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Integration of Bioorthogonal Probes and Q-FRET for the Detection of Histone Acetyltransferase Activity

Zhen Han^[a], Dr. Yepeng Luan^{[a],[b]}, and Dr. Prof Yujun George Zheng^[a]

Yujun George Zheng: yzheng@uga.edu

^[a]Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, 250 W Green St, Athens, GA, 30602

Abstract

Histone acetyltransferases (HATs) are key players in epigenetic regulation of gene function. Recent discovery of diverse HAT substrates implicates a broad spectrum of cellular functions of HATs. Many pathological processes are also intimately associated with dysregulation of HAT levels and activities. However, detection of enzymatic activity of HATs has been challenging and significantly impeded drug discovery. To advance the field, we developed a convenient one-pot mix-and-read strategy that is capable to directly detect the acylated histone product via fluorescent readout. The strategy integrated three technological platforms, bioorthogonal HAT substrate labeling, alkyne-azide click chemistry, and quenching-FRET, into one system for effective probing of HAT enzyme activity.

Graphical Abstract

Keywords

Bioorthogonal chemical probe; protein acetylation; HAT activity; click reaction; Quenching-FRET

Histone acetyltransferases (HATs) are important posttranslational modification enzymes that charge small acetyl groups to substrate lysine residues, with most well studied in nucleosomal histones. Since their genetic identification in the mid-1990s, HATs have gained extensive attention of research in aspects of structural characterization, biochemical properties, and functions in physiology and pathology.^[1] Based on their conserved domain

Correspondence to: Yujun George Zheng, yzheng@uga.edu.

^[b]Current address: Department of Medicinal Chemistry, School of Pharmacy, Qingdao University, Qingdao, Shandong, China, 266000

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sequences, HATs are categorized into several major families, including Gcn5/PCAF family, p300/CBP family, and the MYST (MOZ, Ybf2/Sas3, Sas2, Tip60) family. Each HAT member transfers the acetyl group from acetyl-coenzyme A (acetyl-CoA) to the epsilon amino group of specific lysine residues in their protein substrates. Owing to their capability of acetylating the nucleosomal histones, HATs are important epigenetic players for the control of DNA transcription, replication, and damage repair.^[2] Impressively, in recent years lysine acetylation is more appreciated as a common post-translational modification in proteins, far out of the chromatin realm. Mass spectrometry-based proteomic screening has identified a vast number of non-histone substrates, suggesting the general significance of lysine acetylation in biology.^[3] It is expected that many unknown functions of HATs in the cell remain to be elucidated in the near future. The pathological significance of HATs has become increasingly clear. Deregulation of HAT levels and activities were observed in and associated with myriad disease phenotypes such as inflammation,^[4] diabetes mellitus,^[5] obesity,^[6] neurological disorder,^[7] cancer,^[1c, 8] hematologic diseases,^[1b, 9] cardiovascular disorders,^[10] viral infection,^[11] etc.

Given the physiological and pharmacological significance of HATs, it is imperative to develop isoform-selective HAT inhibitors as either mechanistic tools or therapeutic lead agents. To move this field forward, reliable and efficient biochemical methods that can be applied to detect HAT activity and screen thousands of library compounds are critically needed. Simplicity, sensitivity, dose-dependent linearity, and signal robustness are often important factors to consider in the design of enzymatic assays. Nevertheless, detection of HAT activity is technically challenging: the transferred acetyl group is both chemically and spectroscopically inert so that there are no straightforward detectable readouts under routine assay settings (Scheme 1). Till now, different strategies had been developed to study HAT activity based on different detection principles, each of which owes pros and cons. Radiometric assays relying on isotope-labeled acetyl-CoA represent a gold standard in the characterization of HAT activities because of their relatively high sensitivity.^[12] Handling of radioactive hazards, however, is often a concern for many laboratories. Quantitation of CoA, the side product of HAT reaction, using either chemical- or enzyme-coupled spectroscopic methods are also widely adopted.^[13] Antibody-based immunosorbent assays are sensitive and commonly applied by molecular biology scientists who study biological functions of lysine acetylation.^[14] High cost of antibody production and sacrifice of lab animals limit their use in large-scale applications. Of note, many assay types require separation of products from reaction mixtures before signal detection, which is a painful technical bottleneck in high-throughput study. Therefore, homogeneous, spectroscopic, mix-and-read assays for HAT activity detection are most preferred approaches.^[15]

