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Chemical Probes to Directly Profile Palmitoleoylation of Proteins

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Abstract

Palmitoleoylation is a unique lipid modification of proteins, where a monounsaturated fatty acid, palmitoleic acid (C16:1), is covalently attached to proteins. Wnt proteins are known to be palmitoleoylated by *cis*- 9 palmitoleate at conserved serine residues. O-palmitoleoylation plays critical roles in regulating Wnt secretion, binding to the receptors and the dynamics of Wnt signaling. Therefore, protein palmitoleoylation is important in tissue homeostasis and tumorigenesis. Chemical probes based on saturated fatty acids, such as ω -alkynyl palmitic acid (Alk-14 or Alk-C16), have been used to study Wnt palmitoleoylation. However, such probes require prior conversion to the unsaturated fatty acid by stearoyl-CoA desaturase (SCD) in cells, significantly decreased their selectivity and efficiency to study protein palmitoleoylation. We synthesized and characterized ω -alkynyl *cis*- and *trans*-palmitoleic acids (*cis*- and *trans*-Alk-14:1) as chemical probes to directly study protein palmitoleoylation. We found that *cis*-Alk-14:1 could more efficiently label Wnt proteins in cells. Interestingly, DHHC-family of palmitoyl acyltransferases can charge both saturated and unsaturated fatty acids, potentially using both as acyl donors in protein lipidation. Furthermore, proteomic analysis of protein targets labeled by these probes revealed new *cis*- and *trans*-palmitoleoylated proteins. Our studies provided new chemical tools and revealed new insights into palmitoleoylation in cell signaling.

Target proteins of monounsaturated fatty acids

We synthesized and characterized ω -alkynyl (*cis*- and *trans*-) palmitoleic acids as chemical probes to directly study monounsaturated fatty acid modified proteins. Our studies provided new chemical tools and revealed new insights into palmitoleoylation in cell. signaling.

Keywords

Palmitoleoylation; Wnt; palmitoleic acids; chemical probe

Fatty acylation is an important co-translational or posttranslational modification of proteins, where fatty acids are covalently attached to proteins through amide, ester or thioester linkages on N-terminus, serine or cysteine residues, respectively.^[1] Various fatty acyl-CoAs are intermediates of lipid biosynthesis in cells, and can be utilized as acyl donors for protein fatty acylation. To date, saturated fatty acylations, such as myristoylation (C14), palmitoylation (C16), and stearoylation (C18), have been widely studied. The lipid chain can serve as membrane binding anchors or as structural motifs, regulating protein trafficking, localization, folding and co-factor binding, playing diverse and critical roles in cell signaling.^[2] A large number of proteins have been identified to be myristoylated and palmitoylated through chemoproteomic studies.^[3] For example, myristoylation of c-Abl kinase serves as an autoinhibition switch for the kinase functions,^[4] and palmitoylation of TEAD transcription factors functions as an allosteric regulator for the transcription co-activator YAP binding,^[5] suggesting that fatty acylation plays diverse and important roles in normal physiology. Therefore, targeting protein lipidation might provide new therapeutic opportunities for diseases.

Interestingly, some proteins are uniquely acylated by monounsaturated fatty acid (MUFA), which contains a double bond in the acyl chain, giving a kink conformation of the lipid. For example, Wnt proteins are posttranslationally modified by *cis*-9 palmitoleate (C16:1⁹) at a highly conserved serine residue.^[6] Genetic and biochemical studies revealed that Porcupine (Porcn), a membrane bound O-acyl transferase (MBOAT), is the enzyme dedicated for Wnt palmitoleoylation.^[7] Palmitoleoylation facilitates Wnt secretion and binding to the Frizzled receptors^[8]. The carboxylesterase Notum, which selectively cleaves *cis*-palmitoleate group, negatively regulates Wnt activities.^[9] Taken together, palmitoleoylation is an important molecular switch, which specifically and dynamically regulates Wnt signaling, and plays critical roles in tissue homeostasis, embryonic development and tumorigenesis. In addition, small molecule inhibitors of Porcn, such as LGK974, have shown promising efficacy in clinical studies for Wnt-dependent cancers, highlighting the therapeutic importance of targeting protein palmitoleoylation.^[10]

