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Bioorthogonal proteomics of 15-hexadecynyloxyacetic acid chemical reporter reveals preferential targeting of fatty acid modified proteins and biosynthetic enzymes

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ABSTRACT

Chemical reporters are powerful tools for the detection and discovery of protein modifications following cellular labeling. The metabolism of alkyne- or azide-functionalized chemical reporters in cells can influence the efficiency and specificity of protein targeting. To evaluate the effect of degradation of chemical reporters of protein fatty acylation, we synthesized 15-hexadecynyloxyacetic acid (HDYOA), a reporter that was designed to be resistant to β-oxidation, and compared its ability to label palmitoylated proteins with an established reporter, 17-octadecynoic acid (ODYA). HDYOA was able to label known candidate S-palmitoylated proteins similarly to ODYA. Accordingly, bioorthogonal proteomic analysis demonstrated that 70% of proteins labeled with ODYA were also labeled with HDYOA. However, the proteins observed differentially in our proteomic studies suggested that a portion of ODYA protein labeling is a result of β-oxidation. In contrast, downstream enzymes involved in β-oxidation of fatty acids were not targeted by HDYOA. Since HDYOA can label S-palmitoylated proteins and is not utilized by downstream β-oxidation pathways, this fatty acid chemical reporter may be particularly useful for bioorthogonal proteomic studies in cell types metabolically skewed toward fatty acid breakdown

1. Introduction

Chemical proteomics using bioorthogonal ligation methods has revealed new posttranslational modifications on proteins as well as targets of small molecules.[1–5] The administration of azide- or alkyne-functionalized metabolites or drugs to cell lysates or living cells has enabled covalent labeling of protein targets that can be visualized or identified via copper catalyzed azide-alkyne cycloaddition (CuAAC) with fluorescent or affinity detection tags (Fig. 1A).[1–5] For protein lipidation, our laboratory and others have developed azido- or alkynyl lipid analogs to detect and identify fatty-acylated[6–9] (Fig. 1B), prenylated[10–13] and cholesterylated[14] proteins in mammalian cells as well as lipoproteins in bacteria.[15] These lipid chemical reporters are facilitating the discovery of new lipidated proteins and the characterization of their regulatory mechanisms in diverse biological contexts.

Since protein S-palmitoylation is dynamic and difficult to predict, fatty acid chemical reporters have been particularly useful for evaluating this class of protein lipidation. Protein S-palmitoylation is a posttranslational, covalent addition of a 16-carbon saturated fatty acid chain on cysteine residues via a thioester bond (Fig. 1C). This modification not only increases hydrophobicity and thus membrane affinity, but also can affect protein stability, clustering, trafficking, and activity.[16,17] For example, the oncogenic H/N-Ras small GTPases localize to the plasma membrane or the Golgi apparatus depending on their palmitoylation state, and signal through distinct pathways at each location.[18,19] In addition to H/N-Ras, understanding the dynamic S-palmitoylation of other proteins will be important. For these studies alkynyl-fatty acid reporters in conjunction with azido-fluorescent dyes allow sensitive in-gel fluorescent visualization of S-palmitoylated proteins within minutes.[6] Coupled with amino acid reporters like azidohomoalanine, dual pulse-chase labeling studies of mammalian cells with alkynyl-fatty acid reporters enables tandem imaging of S-palmitoylation and protein turnover.[20] In addition to visualizing S-palmitoylation of known proteins, fatty acid reporters and cleavable affinity tags such as azido-azo-biotin[21] allow the discovery of new S-palmitoylated proteins using bioorthogonal chemical proteomic strategies.[7,22,23] For example, our bioorthogonal profiling studies of dendritic cells with an alkynyl-palmitic acid reporter revealed S-palmitoylation of interferon-induced transmembrane protein 3 (IFITM3) is crucial for its antiviral activity against influenza virus.[7] Chemical proteomics with acyl biotin exchange provides a complementary method and has also allowed the identification of new S-palmitoylated proteins such as a brain-specific Cdc42 isoform.[24] These chemical methods are providing opportunities to discover new S-palmitoylated proteins and characterize their regulatory mechanisms.

