wolman's disease is a G5R mutation in the LIPA signal peptide[4]. This mutation does not affect the activity of LIPA in cell extracts, although it does prevent the secretion of LIPA from *Spodoptera frugiperda* cells. However, LIPA activity was measured in lysates, not intact cells. Therefore, it is unclear if intracellular LIPA with the G5R mutant is active. Unfortunately, it is not possible to measure LIPA activity in cells directly since other enzymes, such as Neutral Cholesterol Ester Hydrolase 1, also hydrolyze cholesterol esters[5]. Consequently, we sought to develop a compound that reports directly on LIPA activity.

To develop a LIPA specific activity probe, we searched for reported LIPA inhibitors that could be developed into activity probes. Compound 1 was previously reported in the literature, and was used to study the consequences of LIPA inhibition (Figure 1A)[6]. We confirmed the inhibition of LIPA with compound 1 utilizing a coupled enzyme assay measuring free cholesterol production with purified LIPA, and found it had an IC50 of 121 nM. (Supporting Figure 1), however the specificity of this probe was uncharacterized[7]. Since we sought to develop a LIPA specific activity probe, we first characterized the specificity of compound 1 against other enzymes in the serine hydrolase family.

Using competitive activity based protein profiling (ABPP) we studied the selectivity of compound 1 in THP1 cells, a macrophage cell line, since LIPA has a role in athereosclerosis[8]. Cells were treated with compound 1, and after lysis unlabeled serine hydrolases were labeled with fluorophosphonate-rhodamine (FP-rho) for in gel analysis or biotin (FP-biotin) probe for mass spectrometry based analysis. Both gel based and mass spectrometry based ABPP profiling showed that compound 1 hit several off targets (Figure 1B-1C). However, as the majority of serine hydrolases did not react with compound 1, we reasoned that we may be able to use compound 1 to develop a more specific inhibitor amenable to bio-orthogonal conjugation

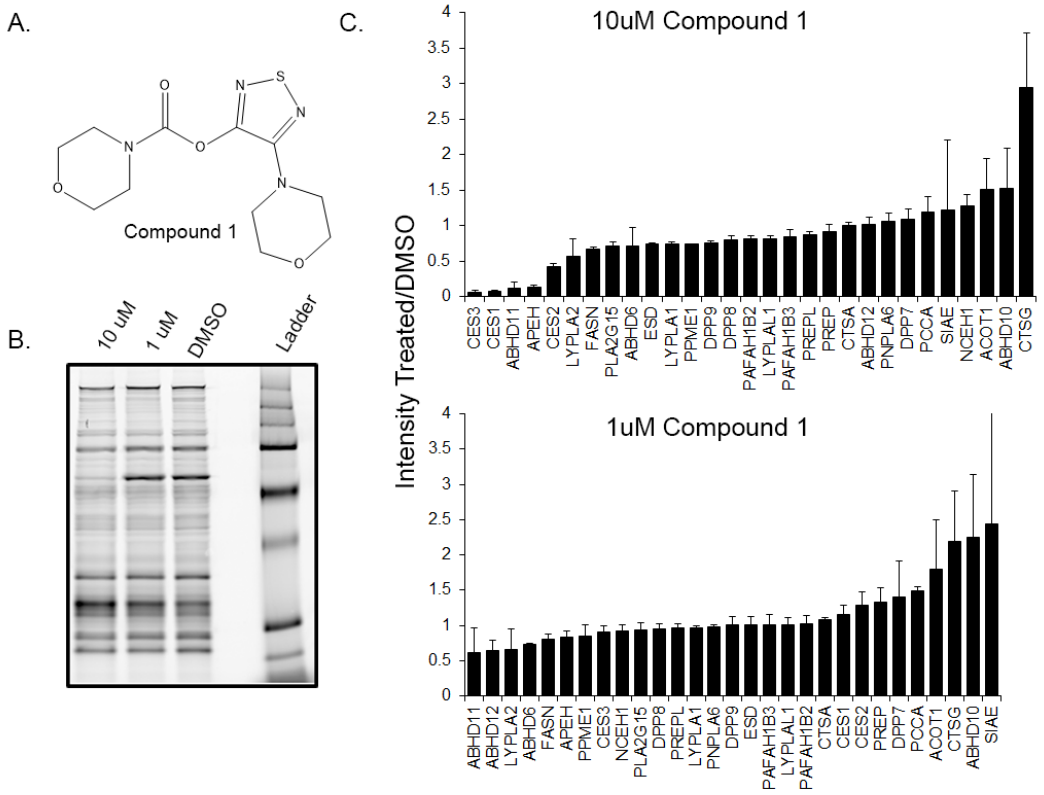


Figure 1: A. The structure of compound 1. B. Gel based activity-based protein profiling with FP-rhodamine shows compound 1 has some off targets at high concentration. C. Mass spec based activity-based protein profiling with FP-biotin confirms that compound 1 has some off targets, although it is somewhat selective amongst serine hydrolases. Bars represent the average of the protein ratios from 3 biological replicates. Error bars represent the standard error of the mean.