In this study, we demonstrate a fluorescent assay strategy for HAT activity detection by aligning several chemical biology techniques into one system: bioorthogonal protein acetylation labeling, alkyne-azide click chemistry, and quenching-fluorescence resonance energy transfer (Q-FRET). As is shown in Scheme 1, the first step is to create a bioorthogonal enzyme-cofactor matching pair so that the HAT enzyme can take up orthogonal acyl-CoA cofactors for histone modification. We recently identified several active bioorthogonal pairs for the HAT members MOF, GCN5 and p300.^[16] Interestingly, 3-azidopropanoyl CoA (3AZ-CoA) is a highly effective surrogate of acetyl-CoA that can be

used as a cofactor by several HAT enzyme forms. We will carry out the enzymatic reaction with a histone substrate containing a fluorescent donor (e.g. fluorescein). Upon HAT-catalyzed acylation modification, the added 3-azidopropanoyl group in the substrate can be reacted with a FRET acceptor chromophore via alkyne-azide cycloaddition click chemistry. In this way, the donor and acceptor will be brought into spatial proximity within the same molecule, leading to FRET signal transduction. The unique advantage of this new strategy is that the intramolecular FRET mechanism occurs in situ as a function of the progress of HAT-catalyzed reaction. This will be a straightforward mix-and-read HAT assay approach, eliminating any tedious washing procedure and possessing high throughput capacity.

As a proof of concept, we chose the MYST enzyme member MOF (male absent on the first, MYST1, HAT8) to demonstrate the FRET detection strategy. MOF is the key acetyltransferase responsible for the acetylation of lysine-16 of histone H4.^[17] We recently determined that active site engineering of MOF by mutating its isoleucine-317 to alanine expanded the cofactor binding site resulting in a consequence that MOF-I317A was able to utilize bulky acetyl-CoA surrogates such as 3AZ-CoA to modify histone substrates.^[16] Indeed, the kinetic characterization of histone H4 peptide modification showed that MOF-I317A mutant exhibited a strong activity toward 3AZ-CoA, two-fold that of acetyl-CoA (Table 1). In contrast, wt-MOF had good activity for the nascent cofactor acetyl-CoA, but with minimal activity toward 3AZ-CoA. Therefore, MOF-I317A—3AZ-CoA is a superior bioorthogonal pair for MOF substrate labeling. Importantly, the clickable functionality of the transferring 3-azidopropanoyl group offers a great power for downstream detection of modified substrates using fluorescent reporters or affinity tags through the copper-catalyzed azide-alkyne cycloaddition (CuAAC)^[18] or strain-promoted azide-alkyne cycloaddition (SPAAC).^[19]

To construct the one-pot FRET assay for MOF activity detection, we adopted a fluorophore-labeled histone peptide substrate (i.e., H4-FL) containing the N-terminal 20 residue of histone H4 with Leu-10 replaced with a fluorescently labeled lysine.^[20] First, 3AZ-CoA and H4-FL were incubated with different concentrations of MOF-I317A for 1 h to allow the acylation reaction to occur. After the reaction, a cocktail of click reagents was added which contained propargyl-Dabcyl, copper sulfate, ligand BTTP, and sodium ascorbate in DMSO. After further incubation for 1 h, guanidine hydrochloride was added with final concentration at 0.25 M in order to eliminate any nonspecific interaction between the cofactor and the histone substrate.^[12c] In the final step, the fluorescence emission spectra were measured with fixed excitation wavelength at 491 nm. As expected, emission of fluorescein decreased as more enzymes were added (Figure 1), which illustrates the effectiveness of this detection platform: the enzyme delivered the 3-azidopropanoyl group to the Lys-16 position of the H4 substrate; then propargyl-Dabcyl specifically reacted with the azide through the CuAAC reaction to form a covalent triazole linkage; in this way the quencher chromophore was brought into close proximity to the fluorescein donor and thus introduced an intramolecular quenching-FRET process between Dabcyl and fluorescein, leading to diminished fluorescence of the donor. Impressively, the fluorescence intensity changed linearly as a function of enzyme concentration (Figure 1b) which indicates that the quenching effect is not only related to but proportional to the yield of acylated histone product.