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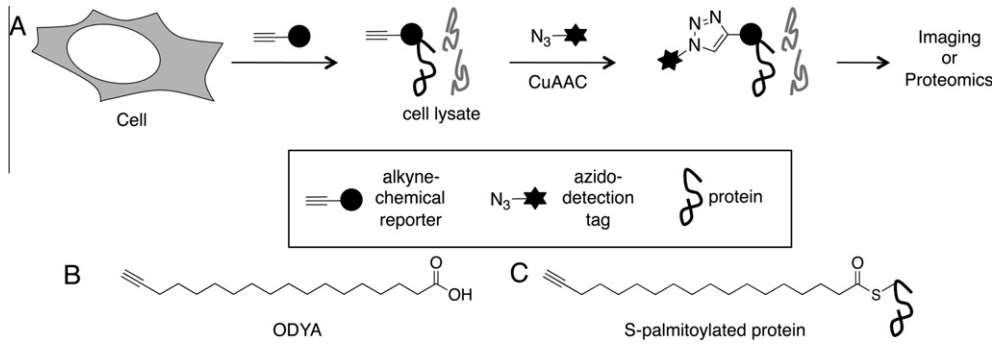


Figure 1. Metabolic incorporation of alkynyl reporters of fatty acylation. (A) Alkynyl reporters of fatty acylation are incorporated by cells and installed onto proteins at sites of palmitoylation. Subsequent reaction of cell lysates with azido detection or affinity tags via CuAAC allows visualization or proteomic identification of labeled proteins. (B) Structure of 17-octadecynoic acid (ODYA), a reporter of protein palmitoylation. (C) Protein labeling with ODYA on a cysteine residue via a thioester bond.

Alkynyl-fatty acid chemical reporters label enzymes associated with fatty acid synthesis and metabolism in addition to fatty-acylated proteins.[7,22,23] Based upon previous studies indicating that polar fatty acid analogs can be tolerated by *N*-myristoyltransferase,[25,26] we evaluated whether an oxygen-bearing analog of 17-octadecynoic acid (ODYA also termed alk-16) could differentially target fatty-acylated proteins compared to metabolic enzymes. Here, we report the synthesis and characterization of 15-hexadecynyloxyacetic acid (HDYOA), an oxy-alkynyl-fatty acid chemical reporter. The analysis of HDYOA labeling of full-length IFITM3 and a triple cysteine mutant demonstrated that this fatty acid reporter specifically labels S-palmitoylated proteins in mammalian cells. Comparative bioorthogonal proteomic analysis with ODYA revealed HDYOA preferentially labels S-palmitoylated proteins and enzymes associated with fatty acid biosynthesis but not downstream enzymes involved in the degradation of fatty acids.

2. Results

2.1. Synthesis and characterization of HDYOA

Proteomic studies from our laboratory revealed that fatty acid chemical reporters also label metabolic enzymes,[7,22] particularly in the β-oxidation pathway of fatty acid breakdown such as acyl-CoA dehydrogenase and mitochondrial trifunctional enzyme subunit β, suggesting that these reporters may be substrates for metabolic degradation. The first enzymatic step of β-oxidation of a fatty acid-CoA molecule is the reduction of the bond between the α and β carbons by acyl-CoA dehydrogenase (Fig. 2). We thus reasoned that inserting an oxygen atom at the β position might prevent reduction by this enzyme (Fig. 2). We therefore synthesized HDYOA (Scheme 1) and characterized its protein labeling to