Based on the X-ray structure of the active form of dog gastric lipase (PDB Code: 1K8Q)[9], we built a homology model of the active form of LIPA, and modeled compound 1 into the catalytic pocket (Figure 2). From the modeled compound 1 structure, we reasoned that a long hydrophobic group may be tolerated in the meta or para position of the morpholine ring, as these

positions likely point into solvent. Hence, we designed a panel of para and meta substituted derivatives that incorporated an alkyne to allow for copper-catalyzed azide-alkyne cycloaddition (CuACC) and fluorescent labeling (Figure 3A). While derivitizations in both the meta and para positions were tolerated, substitution at the para position led to a drastic improvement in IC50 (Figure 3B). For instance, propyne substitution at the para position resulted in a much more potent IC50 than substitution at the meta position. Our most potent derivative, compound 2, had an IC50 of 1 nM (Figure 3B). This is an approximately 120 fold increase in potency over compound 1 and suggested this molecule could be a promising LIPA -ABPP probe (Supporting Figure 1).

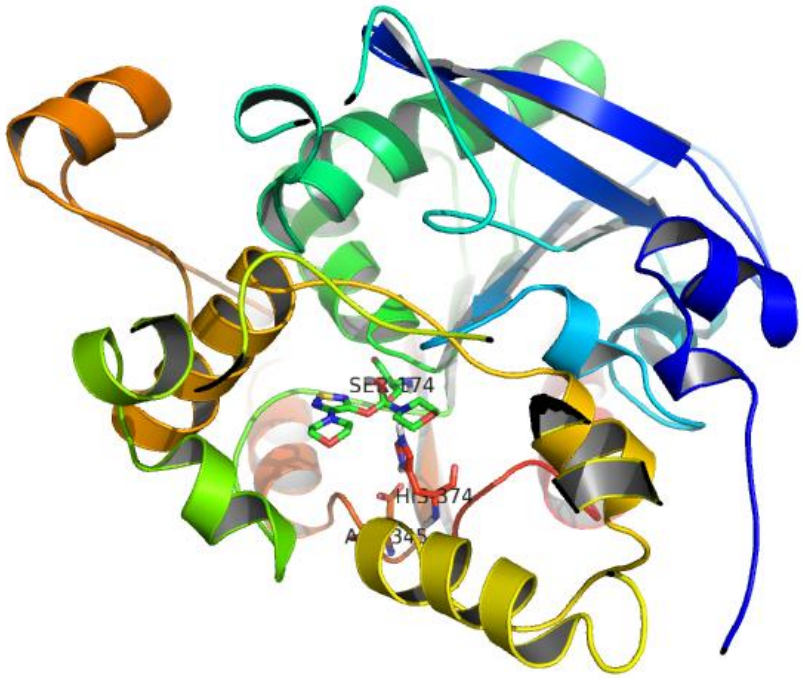
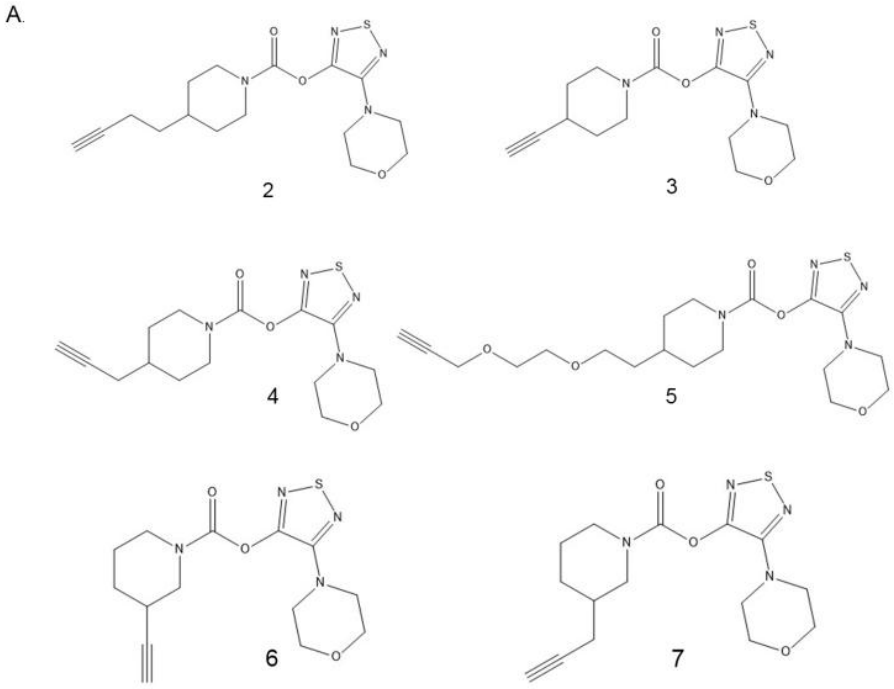


