Published in final edited form as:

ACS Chem Biol. 2013 September 20; 8(9): 1912–1917. doi:10.1021/cb400380s.

Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate

Dahvid Davda¹, Mahmoud A. El Azzouny², Christopher T.M.B. Tom¹, Jeannie L. Hernandez², Jaimeen D. Majmudar², Robert T. Kennedy^{2,3}, and Brent R. Martin^{1,2,*}

¹Program in Chemical Biology, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109, USA

²Department of Chemistry, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109, USA

³Department of Pharmacology, University of Michigan, 930 N. University Ave., Ann Arbor, MI 48109, USA

Abstract

2-bromohexadecanoic acid, or 2-bromopalmitate, was introduced nearly 50 years ago as a non-selective inhibitor of lipid metabolism. More recently, 2-bromopalmitate re-emerged as a general inhibitor of protein S-palmitoylation. Here, we investigate the cellular targets of 2-bromopalmitate through the synthesis and application of click-enabled analogues. In cells, 2-bromopalmitate is converted to 2-bromopalmitoyl-CoA, although less efficiently than free palmitate. Once conjugated to CoA, probe reactivity is dramatically enhanced. Importantly, both 2-bromopalmitate and 2-bromopalmitoyl-CoA label DHHC palmitoyl acyl transferases (PATs), the enzymes that catalyze protein S-palmitoylation. Mass spectrometry analysis of enriched 2-bromopalmitate targets identified PAT enzymes, transporters, and many palmitoylated proteins, with no observed preference for CoA-dependent enzymes. These data question whether 2-bromopalmitate (or 2-bromopalmitoyl-CoA) blocks S-palmitoylation by inhibiting protein acyl transferases, or by blocking palmitate incorporation by direct covalent competition. Overall, these findings highlight the promiscuous reactivity of 2BP, and validate clickable 2BP analogues as activity-based probes of diverse membrane associated enzymes.

INTRODUCTION

2-bromopalmitate (2BP) is an irreversible inhibitor of many membrane-associated enzymes¹. It was initially reported as an inhibitor of β -oxidation^{2,3}, but later shown to inhibit mono-, di-, and triacylglycerol transferases, fatty acyl CoA ligase, glycerol-3-phosphate acyltransferase, as well as non-lipid processing enzymes such as NADPH cytochrome-C reductase and glucose-6-phosphatase at sub-millimolar concentrations¹. Importantly, each of these enzymes has cysteine residues in or near the enzyme active site, suggesting α -halo-carbonyl electrophilic alkylation mediates the observed irreversible inhibition. This reactivity was later confirmed by labeling rat liver fractions with millimolar concentrations of 1-[¹⁴C],2-bromopalmitate¹. After separation by SDS-PAGE and autoradiography, radioactivity was detected across many unique proteins, highlighting the promiscuous reactivity and potential issues associated with this non-specific covalent inhibitor.

*Correspondence should be sent to brentm@umich.edu.

Supporting Information Available: This material is available free of charge via the Internet at <http://pubs.acs.org>.

Despite these issues, 2BP was later shown to block the S-palmitoylation and microdomain recruitment of the Src-family kinases Lck and Fyn⁴. Inhibition with 100 μ M 2BP attenuated Jurkat T-cell calcium activation and blocked tyrosine phosphorylation of LAT, PLC- γ , ZAP-70, and Vav⁴. This finding established 2BP as the only pharmacological tool to block protein S-palmitoylation. Over the last decade, 2BP has become deeply rooted in the palmitoylation field, often referenced as a selective inhibitor of protein S-palmitoylation. Indeed, many studies have used 2BP-induced phenotypes as evidence of the importance of palmitoylation in parasitic infection⁵, differentiation⁶, and various other cellular phenotypes⁷.