In cells, *cis*-palmitoleoyl-CoA can be biosynthesized from palmitoyl-CoA by stearoyl-CoA desaturase-1 (SCD-1), an iron-dependent enzyme catalyzing regiospecific *cis*-9 desaturation of fatty acids.^[11] Monounsaturated fatty acid can also be uptaken from diet, which contains both *cis*- and *trans*-palmitoleate. For example, *trans*-palmitoleate, which cannot be biosynthesized in human body, is abundant in dairy food sources and has been associated with many health benefits.^[12] However, little is known whether *trans*-palmitoleate can be used as an acyl donor for protein lipidation in cells.

Currently, all the Wnt *cis*-palmitoleoylation studies rely on the saturated fatty acid reporters, such as ω -alkynyl palmitic acid (Alk-14, **1**) (Fig. 1).^[13] However, such saturated reporters require prior desaturation by SCD-1^[6b]. It is also noted that in previous proteomic studies, **1** and similar probes (17-ODYA, Alk-16) have failed to enrich and identify Wnt proteins from palmitoylated proteomes, suggesting that such probes were not specific and efficient enough to distinguish palmitoleoylation from high background of palmitoylation without antibody enrichment or proximity ligation approaches.^[3b, 14] Furthermore, these probes could not identify *trans*-palmitoleoylation of proteins. Therefore, new chemical tools are needed to

directly and efficiently label palmitoleoylated proteins. It has been shown that exogenous addition of palmitoleic acid could rescue Wnt acylation blocked by SCD inhibition, suggesting that exogenous palmitoleic acid can be converted to CoA intermediates and used as acyl donor in cells.^[15] Toward this end, we synthesized ω -alkynyl *cis*-palmitoleic acid (cAlk-14:1, **2**) and ω -alkynyl *trans*-palmitoleic acid (tAlk-14:1, **3**) as new chemical probes to directly study protein palmitoleoylation.

We first compared the metabolic labeling efficiency of unsaturated probe **2** with saturated probe **1** on mouse fibroblast L cells that stably express Wnt3a (L-Wnt3a) or vector control (control L cells). L cells or L-Wnt3a cells were treated with **1** or **2** for 6 hours. Wnt3a proteins were then immunoprecipitated (IP) by anti-Wnt3a antibody from the cell lysates, and coupled to biotin-azide using copper-catalyzed 1,3-dipolar cycloaddition reaction. The labeling efficiencies were then evaluated by streptavidin blot. Probe **1** only showed very weak signals. In contrast, **2** showed very strong labeling of Wnt3a protein at the same doses (Fig. 2A). These results confirmed that **2** is a more efficient and direct reporter for Wnt palmitoleoylation. In addition, we expressed HA-tagged wild type Wnt3a or its unacylated (S209A) mutant in HEK293A cells. The cells were then metabolically labeled with **2** for 6 hours. We found that Wnt3a (S209A) mutant cannot be labeled by the probe, confirming that the acylation site is indeed at Ser209 (Fig. 2B). We further tested the dose-dependent and time-dependent labeling of Wnt3a protein by **2**. Wnt3a acylation can be detected using as low as 10 μ M of **2**. Higher concentration of the probe led to enhanced labeling, without apparent cytotoxicity (Fig. S1A). We also observed Wnt3a labeling by **2** after 2 hours of incubation, confirming that the unsaturated probes can be readily uptaken, converted to CoA intermediates, and used as acyl donors in cells (Fig. S1B). It has been postulated that Wnt acylation is regiospecific, but it is not clear whether Wnt protein can be modified by unnatural *trans*-palmitoleic acid. We could not detect Wnt3a acylation using *trans*-palmitoleoylation probe **3**, while **2** gave robust labeling under the same condition, confirming that Wnt acylation is regiospecific for the *cis*-isoform (Fig. 2C).