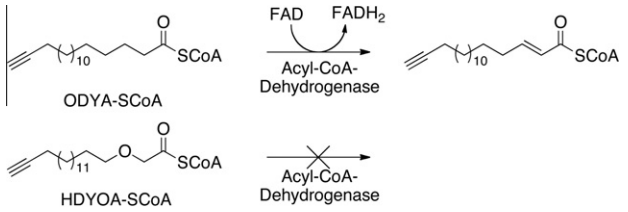
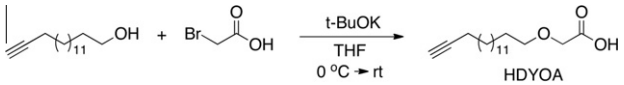


Figure 2. Rationale for the design of HDYOA. Acyl-CoA dehydrogenase initiates fatty acid catabolism through β-oxidation of fatty acyl-CoA substrates resulting in a double bond between the α and β carbons. By inserting an oxygen at the β position in HDYOA, its corresponding acyl-CoA analog should not be a substrate for acyl-CoA dehydrogenase in cells.



Scheme 1. Synthesis of HDYOA. Hexadec-15-yn-1-ol and bromoacetic acid were reacted in the presence of potassium *tert*-butoxide to generate HDYOA.

determine if this fatty acid chemical reporter can function as a substrate for protein S-palmitoylation yet differentially target enzymes involved in fatty acid metabolism compared to ODYA.

We first tested the ability of HDYOA to label proteins in live cells as compared to ODYA. Both reporters showed strong labeling of proteins as visualized by bioorthogonal ligation with azido-rhodamine via CuAAC and fluorescence gel scanning (Fig. 3A). To test whether HDYOA is able to serve as a reporter of palmitoylation, we labeled cells transfected with HA-tagged IFITM3, a S-palmitoylated protein.[7] Immunoprecipitation of IFITM3 from transfected cell lysates followed by click chemistry with azido-rhodamine, SDS–PAGE, and fluorescence gel scanning revealed that HDYOA is indeed able to label IFITM3, though somewhat less efficiently than ODYA (Fig. 3B). Nonetheless, HDYOA provides robust labeling considering that the traditional method of studying protein fatty acylation using radioactive lipids typically requires exposure times of weeks to visualize protein palmitoylation while visualization experiments with HDYOA can be completed in minutes. As expected, mutation of palmitoylated cysteine residues (C71, C72, and C105) to alanine in IFITM3 prevents its labeling by HDYOA demonstrating the specificity of this reporter (Fig. 3C). Likewise, the tetraspanin CD9, another known palmitoylated protein,[27] is also labeled by HDYOA on specific cysteine residues as labeling is abrogated for a mutant bearing alanine at all six of the known CD9 palmitoylation sites (C9, C76, C77, C85, C216, and C217) (Fig. 3D).

2.2. Bioorthogonal proteomic analysis of HDYOA protein targets

Lysates from HeLa cells labeled with ODYA or HDYOA were reacted with azido-azo-biotin[21] allowing selective retrieval of labeled proteins using streptavidin bead pulldown and sodium dithionite elution (Supplementary Fig. 1). Mass spectrometric analysis of selectively retrieved proteins revealed that both reporters were able to label known palmitoylated proteins such as calnexin, transferrin receptor and heat shock proteins (Supplementary Table 1). Of the 53 proteins that were labeled by the fatty acid reporters, 29 were found in both datasets and 10 and 14 were differentially recovered for HDYOA and ODYA, respectively (Fig. 4A). As predicted, the two fatty acid chemical reporters labeled different profiles of metabolic enzymes. HDYOA uniquely labeled acyl-CoA