2BP inhibition is thought to block protein palmitoylation by inhibiting a family of conserved protein acyl transferases (PATs)⁸. Mammals express 23 distinct PAT enzymes that are presumed to regulate the profile of palmitoylated proteins, either by PAT localization, protein interactions, or active site selectivity^{7, 8}. Knockdown of specific PAT enzymes reduces palmitoylation of select substrates. For example, a hypomorphic gene-trap mouse model of DHHC5 demonstrates reduced flotillin-2 palmitoylation, disrupted stem cell differentiation, and defective hippocampal-dependent learning⁹. Other genetic models of PAT enzymes reveal a wide array of phenotypes in cancer, neurodegeneration, hair loss, and amyloidosis⁷. Similarly, overexpression of certain PATs are implicated in cancer progression, malignancy, and metastasis⁷. Given the array of novel biology regulated by PAT enzymes, selective pharmacological reagents are critical for advancing our basic understanding of protein palmitoylation in disease.

Each PAT enzyme contains a highly conserved cysteine-rich domain anchored by the four amino acid Asp-His-His-Cys (DHHC) motif. Mutation of the DHHC-cysteine residue to serine abolishes enzyme activity, suggesting this cysteine is the catalytic nucleophile and site of acyl-transfer^{10, 11}. Addition of palmitoyl-CoA to purified, detergent solubilized PAT enzymes induces auto-palmitoylation and formation of the enzyme-acyl-intermediate¹², which then transfers the palmitoyl group to cysteine residue on the substrate. *In vitro*, 2BP covalently blocks the formation of the PAT acyl-intermediate (IC₅₀ of $\sim$ 10 μ M)¹². Similarly, GAP43-YFP plasma membrane localization is inhibited in live cells by 2BP at nearly the same potency (IC₅₀ = 14.9 μ M)¹³. These experiments strongly suggest DHHC proteins are unlikely to require a CoA conjugate for inhibition.

Bioorthogonal alkyne and azide palmitate analogues have been introduced for metabolic labeling and detection of native sites of protein palmitoylation^{14, 15}. This approach uses the endogenous palmitoylation machinery to covalently tag palmitoylated proteins, which are then labeled using Cu(I) catalyzed click chemistry to azide or alkyne-linked reporters. The successful metabolic incorporation of these analogues demonstrates the broad tolerance of minor terminal acyl modifications in palmitoylation, and suggests α -brominated analogues may be useful mechanistic probes to profile the cellular targets of 2BP alkylation.

Historically, 2BP was recognized as a non-selective inhibitor of lipid metabolism¹. Over the last decade, the promiscuity of 2BP has been overshadowed by significant need for pharmacological tools to study protein palmitoylation. Here, we present the synthesis and evaluation of a click-enabled 2BP analogue and the corresponding CoA conjugate to explore the targets and mechanism of 2BP inhibition in cells, and annotate the consequences of promiscuous 2BP inhibition in palmitoylation analysis.

RESULTS AND DISCUSSION

Given the broad use of 2BP as a palmitoylation inhibitor, we sought to test if this scaffold could be useful as an activity-based probe for DHHC PAT enzymes. Such probes would

covalently label endogenous targets for click chemistry conjugation to alkyne-linked reporters, including fluorescent dyes for gel-based detection, or biotin for mass spectrometry annotation (Figure 1A). Because of the generic scaffold and electrophilic reactive group, we anticipated this approach would likely label a profile of proteins involved in lipid metabolism. The ω -azido analogue of 2BP (2BPN₃) was synthesized and added directly to the growth media of human 293T cells at varying concentrations. After 1 hour, the cells were harvested and lysed for copper-catalyzed azide-alkyne cycloaddition to rhodamine-alkyne for gel-based detection. Membrane fractions demonstrated significant contrast and dose-dependent labeling of 2BPN₃ (Figure 1B). Higher concentrations led to enhanced labeling (Supplementary Figure 1), but also pronounced cytotoxicity. In contrast, analysis of soluble fractions was complicated by non-specific, alkyne-dependent background (Supplementary Figure 2). Copper-free strain-promoted cycloaddition with rhodamine-linked aza-dibenzocyclooctyne (DIBAC)¹⁶ showed dramatically enhanced labeling, but did little to improve the overall contrast (Supplementary Figure 3). These findings corroborate recent reports quantifying thiol cross-reactivity of strain-promoted cycloadditions¹⁷.