Palmitoleoylation of Wnt protein is known to regulate Wnt ligand maturation and secretion.^[6b] Genetic and pharmacological inhibition of Porcn impairs Wnt ligand secretion. To test whether our chemical probes could detect acylated, secreted Wnt, we transfected HEK293 cells with HA-Wnt3a, and treated these cells with different chemical probes. We collected the conditioned media, and detected acylated Wnt3a in conditioned media by Click reaction and streptavidin blot. We found that **2** could strongly label acylated and secreted Wnt3a in the conditioned media, while **1** and **3** failed to detect acylated Wnt in the conditioned media (Fig. 2D). Similar results were observed using L-Wnt3a cells (Fig. S1C).

As Porcn is the dedicated O-acyltransferase for Wnt, the enzymatic activities of Porcn should be required for the probe labeling of Wnt3a. We overexpressed Flag-tagged Porcn in L-Wnt3a cells, and treated the cells with **2**. As expected, Wnt3a acylation levels are markedly enhanced by Porcn expression (Fig. 3A). Next, we test whether pharmacologically inhibition of Porcn can abolish Wnt3a acylation. We treated L-Wnt3a cells with a small molecule inhibitor of Porcn (WntC59, an analogue of the clinical compound LGK974),^[16] and labeled cells with **1** or **2**. Indeed, Porcn inhibition abolished Wnt3a acylation (Fig. 3B).

Taken together, **2** is an effective chemical reporter of Wnt palmitoylation, and can be utilized by O-acyltransferase Porcn as a substrate.

It has been shown that the saturated fatty acids can be converted to monounsaturated palmitoleic acid in cells by SCD-1.^[15] We speculate that SCD-1 activities are required for Wnt3a labeling by **1**, but is dispensable for **2**. We treated L-Wnt3a cells with SCD-1 inhibitor CAY10566, and labeled cells with **1** or **2**. As expected, SCD-1 inhibitor greatly blocked Wnt3a acylation labeled by **1**, while has little effects on the labeling efficiency of **2** (Fig. 3C).^[17] Furthermore, addition of exogenous *cis*-palmitoleic acid or **2**, could markedly rescue HA-Wnt3a secretion inhibited by CAY10566 (Fig. S2). Taken together, our results showed that **2** could bypass the rate-limiting desaturation of saturated fatty acid, and greatly enhance Wnt3a acylation and detection.

The saturated S-palmitoylation can be catalyzed by the DHHC-domain containing palmitoyl acyltransferases (PATs). Those enzymes could bind to acyl-CoA, and form acyl-enzyme adduct intermediates during the catalysis.^[18] It has been postulated that DHHC-proteins can transfer various acyl chains. For example, ZDHHC6 was shown to transfer C18 stearoyl group to proteins.^[19] We speculate that DHHC proteins might also be able to utilize unsaturated fatty acids as acyl donors, when palmitoleoyl-CoA is available.

It has been widely known that N-Ras protein could be palmitoylated by ZDHHC9.^[20] To test whether N-Ras fatty acylation can utilize saturated and unsaturated fatty acid, we expressed HA-N-Ras in HEK293T cells, and treated the cells with **1** or **2**. We observed that N-Ras can be modified by both probes, suggesting that S-fatty acylation of N-Ras does not have selectivity between saturated and unsaturated acyl donors (Fig. S3). The SNARE complex component Syntaxin 8 (STX8) is also palmitoylated and regulates synaptic responses in neurons.^[21] Similarly, we found that both **1** and **2** can efficiently label STX8 protein (Fig. S3). These results suggest that the PATs responsible for N-Ras or Syntaxin palmitoylation might not distinguish saturated and unsaturated fatty acids for acylation. In addition, we overexpressed 23 of mouse ZDHHC proteins in HEK293T cells and incubated cells with **1** or **2**. We found that almost all the DHHC proteins could charge both **1** and **2** (Fig. S4, S5). The labeling of PATs could be through the acyl-enzyme intermediates, or through the palmitoylation at the conserved C-terminal cysteine residues of the DHHC proteins.^[22] Nevertheless, our results suggest that most of PATs might utilize both saturated and unsaturated fatty acyl-donors. Interestingly, ZDHHC18 cannot be labeled by **2**, but can be effectively labeled by **1**. It is possible that ZDHHC18 prefers saturated fatty acylation, and detailed follow-up studies are needed to elucidate the mechanisms.