Figure 2: Compound 1 docked in homology model of LIPA. Catalytic triad residues are shown by stick model.

Next we checked if these inhibitors could bind to LIPA in cells. We incubated compound 2 at a concentration of 100 nM to 10 uM in THP1 cells overexpressing LIPA (THP1-LIPA) in order to clearly observe LIPA labeling, and conjugated compound 2 to TAMRA-azide by CuAAC post cell lysis (Figure 3C). To our surprise the fluorescence signal from compound 2 was much weaker than compound 5 for unknown reasons.



B.

Compound #	IC50
2	1 nM
3	9.6 nM
4	2.6 nM
5	2.2 nM
6	251 nM
7	943 nM

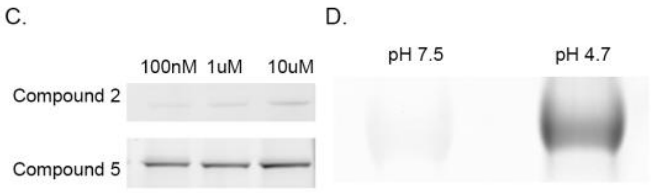


Figure 3: A-B. The structures and IC50s of compounds 2-6. C. Despite the higher affinity of compound 2 over compound 5, compound 5 yielded cleaner labeling in cells. D. Compound 5 labeled LIPA at pH 4.7, but not at pH 7.5. This is consistent with compound 5 labeling only catalytically active LIPA.

We next asked if compound 5 was a true activity probe, and tested whether compound 5 would label only enzymatically active LIPA. To do this we incubated compound 5 with recombinant LIPA at lysosomal pH, where it is known to be enzymatically active, and cytosolic pH where LIPA is enzymatically inactive (Figure 3D). Compound 5 labeled LIPA only at pH 4.7 and not at pH 7.5 indicating it is a true activity probe. We tested the cellular IC50 and selectivity of compound 5 in THP1-LIPA cells and found that the cellular IC50 of compound 5 was similar to the *in vitro* IC50 (7.2 nM vs 2.2 nM) (Figure 4A-B). Remarkably, compound 5 appears to be extremely selective in the cell line tested, and the only fluorescence band detected migrated at the molecular weight of LIPA as determined by western blot (Figured 4C). Notably, by western blot we detect 3 LIPA bands, whereas compound 5 labeling reveals only a single, high molecular weight band. LIPA is known to be heavily glycosylated and multiple molecular weight bands of LIPA, corresponding to different glycosylation states, have previously been observed[10,11]. This led us to believe that compound 5 was specific for glycosylated LIPA. To test this, we treated cells with compound 5 and incubated cell lysate with or without PNGase F, a deglycosylase (Figure 4D). The compound 5 band migrated at a lower molecular weight in the PNGase F treated sample compared to control. This indicates that compound 5 is labeling only a glycosylated form of LIPA. It should be noted that after PNGase F treatment the compound 5 band did not migrate at the lowest molecular weight observed by western blot, indicating that PNGase F induced deglycosylation is not complete under the conditions tested.

