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Bioorthogonal Chemical Reporters for Monitoring Protein Acetylation

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Protein acetylation is a prevalent post-translational modification (PTM) that modulates diverse biological activities in eukaryotes[1a] as well as in bacterial pathogenesis.[1b] In particular, reversible protein acetylation regulated by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) plays key roles in controlling gene expression and is misregulated in many diseases.[1a] Identifying the protein substrates of specific KATs is crucial for elucidating the function(s) of acetylation.[2] Protein acetylation is primarily visualized by employing radiolabeled acetate or acetyl-CoA,[3] but autoradiography exhibits low sensitivity and is hazardous to implement. Alternatively, azide/alkyne-functionalized chemical reporters in conjunction with bioorthogonal ligation methods have afforded new opportunities for imaging and proteomic analysis of PTMs.[4] Herein, we report alkyne-derivatized chemical reporters that enable rapid detection and identification of acetylated proteins *in vitro* and in mammalian cells via Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC)[5] (Figure 1a).

We first investigated whether alkynyl-acetyl-CoA could be utilized by KATs *in vitro*. Three alkynyl-acetyl-CoA analogs, 3-butynoyl-CoA (**1**), 4-pentynoyl-CoA (**2**), and 5-hexynoyl-CoA (**3**), were therefore synthesized and evaluated as acyl-CoA donors for full-length p300-KAT.[6] Mass spectrometry (MS) analysis of p300-acylation reactions with **1**−**3** using H3 peptide as the acceptor substrate revealed that **2** is readily utilized by p300 while **3** is a less efficient acyl-donor substrate (Figure S1a). Compound **1** did not function as a p300 substrate. Examining the stability of these analogs revealed that half of the amount of **1** decomposed within 30 min, while **2** and **3** remained stable for up to 4 h under p300 reaction conditions (Figure S1b). We hypothesize that the relatively acidic propargylic protons adjacent to the thioester of **1** render it an unstable acetyl-CoA analog.

To verify that **2** and **3** were transferred onto lysine (Lys) residues, we performed p300-catalyzed acylation with histone H3 protein followed by in-gel trypsin digestion for MS/MS analysis (Figure S1c,d). While alkynyl-acetyl-CoA analogs exhibited low levels of chemical acylation in the absence of KAT, the addition of 4-pentynoyl and 5-hexanoyl groups from **2** and **3**, respectively, onto Lys residues significantly increased upon addition of p300 (Table S1). These results suggest that **2** and **3** can be utilized by p300 with similar acceptor specificities *in vitro*, albeit with reduced efficiency for multiple acylation. These data are consistent with the previous observations of p300 acetyl-donor promiscuity that enables Lys propionylation and butyrylation of histones *in vitro* and in cells[7a,b] as well as yeast Gcn5-KAT utilization of chloroacetyl-CoA *in vitro*.[7c]

We then evaluated fluorescent detection of KAT activity by CuAAC of the *in vitro* acylation reactions with azido-rhodamine[8] (az-Rho). Both **2** and **3** served as sensitive reagents for visualizing

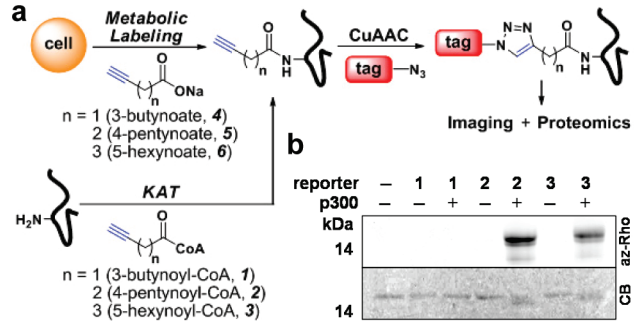

Figure 1. (a) Two-step labeling strategy for detecting protein acetylation *in vitro* and in cells. (b) In-gel fluorescent detection of p300-catalyzed histone H3 acylation. The Coomassie Blue (CB) gel shows the protein loading control.

p300-catalyzed acylation of histone H3 (Figure 1b). In the absence of p300, only minimal fluorescent labeling of histone H3 was observed. Quantifying the observed fluorescent intensities demonstrated that p300 acylation with **2** was time- and enzyme-concentration-dependent (Figure S1e,f). Using this fluorescence assay and a standard curve for product formation, we estimated the K_M and k_{cat} for **2** as 0.69 ± 0.12 μM and 2.10 ± 0.06 s^{-1}, respectively (Figure S2). These values are comparable to those determined for acetyl-CoA by radioactivity assay.[9] These studies demonstrate that **2** and CuAAC enables rapid fluorescence detection of KAT activity with picomolar sensitivity within minutes, in contrast to days or weeks as required for radioactivity.

To investigate whether the alkynyl-acetate analogs can metabolically label proteins in living cells, the alkynyl-acetate analogs 3-butynoate (**4**), 4-pentynoate (**5**), and 5-hexynoate (**6**) were prepared as sodium salts (Figure S3) and examined for incorporation into Jurkat T cells by CuAAC−fluorescent detection (Figure 1a). The results showed that metabolic labeling with **4**−**6** was both dose- and time-dependent and was optimal at 2.5−10 mM acetate analogue for 6−8 h (Figure S4). The selective labeling of core histones and histone H3 derived from metabolically labeled Jurkat T cells demonstrate that **4**−**6** can be installed onto known Lys-acetylated proteins (Figure 2a,b). Fluorescent profiling of total cell lysates revealed many proteins labeled by **4**−**6** (Figure 2c). The majority of the selectively labeled proteins were insensitive to cycloheximide treatment and largely distinct from those targeted by longer-chain alkynyl fatty acid analogs[8] (Figure S5a,b). Co-incubation with a KDAC inhibitor, suberoylanilide hydroxamic acid (SAHA),[10a] slightly decreased the addition of **4**−**6** onto proteins (Figure S5c), suggesting that blocking the removal of protein acetylation prevents metabolic incorporation of these acetate analogs. Cells treated with curcumin,[10b] a p300 inhibitor, reduced the acetate-analog labeling of known p300 substrates such as core histones (Figure S5d). Further analysis of the total cell lysates showed that not all of the labeled protein signals were reduced by