Surprisingly, 2BPN₃ labeling occurs within 5 minutes, and gradually increases until ~60 minutes (Figure 1C). This rapid labeling suggests direct action of the native probe, but also may involve metabolic activation to the CoA conjugate. Based on these results, we selected 50 μ M 2BPN₃ for 1 hour as an effective protocol for labeling live cells. These conditions are consistent with cell-based experiments of palmitoylation inhibition and the reported IC₅₀ for PAT inhibition of 10 – 15 μ M¹³. In cells, pre-incubation with 50 μ M 2BP reduced, but did not eliminate 2BPN₃ metabolic labeling (Figure 1D). *In vitro*, higher 2BP concentrations could effectively compete with 2BPN₃ (Supplementary Figure 4), suggesting poor cellular uptake and distribution in live cells. Moreover, 2BPN₃ addition reduced but did not eliminate metabolic incorporation of 17-octadecynoic acid (17-ODYA), suggesting a significant fraction of protein palmitoylation is highly stable or non-enzymatic (Supplementary Figure 5).

2BP is believed to undergo activation to the CoA intermediate in live cells, which then directs reactivity towards CoA-dependent enzymes. Metabolic labeling with 2BPN₃ is ~10-fold less efficient than *in vitro* labeling (Figure 2A), but the profile of labeled proteins appears largely similar. This discrepancy may be due to insufficient probe solubility and uptake in culture. In order to establish metabolic activation of 2BP to the CoA conjugate, cells were incubated with 2BP or palmitic acid for 30 minutes, followed by extraction, chromatographic separation, and high-resolution mass spectrometry analysis (Figure 2B). Synthetic 2BP-CoA was shown to ionize similarly (99.8 $\pm$ 0.3%, standard error) to palmitoyl-CoA across a series of dilutions (Supplementary Figure 6). Basal palmitoyl-CoA levels were measured as 250 $\pm$ 31 pmol/mg of protein, yet were elevated 3.2-fold (805 $\pm$ 101 pmol/mg) after a 30 minute incubation with of 50 mM palmitic acid. Addition of 50 μ M 2BP led to the formation of 36 $\pm$ 4 pmol/mg of 2BP-CoA, but had no major effect on palmitoyl-CoA (205 $\pm$ 17 pmol/mg). 2BP-CoA was nearly 6-fold lower than endogenous levels of palmitoyl-CoA, which likely competes with 2BP-CoA for access to CoA processing enzymes. These data confirm 2BP metabolic conversion in live cells to the CoA conjugate within 30 minutes, although at reduced efficiency as compared to palmitoyl-CoA.

Next, a 2BPN₃-CoA conjugate was synthesized for analysis in cell lysates. 2BPN₃-CoA (cLogP = 4.4) is predicted to be much more water-soluble than 2BPN₃ (cLogP = 7.7). Furthermore, 2BP is negatively charged at physiological pH, which increases the electron density and delocalization at the carboxylate and reduces the electrophilic character of the α -carbon. Thioesterification is predicted to reduce the delocalization and enhance the carbonyl dipole to promote α -substitution, resulting in improved probe reactivity. Experimentally, the CoA conjugate achieved equivalent labeling with 10-fold less probe (Figure 2C), and

unexpectedly labeled a similar profile of targets as the free acid. Furthermore, 2BPN₃ and 2BPN₃-CoA react with different targets than 2-iodoacetamide-rhodamine (Figure 2D). Whether the enhanced reactivity of 2BPN₃-CoA is a function of the CoA motif, or a manifestation of the thioester linkage remains to be investigated. Overall, we observe that 2BP is coupled to CoA in cells at a reduced efficiency, yet the conjugate is exceptionally reactive.