The fluorescent changes in response to the enzymatic reaction time were also tested. At different time points of reaction, a DMSO solution of click reagent was added to quench the enzymatic reaction and meanwhile initiated the alkyne-azide cycloaddition reaction. Next, 0.25 M of guanidine hydrochloride was added and fluorescence intensity was measured. As shown in Figure 2, the fluorescence intensity decreased linearly as a function of enzymatic reaction time, which further supports that this assay offers a quantitative approach for detecting the product of MOF catalysis. We further tested if this method could be used for determining kinetic parameters of substrates. So, a range of 3AZ-CoA concentrations were selected for the enzymatic reaction followed by fluorescent measurement. Increase of 3AZ-CoA concentration led to a hyperbolic decrease of fluorescence intensity because more azidoacyl groups were transferred to H4-FL (Figure 3). The hyperbolic curve was fitted to a modified Michaelis-Menten equation (Equation S1). An apparent K_m value of $1.5 \pm 0.1 \mu\text{M}$ was derived, which was in the close range of the K_m of $2.7 \pm 0.2 \mu\text{M}$ that was measured using regular 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM)-CoA fluorogenic assay.^[21]

The excellent performance of this FRET strategy for HAT activity detection suggests that it can be well suited for HAT inhibitor screening and optimization. Anacardic acid is a known inhibitor of the MYST HATs^[22] and p300.^[23] We attempted to test if our new approach can be used to quantify the potency of anacardic acid in MOF inhibition. In this experiment, different concentrations of anacardic acid were present in the H4 acylation reaction catalyzed by MOF-I317A. We anticipated that increasing inhibition of MOF activity by higher concentrations of anacardic acid would reduce the transfer rate of 3-azidopropanoyl group to H4-FL. Consequently, less propargyl-Dabcyl would be recruited to the substrate, and the FRET interaction would be reduced, with a manifestation of higher fluorescence emission. Indeed, the fluorescence intensity increased as a function of anacardic acid concentration (Figure 4a). By fitting the dose response curve with the inhibitor concentration—response equation (Equation S3), the IC_{50} value was calculated to be $3.2 \pm 0.5 \mu\text{M}$. This value is almost the same as the IC_{50} value ($3.4 \pm 0.5 \mu\text{M}$) measured using the standard radiometric acetylation assay in which [^{14}C]Ac-CoA was the cofactor (Figure 4b). Besides, the IC_{50} value of anacardic acid towards p300 was measured using both quenching-FRET assay and radiometric acetylation assay and both methods gave comparable results (Figure S4). Therefore, the quenching-FRET assay is a valid approach for HAT inhibitor study. Given that development of selective chemical probes of HATs has greatly lagged in the epigenetic drug discovery, biochemical assays that effectively detect HAT activity are of significant value in the course of discovering HAT probes in the high throughput manner. We expect that the new design will help fill the gap in this important biomedical arena.

In summary, by combining several technologies, i.e. bioorthogonal HAT substrate labeling, CuAAC click chemistry and quenching-FRET, into one platform, we have developed a homogeneous one-pot fluorescent assay approach for HAT activity detection. This method directly measures the acylated histone product, which is advantageous compared to the current CPM fluorescent method that measures the by-product CoA.^[13b] The latter assay is easily susceptible to background fluorescence interference and the instability of acetyl-CoA can lead to false positive results. With our new approach, the fluorescent readout is linearly proportional to the yield of the acylated product. The distance-dependent intramolecular

FRET working mechanism suggests that this assay type is more suitable to organic dyes in inhibitor screenings. Protein acylation is an emerging area of science,^[24] and the high-performance assay strategy presented here has great potential to be widely applied to other HATs and acyltransferases.

Experimental Section

Preparation of 3AZ-CoA, MOF mutagenesis, protein expression of MOF-wt and MOF-I317A in BL21(DE3) bacteria cells were done following previous procedures.^[16] Histone peptides H4-20 (Ac-SGRGKGGKGLGKGGAKRHRK) and H4-FL (Ac-SGRGKGGKGGK(Fluorescein)GKGGAKRHRK) peptides were synthesized with the standard solid-phase peptide synthesis (SPPS) protocols, purified with C-18 RP-HPLC and characterized with MALDI-MS as previously described.^[20]

Synthesis and characterization of propargyl-DabcyL

Propargyl-DabcyL was synthesized by reacting 4-([4-(Dimethylamino)phenyl]azo) benzoic acid succinimidyl ester (DABCYL SE) with propargyl amine under mild condition. Product was purified with silicon chromatography and characterized with ESI-MS and NMR (Figure S1–S3). The yield of final product is 86%.

Enzyme-cofactor kinetic characterization

H4-20 peptides and cofactors at varied concentrations were incubated in reaction buffer containing 50 mM of pH 8.0 HEPES and 0.1 mM of EDTA at 30°C. 0.05 μ M of enzyme was added to initiate the reaction which lasted for 10 minutes. After that, 10 μ M of CPM in DMSO was added to both quench enzymatic reaction and initiate CoA CPM reaction. After 20 minutes, fluorescence intensities were measured using microplate reader with the fixed excitation wavelength at 392 nm and emission wavelength at 482 nm. Fluorescence readouts were plotted over cofactor concentrations with Michaelis-Menten equation to get the apparent K_m value.