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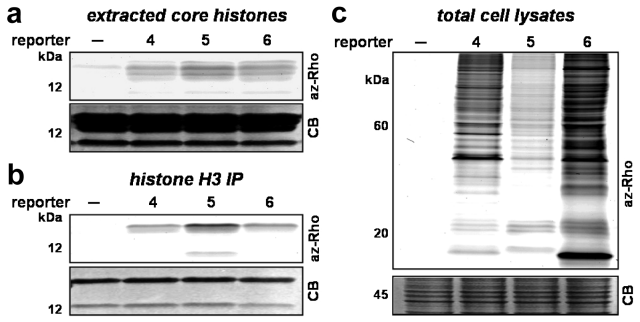

Figure 2. Fluorescent detection of acetate reporter (**4**, **5**, or **6**)-labeled (a) core histones, (b) purified histone H3, and (c) total cell lysates from Jurkat T cells.

curcumin (Figure S5d), which suggests these acetate analogs may also be utilized by other KATs or acyltransferases or incorporated by chemical acylation. Nonetheless, these cellular experiments suggest that **4**, **5**, and **6** can be converted into active alkynyl-acetyl-CoA analogs in cells by promiscuous acetyl-CoA[11] or acyl-CoA synthetases and post-translationally installed onto proteins in living cells.

To identify proteins metabolically labeled by **4**, **5** and **6**, Jurkat T cell lysates were subjected to CuAAC with the cleavable azido-diazo-biotin tag (Scheme S4) followed by affinity purification on streptavidin beads (Figure S7). Subsequent treatment of the streptavidin beads with sodium dithionite enabled efficient elution of the captured proteins for gel-based proteomics using an LTQ-Orbitrap mass spectrometer. A survey of the protein hits from the cell lysates metabolically labeled with **4**, **5**, and **6** revealed many reported (86%) as well as new candidate (14%) Lys-acetylated proteins (Table S2). Our p300-acylation studies and cellular labeling and proteomics data suggest that both 4-pentynoate derivatives (**2** and **5**) are the optimal chemical reporters for detecting protein acetylation *in vitro* and in cells. Though **4** and **6** can also metabolically label Lys-acetylated proteins in cells, the CoA derivative of **4**, 3-butynoyl-CoA (**2**) is an unstable substrate for *in vitro* reactions, and **6** may also target long-chain fatty-acylated proteins in cells (i.e., transferrin receptor, SNAP-23) as indicated in the MS/MS-identified proteins lists (Table S2). We therefore focused on **5** for additional proteomic studies. From three independent proteomic experiments (Figures S7 and S8), we identified approximately 194 4-pentynoate-labeled proteins from Jurkat T cells (Tables S2 and S3), 86% of which were also identified by anti-acetyl-Lys proteomic studies.[12a,b] We confirmed the enrichment of several MS/MS-identified acetylated proteins, including Ku70, moesin, cofilin, coronin-1A, Hsp90, HMG-1, and adenosine deaminase by Western blotting analysis of the affinity-enriched proteins (Figure S8b). Bioinformatic analysis of our data set suggests that the majority of acetylated proteins reside in the nucleus and cytoplasm and are associated with diverse cellular functions ranging from metabolism and signal transduction to gene expression (Figure S8c).

To verify that **5** targets Lys residues on proteins in cells, 4-pentynoate-labeled Jurkat T cell lysates were CuAAC-biotinylated, trypsin-digested in solution, purified using streptavidin beads, and eluted with $Na_2S_2O_4$ for MS/MS sequencing. Analysis of the recovered peptides demonstrated that **5** is metabolically incorporated onto known sites of Lys acetylation on histones H2B, H3, and H4 (Figure S9). The characteristic marker ion (mass = 259 Da) corresponding to the fragmentation peak of the modified lysine residue (4-pentynoate + CuAAC/$Na_2S_2O_4$ cleavage adduct) was observed in all of the MS/MS spectra. These experiments collectively demonstrate that alkynyl-acetate analogs **2** and **5** function as efficient chemical reporters for protein acetylation *in vitro* and in cells, respectively. Notably, fluorescent profiling of various mammalian cell lines with **4**, **5**, and **6** revealed distinct and analog-specific patterns of acetylomes in diverse cell types, which highlights the generality and utility of these bioorthogonal chemical reporters for protein acetylation detection (Figure S6).

Unraveling the functions of protein acetylation remains a challenging task. The bioorthogonal chemical reporters presented here provide readily accessible non-radioactive reagents for fluorescence profiling and large-scale analysis of protein acetylomes. Moreover, alkynyl-acetyl-CoA analogs enable rapid and sensitive detection of KAT activities that should be useful for assigning protein substrates in complex mixtures. This chemical approach provides experimental tools complementary to anti-acetyl-Lys antibodies,[12a,b] MS/MS,[12a,b] bioinformatic methods,[12c] and affinity-based CoA probes.[13] The functional analysis of KAT-regulated acetylation will be essential going forward. The incorporation of quantitative proteomic methods[12b] and bump-hole strategies[14] in the future should expand the utility of these chemical tools and facilitate the functional analysis of protein acetylation in physiology and disease.

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Supporting Information Available: Experimental procedures and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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