The DHHC PAT family contains a cysteine-rich domain with 7 highly conserved cysteines, including the cysteine present in the Asp-His-His-Cys (DHHC) catalytic motif⁸. Mutation of the conserved DHHC cysteine to serine (DHHS) abolishes acyl-transferase activity, and prevents palmitoyl-CoA dependent formation of the enzyme-acyl intermediate^{10, 11}. Other residues may also be involved in catalysis, potentially by acyl-transfer between other active site catalytic cysteines. To explore the mechanism of DHHC catalysis, 2BPN₃ was added to cells over-expressing epitope-tagged DHHC2 or DHHS2 (C157S), and conjugated to rhodamine-alkyne for gel-based analysis. DHHC2 was efficiently labeled in transfected cells (Figure 3A), but labeling of the catalytic dead DHHS2 mutant was nearly abolished. DHHC2 labeling was not affected by hydroxylamine addition, demonstrating the absence of the acyl-intermediate and formation of the covalent alkylation product. Further detailed analysis of selected proximal cysteines (C146S, C149S, C163S) showed reduced 2BPN₃ labeling (Supplementary Figure 7). These results confirm that other cysteine residues are functionally important in the maturation and/or mechanism of DHHC PAT-catalyzed protein palmitoylation¹⁸. Importantly, these results reinforce existing evidence for more complex catalysis in the DHHC PAT active site¹⁸, potentially through multiple reactive thiolates. Finally, these experiments validate 2BPN₃ as an activity-based probe for select DHHC PAT enzymes, which may be useful for competitive assays to profile active site occupancy. Indeed, transfection studies show robust 2BPN₃ alkylation of six of nine tested epitope-tagged DHHC PATs (Supplementary Figure 8).

Metabolic conversion of 2BP to 2BP-CoA is thought to guide inhibition to CoA-dependent enzymes. To examine the role of CoA in DHHC PAT inhibition, membrane lysates were prepared from cells overexpressing DHHC2 and DHHS2, and labeled *in vitro* with 2BPN₃ or 2BPN₃-CoA (Figure 3B). Similar to metabolic labeling, *in vitro* labeling showed DHHC2 reactivity, but not DHHS2. Importantly, both 2BPN₃ and 2BPN₃-CoA demonstrate activity-dependent labeling of DHHC2, although at different optimal probe concentrations. These results corroborate previous reports that DHHC2 is directly inhibited by 2BP^{12, 19}, and suggests CoA conjugation accelerates inhibition without altering the profile of labeled targets. Furthermore, these findings confirm the predominant reactivity of the catalytic DHHC cysteine over other proximal cysteine residues.

In order to annotate the targets of 2BPN₃, cells were metabolically labeled with 2BP or 2BPN₃ for 1 hour. After lysis, membrane proteomes were conjugated to biotin-alkyne for streptavidin enrichment, trypsin digestion, and mass spectrometry analysis. Biotin-alkyne conjugation required careful optimization of labeling concentrations to reduce alkyne-dependent non-specific reactions (Supplementary Figure 9). Mass spectrometry results were filtered to include proteins with ≥ 2 average spectral counts, identified in 2 of 3 replicates, and ≥ 5-fold enrichment as compared to the 2BP controls. Approximately 450 protein targets of 2BPN₃ were annotated (Supplementary Table 1), including carriers, transporters, and channels. The most frequently identified targets in 293T cells of 2BPN₃ were the voltage-dependent anion channel, SERCA Ca²⁺ ATPase, heme oxygenase 2, and the palmitoylated protein CKAP4, all validated palmitoylated proteins (Table 1)²⁰. Importantly, five palmitoyl transferases were annotated, including DHHC5, DHHC6, DHHC7, DHHC17, and DHHC20. In addition, another 9 PAT enzymes were identified that failed to pass the stringent thresholds, likely due to their low abundance (Supplementary Table 2). This list

does not include DHHC1, DHHC8, and DHHC23, which similarly failed to label with 2BPN₃ when overexpressed in 293T cells (Supplementary Figure 8). While this enzyme family is broadly inhibited by 2BP, it is by no means privileged or selective. Early reports demonstrated that 2BP inhibition of carnitine palmitoyl transferase activity blocked mitochondrial long-chain fatty acid oxidation in cells^{3, 21}. Our results confirm that CPT1 is indeed labeled with 2BPN₃ in cells, suggesting broad disruption of cellular lipid metabolism. Other identified targets include lipid-modifying enzymes, detoxifying enzymes, regulators of redox stress. The surprising breadth of targets demonstrates the broad reactivity of this promiscuous inhibitor in cells.