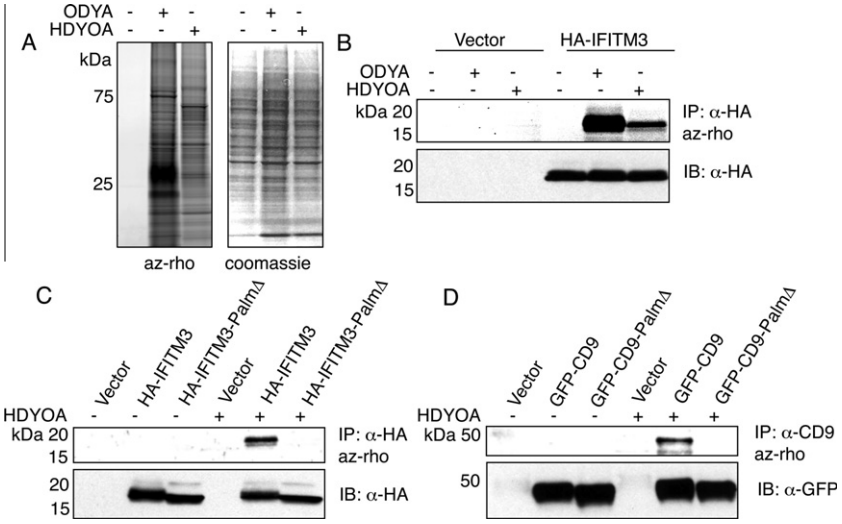


Figure 3. Comparison of protein labeling by ODYA and HDYOA. (A) HeLa cells were labeled with 50 μM ODYA or HDYOA for 2 h. Cell lysates were reacted with azido-rhodamine via CuACC and visualized by fluorescence gel scanning. Coomassie blue staining of the same gel demonstrates comparable loading. (B–D) HeLa cells in 6-well plates were transfected with plasmids encoding the indicated proteins and labeled with 50 μM ODYA or HDYOA for 2 h. Immunoprecipitated proteins were reacted with azido-rhodamine via CuAAC and visualized by fluorescence gel scanning. Western blots serve as loading controls. Cells were treated with DMSO as a solvent control in all experiments.

dehydrogenase while ODYA selectively labeled the trifunctional mitochondrial enzyme involved in subsequent steps of β-oxidation. These results suggest that while ODYA may be degraded by β-oxidation, HDYOA halts this process at the first enzymatic dehydrogenation (Fig. 4B). In support of this notion, we found that histone H4, which is not likely palmitoylated since it lacks cysteine residues, was selectively labeled by ODYA (Supplementary Table 1). These results suggest that known acetylated proteins like histone H4 may be labeled by a degradation product of ODYA. Indeed, our laboratory has demonstrated that shorter alkynyl-fatty acids can function as bioorthogonal reporters of protein acetylation.[28] Accordingly, labeling of histone H4 was not seen with HDYOA (Supplementary Table 1). Lack of HDYOA degradation may lead to a buildup of HDYOA-CoA as demonstrated by labeling of fatty acid CoA ligase (Fig. 4B, Supplementary Table 1). As such, HDYOA-CoA appears to be shuttled into other pathways such as the production of membrane lipids as seen by specific labeling of 1-acylglycerophosphocholine *O*-acyltransferase by HDYOA (Fig. 4B, Supplementary Table 1). Overall, these proteomics results demonstrate that manipulation of the structure of chemical reporters of protein palmitoylation can lead to changes in metabolic pathways targeted by these reporters while still allowing their utilization in palmitoylation pathways.

3. Discussion

Fatty acid chemical reporters provide powerful reagents to analyze the specificity of protein fatty-acylation. Previous work has demonstrated that *N*-myristoyl transferase and palmitoyl transferases have unique preferences for utilizing alkynyl-fatty acids of different lengths.[6,22] These reporters also serve as substrates for metabolic degradation as indicated by labeling and selective retrieval of mitochondrial β-oxidation enzymes seen in various proteomic studies.[7,22,23] We now demonstrate that alkynyl fatty acid reporters can be manipulated structurally such that they should not undergo breakdown by β oxidation, are shuttled to anabolic pathways, and still label S-palmitoylated proteins effectively. Indeed, HDYOA targets a different set of metabolic enzymes as compared to ODYA while both compounds label palmitoylated proteins such as IFITM3, CD9, and calnexin. The differential labeling of acyl-CoA dehydrogenase by HDYOA and trifunctional mitochondrial enzyme by ODYA, respectively, provides evidence that HDYOA is not a substrate for β-oxidation. Thus, similarly to thia-substituted fatty acids previously shown to inhibit acyl-CoA dehydrogenase,[29] HDYOA may be a useful inhibitor of fatty acid degradation in vivo.