Inhibitor assays

For filter binding assay, 30 μ L reaction mixture includes varied concentrations of anacardic acid, 0.5 μ M of MOF-I317A, 5 μ M of [¹⁴C]Ac-CoA and 5 μ M of H4-FL was incubated at 30 °C for 30 min. Then, 20 μ L of sample was spotted on p81 filter paper disk. Paper disks were washed with 50 mM NaHCO₃ (pH 9.0) and air dried. Liquid scintillation counting was performed to quantitate the amount of acetylated peptides. IC₅₀ value was calculated with inhibitor concentration-response equation (Equation S2). For Q-FRET assay, anacardic acid at varied concentrations were incubated with 0.5 μ M of MOF-I317A, 5 μ M of 3AZ-CoA and 5 μ M of H4-FL at 30 °C for 30 min in 20 μ L reaction mixture. Then, 25 μ L click reagent DMSO solution was added, followed by 1 hour incubation at room temperature in darkness. Fluorescence intensity was measured with CLARIO star microplate reader (BMG LABTECH) at fluorescein channel after addition of guanidine hydrochloride with final concentration at 0.25 M. Fluorescence intensities were plotted versus inhibitor concentrations with modified inhibitor concentration-response equation (Equation S3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The quenching FRET is dependent on the enzyme concentration. MOF-I317A at varied concentrations were incubated with 5 μM of H4-FL and 10 μM of 3AZ-CoA followed by incubation with propargyl-DabcyI. Then, mixture was excited at 491 nm and emission spectra were collected at each enzyme concentration. a). Fluorescence spectra with excitation wavelength at 491 nm; b). Linear relationship between fluorescence intensity at 520 nm and enzyme concentration.

Figure 2.

Fluorescent changes in response to enzymatic reaction time. Enzymatic reaction was initiated with mixing of MOF-I317A, H4-FL and 3AZ-CoA and was quenched at different time points. Excess alkyne Dabcyl was added to react with the modified peptides to form quenching-FRET pair with the anchored fluorescein. a). Fluorescence spectra changes in response to reaction time with excitation wavelength at 491 nm; b). Linear relationship between fluorescence intensity at 520 nm and enzymatic reaction time.

Figure 3.

The function of fluorescence intensity with the concentration of 3-azidopropanoyl CoA (3AZ-CoA). 3AZ-CoA at different concentrations were incubated with 0.7 μM of MOF-I317A and 5 μM of H4-FL to produce varied amount of azide labeled H4-FL. Propargyl-DabcyI are then conjugated to modified peptides, followed by fluorescence measurement. Fluorescence decreases with increasing concentration of 3AZ-CoA due to increase of Q-FRET effect. The K_m value of MOF-I317A binding with 3AZ-CoA is calculated to be $1.5 \pm 0.1 \mu\text{M}$ with modified Michaelis-Menten equation.

Figure 4.

Quantitation of anacardic acid potency for HAT inhibition. a) Using Q-FRET to measure IC_{50} value of anacardic acid (enzymatic reaction components included MOF-I317A, H4-FL and 3AZ-CoA) and the obtained IC_{50} was $3.2 \pm 0.5 \mu\text{M}$; b) Using filter binding assay to measure IC_{50} value of anacardic acid (enzymatic reaction components included MOF-I317A, H4-FL and $^{14}\text{Ac-CoA}$) and the measured IC_{50} of anacardic acid was $3.4 \pm 0.5 \mu\text{M}$.

Scheme 1.

Using quenching fluorescence resonance energy transfer (Q-FRET) assay in conjugation with bioorthogonal substrate labeling to detect HAT activity. wt-HAT= wild type histone acetyltransferase, mt-HAT=mutant histone acetyltransferase, FL=Fluorescein.

Table 1

Kinetic parameters of MOF to the nascent and orthogonal cofactors. “——” means the values were unmeasurable because of the low reading.

Enzyme	Cofactor	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1}.min^{-1}$)
MOF-wt	Ac-CoA	1.3 ± 0.1	5.2 ± 0.1	4.0 ± 1.5
	3AZ-CoA	——	——	——
MOF-I317A	Ac-CoA	3.5 ± 0.2	2.2 ± 0.0	0.6 ± 0.2
	3AZ-CoA	2.7 ± 0.2	3.2 ± 0.1	1.2 ± 0.5