Importantly, a significant fraction of the metabolically labeled targets are themselves palmitoylated proteins, such as MTDH, HRAS, GNAI2, and FAM108B¹⁵. Further investigation of validated palmitoylated proteins showed robust 2BPN₃ labeling of the unannotated serine hydrolase ABHD16A (ABHGA), EF-hand calcium-binding domain-containing protein 14 (EFCAB14), FAM108A, KIAA0152, and Thioredoxin-related transmembrane protein 1 (TXNDC1) (Supplementary Figure 10 and 11). FAM108A is palmitoylated at 5 N-terminal cysteines and is readily labeled by 2BPN₃ in cells. Deletion of the N-terminal 19 residues blocked 2BPN₃ labeling, despite the presence of 7 additional cysteines present in the remaining 291 amino acids (Supplementary Figure 11). In addition, 2BP labeling of ABHD16A did not reduce fluorophosphonate reactivity (Supplementary Figure 12), suggesting 2BP alkylation occurs outside of the conserved α/β -hydrolase active site. Even though they label with 17-ODYA²², neither H-Ras or H-Ras-(G12V) labeled effectively with 2BPN₃ when over-expressed (Supplementary Figure 13). Based on these findings, we propose that 2BPN₃ labeling of annotated palmitoylated proteins may instead highlight sites of non-enzymatic palmitoylation, such as activated thiol residues that preferentially react with palmitoyl-CoA by thioester exchange. Thus, these labeled proteins may represent a pool of DHHC-independent palmitoylated proteins. Indeed, G α_{11} is reported to undergo auto-acylation at sites of palmitoylation by direct, non-enzymatic, thioester exchange with palmitoyl-CoA²³. It is therefore not surprising to find the homologues GNAI2 and GNAI3 as targets of 2BP in cells.

Farnesylated Ras-family GTPases are carboxymethylated at their C-termini, which acts to reduce charge repulsion²⁴ and enhance membrane anchoring. 2BP alkylation products yield a negatively charged free carboxylate, which may itself attenuate membrane association. Accordingly, our data supports at least two modes of palmitoylation inhibition by 2BP. First, 2BP can block palmitoylation of select proteins both by inhibiting DHHC PATs, but secondly, 2BP can also directly compete for palmitoylation at select cysteine residues. The functional consequences of each mechanism are presumably very different, since some proteins are left un-palmitoylated, but others are irreversibly alkylated. Importantly, 2BP may block incorporation of [³H]-palmitate onto palmitoylated proteins, which superficially suggests a reduction in palmitoylation, but may in reality report alkylated by 2BP at the site of palmitoylation. Future experiments will examine the competition between 2BP and palmitoylation to shed light on the predicted role of non-enzymatic palmitoylation.

Overall, we present click-enabled activity-based probes for profiling the targets of 2BP inhibition. The probes preferentially label the active site of DHHC PATs, but similarly label hundreds of other proteins, including transporters, channels, enzymes, and chaperones. Nonetheless, 2BPN₃ labeling reports the active state of DHHC PATs, and thus may be useful in inhibitor discovery or selectivity profiling in DHHC over-expressing cells. Importantly, 2BP has been used in nearly every paper in the last 15 years studying protein palmitoylation. In light of this study, and a recent report describing ω -alkynyl 2BP analogues¹⁹, 2BP is clearly a non-selective probe with many targets beyond palmitoyl transferases. This finding has broad implications in the palmitoylation field, given the

widespread use of 2BP in hundreds of published reports. Clearly, greater caution should be used when interpreting the phenotypic consequences of 2BP-inhibition in cell-based experiments.

METHODS

Synthetic methods, materials and methods are included in the supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Z. Tickner (U. Michigan) for research assistance and C. Fierke (U. Michigan) for helpful discussions. Funding is provided by the University of Michigan Rackham Merit Fellowship (D.D.), the National Science Foundation Alliance for Graduate Education and the Professorate (J.L.H.), the National Institutes of Health R00 CA151460 (B.R.M.), R37 DK046960 (R.T.K.), Core Services supported by DK097153, and the University of Michigan.