HDYOA should also be useful for in vitro analysis of cell types metabolically skewed towards β-oxidation such as liver cells or in vivo studies of palmitoylation. To our knowledge, no live animal studies have been reported using alkynyl chemical reporters of fatty acylation, likely due to difficulties in avoiding breakdown of the reporters in the liver. HDYOA presents an exciting tool for such in vivo studies in the future. In summary, the synthesis and characterization of HDYOA provides a novel fatty acid reporter to explore protein fatty-acylation and fatty acid metabolism in physiology and disease.

4. Materials and methods

4.1. Chemicals

Synthesis of ODYA,[6] azido-rhodamine,[6] and azido-azo-biotin[21] have been described previously. All chemicals for synthesis were obtained either from Sigma–Aldrich, MP Biomedicals, Alfa Aesar, TCI, Fluka or Acros and were used as received unless otherwise noted. The silica gel used in flash column chromatography was Fisher S704 (60–200 mesh, Chromatographic Grade). Analytical thin layer chromatography (TLC) was conducted on Merck silica gel plates with fluorescent indicator on glass (5–20 μm, 60 Å) with detection by ceric ammonium molybdate. The ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AVANCE-600 spectrometer equipped with a cryoprobe. Chemical shifts are reported in δ ppm values downfield from tetramethylsilane and J values are reported in Hz. MALDI-TOF mass spectra were obtained on an Applied Biosystems Voyager-DE.

2-(Hexadec-15-yn-1-yloxy)acetic acid (HDYOA): In a flame-dried round-bottom flask under inert atmosphere hexadec-15-yn-1-ol[6] (100 mg, 0.419 mmol) and bromoacetic acid (87 mg, 0.629 mmol)

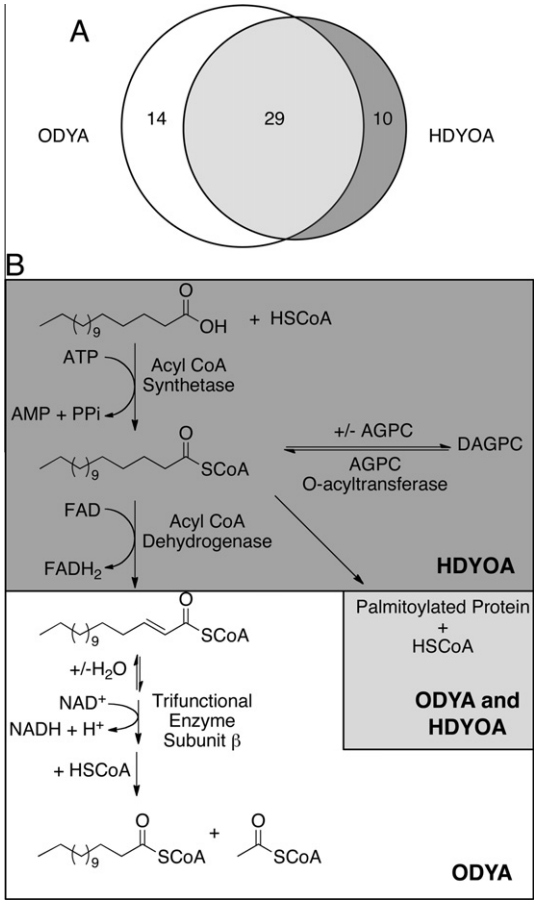


Figure 4. Proteomic comparison of proteins labeled by ODYA and HDYOA. (A, B) HeLa cells were labeled with 50 μM ODYA or HDYOA or treated with DMSO as a control for 2 h. Cell lysates were reacted with azido-azo-biotin via CuAAC and labeled proteins were enriched using streptavidin beads. Sodium dithionite treatment of beads allowed selective retrieval of labeled proteins for mass spectrometric identification. (A) Proteins identified in samples treated with ODYA and HDYOA were compared and represented in a Venn diagram. (B) Proteins identified in samples treated with ODYA and HDYOA, and the cellular pathways in which they are active. Dark grey shading indicates proteins selectively identified in HDYOA samples, lack of shading indicates proteins selectively identified in ODYA samples, and light grey shading indicates overlapping proteins identified in both samples. AGPC, acylglycerophosphocholine; DAGPC, diacylglycerophosphocholine.e.