Since compound 5 only reacts with active LIPA (Figure 3D) and only a single glycosylated band of LIPA was labeled by compound 5 in cells this suggests that LIPA must be

glycosylated to be active in the cellular environment. Glycosylation might be necessary for LIPA activity if it is required for LIPA to assume an enzymatically active conformation. In order to answer this question, we attempted to generate glycosylation free LIPA by mutating the LIPA glycosites. However, we were unable to express LIPA bearing N140Q and N252Q mutations. Instead we expressed and purified partially glycosylation free LIPA bearing mutated glycosites N51Q, N80Q, and N300Q. Compound 5 was able to label partially glycosylation-free LIPA at pH 4.7, but not at pH 7.5 (Figure 4E). This suggests that glycosylation at these glycosites is not *per se* necessary for LIPA enzymatic activity. Our results with compound 5 indicate that our compound only labels LIPA at pH 4.7 and that in cells compound 5 only labels the most glycosylated LIPA glycoform. Together these results indicate that proper glycosylation of LIPA is critical for LIPA activity in cells. Glycosylation is a post translational modification that is often critical for proper cellular localization. While these data are consistent with the explanation that LIPA glycosylation is necessary for translocation to the lysosome we cannot rule out that glycosylation is necessary for LIPA activity due to another mechanism. To our knowledge this is the first time the role of glycosylation on LIPA activity has been studied in intact mammalian cells.

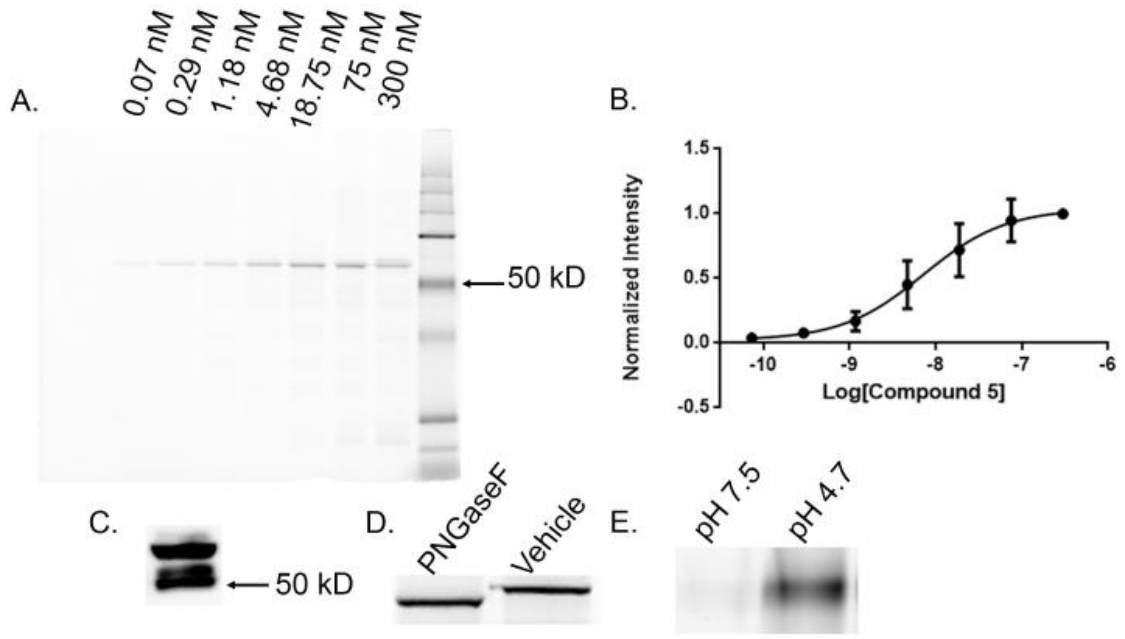


Figure 4: A-B. Compound 5 had high affinity for LIPA in cells, and by gel-analysis is selective. C. Western blot for LIPA reveal three bands for LIPA, the heaviest of which has the same mass as observed with compound 5 labeling. D. Treatment of labeled LIPA with PNGase F shows that the high molecular weight band of LIPA is glycosylated. E. Compound 5 labels partially glycosylated LIPA at pH 4.7, but not pH 7.5.

It has previously been suggested that glycosylation can affect LIPA activity, although the mechanisms by which this occurs are poorly understood[10-13]. Previous reports have suggested LIPA glycosylation could increase LIPA activity. Our results support the link between LIPA glycosylation and activity. Moreover, the development of this probe paves the way for studies that relate LIPA activity to mutations in the LIPA signal peptide or near the LIPA gene. Ultimately, the development of a LIPA specific activity probe provides a way to determine LIPA activity in different cell lines and potentially in clinical samples.

METHODS

Experimental procedures are described in detail in the supporting information.

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Author Contributions

A.G.S., W.R., and A.R. performed the experiments. S.C. oversaw probe synthesis, F.R. expressed and purified protein. All authors discussed and designed experiments. A.G.S. wrote the manuscript.

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Graphical Abstract