REFERENCES

1. Coleman RA, Rao P, Fogelson RJ, Bardes ESG. 2-Bromopalmitoyl-CoA and 2-bromopalmitate: Promiscuous inhibitors of membrane-bound enzymes. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism*. 1992; 1125:203–209.
2. Burges RA, Butt WD, Baggaley A. Some effects of alphabromopalmitate, an inhibitor of fatty acid oxidation, on carbohydrate metabolism in the rat. *Biochem J*. 1968; 109:38P–39P.
3. Chase JF, Tubbs PK. Specific inhibition of mitochondrial fatty acid oxidation by 2-bromopalmitate and its coenzyme A and carnitine esters. *Biochem J*. 1972; 129:55–65. [PubMed: 4646779]
4. Webb Y, Hermida-Matsumoto L, Resh MD. Inhibition of Protein Palmitoylation, Raft Localization, and T Cell Signaling by 2-Bromopalmitate and Polyunsaturated Fatty Acids. *J. Biol. Chem*. 2000; 275:261–270. [PubMed: 10617614]
5. Jones, Matthew L.; Collins, Mark O.; Goulding, D.; Choudhary, Jyoti S.; Rayner, Julian C. Analysis of Protein Palmitoylation Reveals a Pervasive Role in Plasmodium Development and Pathogenesis. *Cell Host & Microbe*. 2012; 12:246–258. [PubMed: 22901544]
6. Leong WF, Zhou T, Lim GL, Li B. Protein palmitoylation regulates osteoblast differentiation through BMP-induced osterix expression. *PLoS One*. 2009; 4:e4135. [PubMed: 19125191]
7. Tom CT, Martin BR. Fat chance! Getting a grip on a slippery modification. *ACS Chem Biol*. 2013; 8:46–57. [PubMed: 23215442]
8. Fukata M, Fukata Y, Adesnik H, Nicoll RA, Brecht DS. Identification of PSD-95 palmitoylating enzymes. *Neuron*. 2004; 44:987–996. [PubMed: 15603741]
9. Li Y, Martin BR, Cravatt BF, Hofmann SL. DHHC5 protein palmitoylates flotillin-2 and is rapidly degraded on induction of neuronal differentiation in cultured cells. *J Biol Chem*. 2012; 287:523–530. [PubMed: 22081607]
10. Roth AF, Feng Y, Chen L, Davis NG. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. *The Journal of Cell Biology*. 2002; 159:23–28. [PubMed: 12370247]
11. Lobo S, Greentree WK, Linder ME, Deschenes RJ. Identification of a Ras palmitoyltransferase in *Saccharomyces cerevisiae*. *J Biol Chem*. 2002; 277:41268–41273. [PubMed: 12193598]
12. Jennings BC, Nadolski MJ, Ling Y, Baker MB, Harrison ML, Deschenes RJ, Linder ME. 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b]thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro. *J. Lipid Res*. 2009; 50:233–242. [PubMed: 18827284]
13. Mikic, I.; Planey, S.; Zhang, J.; Ceballos, C.; Seron, T.; von Massenbach, B.; Watson, R.; Callaway, S.; McDonough, PM.; Price, JH.; Hunter, E.; Zacharias, D. A Live Cell, Image-Based Approach to Understanding the Enzymology and Pharmacology of 2-Bromopalmitate and Palmitoylation. In: James, I., editor. *Methods in Enzymology*. Academic Press; 2006. p. 150-187.