were suspended in anhydrous THF (2 mL) at 0 °C. Potassium *tert*-butoxide (236 mg, 2.1 mmol) was added in one portion and the reaction mixture was allowed to warm to room temperature and then stirred overnight at 60 °C. The reaction was quenched with an ice-cold saturated aqueous solution of ammonium chloride and extracted with EtOAc (3 × 30 mL). The combined organic layers were dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude mixture was purified by silica gel flash chromatography (20% EtOAc/80% hexanes to 100% EtOAc) to yield HDYOA (33 mg, 30% conversion, 90% yield based on recovered starting material) as a white solid. ^{1}H NMR (Supplementary Fig. 2) (600 MHz, $CDCl_3$): δ 1.18–1.33 (m, 18H), 1.33–1.40 (m, 2H), 1.50 (quintuplet, 2H, J = 7.1), 1.59 (quintuplet, 2H, J = 7.1), 1.91 (t, 1H, J = 2.6), 2.15 (dt, 2H, J = 2.6, 7.1), 3.53 (t, 2H, J = 6.7), 4.06 (s, 2H). ^{13}C NMR (Supplementary Fig. 3) (150 MHz, $CDCl_3$): δ 18.4, 25.9, 28.5, 28.8, 29.1, 29.3, 29.4, 29.5, 29.6, 29.6, 29.6, 29.6, 29.6, 67.9, 68.0, 72.1, 84.2, 174.4. High resolution MS: 297.2 $[M+H]^+$ (Supplementary Fig. 4).

4.2. Cells, transfections, immunoprecipitations, and western blots

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (Omega Scientific) at 37 °C in a humidified atmosphere of 5% CO_2. Cloning of IFITM3 into the pCMV-HA vector and CD9 into the pEGFP-C1 vector has been described previously.[7] Cells at 90% confluence in 6-well plates were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with 2 μg plasmid per well. Immunoprecipitations were performed with 500 μg protein using anti-HA conjugated agarose (Sigma) for HA-IFITM3 or anti-CD9 (H-110, Santa Cruz Biotechnology) with protein G agarose (Roche) for GFP-CD9. Western blots were performed with anti-HA (rabbit polyclonal, Clontech) or anti-GFP (JL-8, Clontech).

4.3. Metabolic labeling, bioorthogonal ligation, and proteomics

Cells were metabolically labeled for 2 h with 50 μM ODYA or HDYOA in DMEM supplemented with 2% charcoal filtered fetal bovine serum (Omega Scientific). Labeling with media containing an equal volume of DMSO was used as a solvent control. Cells were lysed with 1% Brij Buffer (0.1 mM triethanol amine, 150 mM NaCl, 1% Brij97, pH 7.4) containing EDTA-free protease inhibitor cocktail (Roche). Bioorthogonal ligation via CuAAC with azido-rhodamine or azido-azo-biotin was performed as previously described.[7] In short, CuAAC reactions contained 100 μM azido reagent, 1 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 100 μM tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA), and 1 mM $CuSO_4{\cdot}5H_2O$. For proteomic experiments, 2.5 mg protein was used. In-gel fluorescence scanning was performed using a Typhoon 9400 imager (Amersham Biosciences) (excitation 532, 580 nm detection filter). Data presented in Supplementary Table 1 are the results of a single proteomic run. Detailed description of proteomic analysis of selectively retrieved alkyne-reporter labeled proteins with streptavidin beads and sodium dithionite elution has been previously published.[7]

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.03.062.

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