To date, Wnt proteins are the only known palmitoleoylated proteins. We speculate that other proteins could also be *cis*- or *trans*- palmitoleoylated specifically. Current methods of using **1** to label palmitoylated proteome could not effectively distinguish palmitoleoylation vs. palmitoylation. Therefore, we carried out metabolic labeling and mass spectrometry studies of palmitoleoylated proteins directly using **2** and **3**. In addition, we co-treated HEK293T cells with 50 μ M of **1** and 100 nM of SCD-1 inhibitor (CAY10566), which should block desaturation of **1**. Therefore, only saturated palmitoylation will be detected. The cell lysates were then subjected to Click chemistry using biotin-azide and enriched by streptavidin

beads. The labeled proteins were digested on beads by trypsin and analyzed by LC-MS/MS. We filtered the mass spectrometry results for proteins with >2 spectra counts in duplicate and with >5-fold enrichment over the DMSO control samples. More than 400 total proteins are putatively palmitoleoylated and labeled by **2** and **3** (Table S1). About 70% of them are overlapping with the proteins labeled by **1** (Fig. S6A), suggesting that those proteins are generally fatty acylated, and do not have specific requirement for saturated or unsaturated fatty acyl donors. Such proteins include G-proteins (GNA11, GNA13, GNAQ), SNARE proteins (SNP23 and Syntaxin), kinases (PI4K2A and PI4K2B) etc. Molecule function analysis showed that many proteins are membrane binding proteins or with transporter functions, which are consistent with the known function of protein palmitoylation in membrane association and trafficking (Fig. S6B).

More interestingly, we identified proteins which are uniquely labeled by each probe (Table 1). Most of the palmitoylated proteins identified by **1** combined with SCD-1 inhibition are consistent with previous studies, including FAM171A2 (F1712), G-protein α 12 (GNA12), LRC57, Golgi-associated protein GAPR1, DCN1-like protein 3 (DCNL3) and Glypican-1 (GPC1). In addition, unique hits labeled by **2** or **3** represent proteins which could be palmitoleoylated (Table 1). We identified Wnt11 from the proteomic hits of **2** labeling, which validated our approaches as effective ways to identify protein palmitoleoylation as all the Wnt proteins are known to be palmitoleoylated.^[23] We did not detect Wnt3a in the mass spec studies, likely due to the low abundance of the protein. We found that abhydrolase domain-containing protein 2 (ABHD2), Vesicle transport protein SFT2A (SFT2), and atlastin-3 (ATLA3) etc. are *cis*-palmitoleoylated protein. In addition, myoferlin (MYOF), transcription elongation factor B (ELOB), and ubiquitin-protein ligase AMFR (AMFR) etc. are *trans*-palmitoleoylated. These results suggest that dietary *trans*-monounsaturated fatty acid could indeed be used to modify cellular protein. Detailed studies would be needed to elucidate their molecular functions, and to determine whether *trans*-palmitoleoylation is linked to the health benefit of the dietary lipids.

In summary, we synthesized and characterized ω -alkynyl (*cis*- and *trans*-) palmitoleic acids as chemical probes to directly study protein palmitoleoylation. The labeling efficiency of **2** is much higher than the saturated fatty acid probe **1**, and does not require the rate-limiting desaturation by SCD-1 to detect *cis*-palmitoleoylation. Our probe provided an efficient chemical method to detect Wnt acylation in cells and in conditioned media, significantly improving the detection of the dynamics of Wnt signaling. Furthermore, we also observed that DHHC-family of palmitoyl acyltransferases can charge both saturated and unsaturated fatty acyl-CoAs. Therefore, it is possible that many S-acylation can have both saturated and unsaturated fatty acids, depending on the availability of the fatty acyl-CoA. Consistently, known palmitoylated protein, such as N-Ras and STX8 can indeed be palmitoleoylated. It would be interesting to further investigate whether saturated and unsaturated acylation of N-Ras could have different signaling activities. In addition, we have investigated the unnatural *trans*-palmitoleic acid, which is abundant in dairy food, including milk, cheese, and yogurt etc., and has been linked to many healthy benefits. Our results suggest that dietary *trans*-palmitoleic acid could be uptaken, converted to acyl-CoA in cells, and used as acyl donor in protein lipidation. Indeed, through proteomic studies using **3**, we found several proteins,