14. Hang HC, Geutjes EJ, Grotenbreg G, Pollington AM, Bijlmakers MJ, Ploegh HL. Chemical probes for the rapid detection of Fatty-acylated proteins in Mammalian cells. *J Am Chem Soc.* 2007; 129:2744–2745. [PubMed: 17305342]
15. Martin BR, Cravatt BF. Large-scale profiling of protein palmitoylation in mammalian cells. *Nat Methods.* 2009; 6:135–138. [PubMed: 19137006]
16. Debets MF, van Berkel SS, Schoffelen S, Rutjes FP, van Hest JC, van Delft FL. Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+2) cycloaddition. *Chem Commun (Camb).* 2010; 46:97–99. [PubMed: 20024305]
17. van der Linden WA, Li N, Hoogendoorn S, Ruben M, Verdoes M, Guo J, Boons GJ, van der Marel GA, Florea BI, Overkleeft HS. Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: a comparative study. *Bioorg Med Chem.* 2012; 20:662–666. [PubMed: 21757357]
18. Mitchell DA, Hamel LD, Ishizuka K, Mitchell G, Schaefer LM, Deschenes RJ. The Erf4 subunit of the yeast Ras palmitoyl acyltransferase is required for stability of the Acyl-Erf2 intermediate and palmitoyl transfer to a Ras2 substrate. *J Biol Chem.* 2012; 287:34337–34348. [PubMed: 22904317]
19. Zheng B, DeRan M, Li X, Liao X, Fukata M, Wu X. 2-Bromopalmitate analogues as activity-based probes to explore palmitoyl acyltransferases. *J Am Chem Soc.* 2013; 135:7082–7085. [PubMed: 23631516]
20. Kang R, Wan J, Arstikaitis P, Takahashi H, Huang K, Bailey AO, Thompson JX, Roth AF, Drisdel RC, Mastro R, Green WN, Yates Iii JR, Davis NG, El-Husseini A. Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature.* 2008; 456:904–909. [PubMed: 19092927]
21. Buechler KF, Lowenstein JM. The involvement of carnitine intermediates in peroxisomal fatty acid oxidation: a study with 2-bromofatty acids. *Arch Biochem Biophys.* 1990; 281:233–238. [PubMed: 2393299]
22. Martin BR, Wang C, Adibekian A, Tully SE, Cravatt BF. Global profiling of dynamic protein palmitoylation. *Nat Methods.* 2012; 9:84–89. [PubMed: 22056678]
23. Duncan JA, Gilman AG. Autoacylation of G Protein alpha Subunits. *J. Biol. Chem.* 1996; 271:23594–23600. [PubMed: 8798571]
24. Philips MR, Pillinger MH, Staud R, Volker C, Rosenfeld MG, Weissmann G, Stock JB. Carboxyl methylation of Ras-related proteins during signal transduction in neutrophils. *Science.* 1993; 259:977–980. [PubMed: 8438158]

Figure 1. Metabolic labeling with 2BPN3 allows click-enabled detection of cellular targets

(A) Schematic of 2BPN₃ alkylation of cysteine thiols on proteins by attack of the α-halo-carbonyl group, followed by click chemistry detection of labeled proteins. (B) Concentration-dependent metabolic labeling with 2BPN₃ in 293T cells. (C) Time-dependent metabolic labeling with 2BPN₃ in 293T cells. (D) Pre-incubation with 2BP attenuated 2BPN₃ labeling at higher concentrations. Each inhibitor was added for 1 hour at the described concentrations. For each gel, lysates were reacted with rhodamine-alkyne and separated by SDS-PAGE gel followed by fluorescence analysis.

Figure 2. 2BPN₃ is conjugated to CoA in cells, resulting in an increase in probe reactivity
 (A) Comparison of 2BPN₃ metabolic labeling and *in vitro* labeling. Cells or lysates were labeled for 1 hour with 2BPN₃. Lower probe concentrations were required for equivalent labeling *in vitro* compared to metabolic labeling. (B) 2BP is marginally converted to 2BP-CoA in cells. Control samples were collected immediately after transfer from RPMI to ringer's solution. The remaining cells were left for 30 minutes in the presence of fatty acid free BSA, BSA bound to 50 μM palmitic acid, or BSA bound to 50 μM 2BP. Cells were quenched and metabolites were extracted and analyzed in quadruplicate by high-resolution LC-MS. Samples were run as biological quadruplicates and standard errors are shown. Synthetic palmitoyl-CoA and 2BP-CoA were used to generate a standard curve to calculate endogenous metabolite concentrations. (C) Labeling of cell lysates with increasing concentrations of 2BPN₃-CoA *in vitro*. The control lane was lysate incubated with 50 μM 2BP-CoA, and is not labeled following the click chemistry reaction. (D) Comparison of 2BPN₃, 2BPN₃-CoA, and 2-iodoacetamide-rhodamine (2IA-Rh) shows probe-specific labeling *in vitro*. The control lanes were incubated with 50 μM 2BP (left) and 2.5 μM 2BP-CoA (center). 2BPN₃-CoA labeling is more reactive, but labels a similar pattern of proteins. 2IA-Rh labels a distinct pattern of proteins.

Figure 3. 2BPN₃ and 2BPN₃-CoA are activity-based probes for DHHC2

(A) 2BPN₃ labels FLAG epitope-tagged DHHC2, but not DHHS2 (C157S) by metabolic labeling in live cells. Labeling is resistant to hydroxylamine, demonstrating the absence of a stable acyl-intermediate. (B) 2BPN₃ and 2BPN₃-CoA both label DHHC2, but not DHHS2 (C157S) *in vitro*. Anti-FLAG western blots show recombinant protein expression levels.

Table 1
Most abundant 2BPN₃-labeled proteins identified by mass spectrometry

293T cells were labeled with 2BPN₃ and reacted with biotin-alkyne for enrichment, trypsin digestion, and mass spectrometry analysis. Proteins analyzed by spectral counting, a label-free quantification method for measuring relative abundance based on the number of fragmentation scans assigned to peptides from a specific protein. Standard errors are shown.

Protein	Description	2BPN ₃	2BP
VDAC2	Voltage-dependent anion channel 2	255 ± 51	11 ± 3
ATP2A2	SERCA Ca(2+)-ATPase	167 ± 14	3 ± 2
HMOX2	Heme oxygenase 2	134 ± 29	0 ± 0
CKAP4	Cytoskeleton-associated protein 4	128 ± 32	2 ± 0
SLC25A5	ADP/ATP translocase 2	89 ± 6	17 ± 5
MGST3	Microsomal glutathione S-transferase 3	77 ± 10	2 ± 0
COMT	Catechol O-methyltransferase	69 ± 27	1 ± 1
VDAC3	Voltage-dependent anion-selective channel protein 3	69 ± 9	6 ± 0
CYB5B	cytochrome b5	63 ± 25	1 ± 1
RPL38	60S ribosomal protein L38	56 ± 11	2 ± 0
MTCH2	Mitochondrial carrier homolog 2	54 ± 17	1 ± 1
ACAA1	3-ketoacyl-CoA thiolase	46 ± 13	3 ± 1
SLC25A5	Adenine nucleotide translocator 2	45 ± 10	0 ± 0
AGPAT6	1-acyl-sn-glycerol-3-phosphate acyltransferase zeta	42 ± 10	0 ± 0
RTN4	Reticulon-4	42 ± 9	3 ± 2
HSDL1	Hydroxysteroid dehydrogenase-like protein 1	40 ± 6	1 ± 1
FAM62B	FAM62B	37 ± 4	1 ± 1
RTN3	Reticulon-3	37 ± 10	1 ± 1
AYTL2	1-acylglycerophosphocholine O-acyltransferase 1	35 ± 10	1 ± 0
SCP2	sterol carrier protein 2	35 ± 9	1 ± 0
TOMM40	mitochondrial import receptor subunit TOM40	34 ± 2	2 ± 1
REEP5	Receptor expression-enhancing protein 5	31 ± 2	0 ± 0
A8K3B9	Uncharacterized reticulon-like protein	29 ± 16	1 ± 1
GNPAT	Dihydroxyacetone phosphate acyltransferase	29 ± 10	1 ± 1
SLC25A4	ADP/ATP translocase 1	29 ± 3	0 ± 0
PRAF2	PRA1 family protein 2	28 ± 9	0 ± 0
MTDH	Protein LYRIC	27 ± 9	2 ± 0
PTPLAD1	Protein-tyrosine phosphatase-like A domain-containing protein 1	26 ± 5	1 ± 1
SCAMP3	SCAMP3	25 ± 7	1 ± 1
TFRC	Transferrin receptor protein 1	25 ± 7	1 ± 0