which are uniquely *trans*-palmitoleoylated. Further studies are needed to investigate the biological functions of protein *cis*- and *trans*-palmitoleoylation. Recently, bioorthogonal analogue of oleic acid (Alk-16:1) has also been used to probe monounsaturated fatty acylation of proteins.^[24] Together, these studies provided unique chemical tools to explore protein palmitoleoylation and other monounsaturated fatty acylations, and have revealed proteins previously unknown to be *cis*- or *trans*-palmitoleoylated, expanding our understandings of palmitoleoylation in cell signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Chemical probes to study protein palmitoleoylation.

Figure 2.

(A) **2** (*cAlk-14:1*) more effectively labels Wnt3a proteins in cells. (B) **2** labels WT, but not the S209A mutant of Wnt3a. (C) Regioselectivity of Wnt3a palmitoleoylation. Wnt3a can be labeled by **2** (*cis*-) but not **3** (*trans*-) probes. (D) **2** effectively detects secreted, palmitoleoylated Wnt3a protein in the conditioned media.

Figure 3.

(A) Expression of Porcn enhances Wnt3a labeling by **2**. (B) Porcn inhibitor C59 blocks Wnt3a labeling by both **1** and **2**. (C) SCD-1 inhibitor CAY10566 blocks Wnt3a labeling by **1**, but does not block labeling by **2**.

Table 1

Representative proteins uniquely labeled by **1**, **2** or **3** in HEK293T cells and identified by mass spectrometry. The number of total matched peptides spectra from two independent experiments is listed. SCD1 inhibitor (CAY) is used to block desaturation of **1** in cells.

Symbol	Protein name	1 + CAY	2	3
F1712	Protein FAM171A2	7/6	3/0	0/0
GNA12	G- protein subunit alpha-12	6/6	0/0	0/0
EFNA5	Ephrin-A5	5/6	0/0	0/0
LRC57	Leucine-rich repeat-containing protein 57	4/7	1/0	0/0
GAPR1	Golgi-associated plant pathogenesis-related protein 1	5/5	1/1	0/0
NDUF4	NADH dehydrogenase 1 alpha factor 4	2/6	0/1	0/0
HS904	Putative heat shock protein HSP 90-alpha A4	7/0	0/0	0/0
L12R1	Loss of heterozygosity 12 chromosomal region 1 protein	2/5	0/0	0/0
DCNL3	DCN1-like protein 3	2/5	0/0	0/0
GPC1	Glypican-1	3/3	0/0	0/0
ABHD2	Abhydrolase domain-containing protein 2	0/0	10/11	6/0
MASU1	Mitochondrial ribosomal large subunit protein 1	0/0	4/6	0/0
SFT2A	Vesicle transport protein SFT2A	0/0	5/2	1/0
ATLA3	Atlastin-3	0/2	5/2	3/0
UFM1	Ubiquitin-fold modifier 1	0/0	2/4	0/2
DDX20	RNA helicase DDX20	1/0	4/2	0/0
SPRE	Sepiapterin reductase	1/0	2/4	0/0
WNT11	Protein Wnt-11	0/0	3/2	0/0
MYOF	Myoferlin	0/0	0/0	30/7
SAFB1	Scaffold attachment factor B1	0/0	0/2	3/4
ELOB	Transcription elongation factor B polypeptide 2	1/0	0/0	4/3
OCTC	Peroxisomal carnitine O-octanoyltransferase	2/0	0/1	7/0
C9JG07	Vacuolar protein sorting-associated protein 8	0/0	0/0	3/3
IMDH1	Inosine-5'-monophosphate dehydrogenase 1	0/0	0/0	3/3
AMFR	E3 ubiquitin-protein ligase AMFR	1/0	1/1	4/2