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Rational Design of Substrate-Based Multivalent Inhibitors of the Histone Acetyltransferase

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Abstract

Tip60 (the 60-kDa HIV-1 Tat-interactive protein) is a key member of the MYST family of histone acetyltransferases (HATs) and plays critical roles in apoptosis and DNA repair. Potent and selective inhibitors for Tip60 are valuable tools for studying the functions of the enzyme. In this work, we designed, synthesized and evaluated a new set of substrate-based inhibitors containing multiple binding modalities. In addition to the CoA moiety conjugated to H3 peptide, mono- and trimethylated lysine residues were incorporated at Lys-4 and/or Lys-9 sites in the H3 peptide substrate. The biochemical assay results showed that the presence of methyl group(s) on the substrate resulted in more potent inhibitors to Tip60, relative to the parent H3-CoA bisubstrate inhibitor. Importantly, by comparing the inhibitory properties of the ligands against full-length Tip60 versus the HAT domain, we determined that the K4me1 and K9me3 marks contributed to the potency augmentation by interacting with the catalytic region of the enzyme.

Keywords

Tip60; bisubstrate inhibitor; multivalency; HAT; methyllysine

Lysine acetylation of the core histone proteins in eukaryotic cells is an evolutionarily conserved posttranslational modification and constitutes a fundamental mechanism for gene function regulation.^[1] The acetylation reaction entails the transfer of acetyl groups from cofactor acetyl-Coenzyme A (AcCoA, acetyl-CoA) to the ε-amino group of the side chain, and is enzymatically catalyzed by histone acetyltransferases (HATs, also named as lysine acetyltransferases, KATs). Several HATs have been identified and characterized which includes GNAT family, MYST family (MOZ, Ybf2/Sas3, Sas2, Tip60), and p300/CBP.^[2] Histone acetylation is critical for chromatin-associated functions such as gene transcription, replication, and DNA damage repair.^[3] Moreover, recent proteomics studies reveal that hundreds and thousands of nonhistone targets exist in cellular contexts, supporting that HATs also regulate broad biological processes such as cell cycle, cytoskeleton remodeling, ribosomal translation, and metabolic pathways.^[4]

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The MYST family contains the largest number of HAT proteins in mammals. The key MYST member, the 60 kDa HIV-1 Tat-interacting protein (Tip60), was found to acetylate nucleosome core histones (H2AK5, H3K14, H4K-5, -8, -12, -16)^[5] as well as a variety of nonhistone targets including androgen receptor (AR),^[6] ATM,^[7] p53,^[8] STAT3,^[9] Myc,^[10] NF-κB,^[11] etc. Tip60 participates in the regulation of gene transcription, cell signalling, apoptosis and DNA damage repair.^[3c] In addition, Tip60 also has been linked to human diseases such as HIV infection and AIDS, Alzheimer's disease, and cancer.^[11a, 12] For example, the androgen receptor is acetylated by Tip60 at the hinge region^[13] and upregulation of Tip60 activity facilitates the progression of prostate cancer.^[13–14] In Alzheimer's disease, Tip60 forms a regulatory complex with the intracellular domain of amyloid precursor protein,^[15] and it was thought that this complex regulates the transcription of genes responsible for the neurogenesis.^[15b] Given Tip60's critical functions in disease initiation and progression, it is of great necessity to develop novel strategies for enzyme inhibition.

We previously reported that conjugation of histone substrate peptides with CoA resulted in bisubstrate inhibitors with amenable potency for the MYST HATs such as Tip60 and Esa1 (e.g. H3-CoA and H4-CoA).^[16] Such bivalent inhibitors are useful chemical tools to facilitate cocrystallization of enzyme-substrate complexes and improve understanding of substrate recognition mechanism of HATs. In this work, we attempt to further improve the potency of these inhibitors for Tip60 by introducing new binding modalities. Recent studies from two research groups showed that the chromodomain of Tip60 preferentially binds to monomethylated lysine-4 and/or trimethylated lysine-9 on the histone H3 tail, i.e. H3K4me1 and H3K9me3.^[17] These new findings promoted us to design a second generation of bisubstrate inhibitors which incorporates K4me1 and/or K9me3 marks in the peptide region of the H3-CoA inhibitor. Under this scheme, three binding modules will be present in the designed ligand: CoA, the peptide sequence, and the methyl marks (Figure 1). The presence of multivalency is expected to produce higher potency than the parent H3-CoA inhibitor.

We selected a 20-residue H3 N-terminal peptide as the framework for the inhibitor design. The CoASH moiety was covalently linked to the side-chain amino group of lysine-14 in the peptide (H3K14) because this is the major site of acetylation in H3 by Tip60. The coupling was achieved via a bromoacetyl linker (Figure 2). In the peptide synthesis, Fmoc-Lys(me, Boc)-OH and Fmoc-Lys(me3)-OH were used to introduce K4me1 and K9me3 groups, respectively. We also prepared H3 peptides containing bromoacetyl or methylthioacetyl group at the K14 site as negative controls (Table 1). The general synthetic route of these compounds followed solid-phase peptide synthesis first and then solution-phase coupling of CoASH to the peptides (Figure 2). All the compounds were purified by reverse-phased HPLC on a semi-preparative C18 column to a purity greater than 95% and characterized by MALDI-MS. The sequence and mass spectrometric data are listed in Table 1.

After obtaining the synthetic compounds, we tested their inhibitory effect on the acetyltransferase activity of Tip60 using the standard radiometric filter binding assays. Both recombinant full-length Tip60 (FL-Tip60, 1—512 aa) and the catalytic domain (CAT-Tip60, 221—512 aa) were used as the enzyme source in order to evaluate inhibition differences. The inhibition assays were carried out with varying concentrations of individual inhibitors

and IC_{50} values were determined from the dose-responsive curves. As shown in Table 2, IC_{50} of CoASH and H3K14CoA (**a**) for Tip60 were very close, $9.1 \pm 1.0 \mu\text{M}$ and $9.0 \pm 1.1 \mu\text{M}$, respectively. This is indicative that the CoA moiety is the dominant factor contributing to the binding affinity of H3K14CoA (**a**) to Tip60, and the H3 part has marginal binding contribution in the ligand-enzyme interaction. No significant inhibition was observed at 500 μM of H3K14Br (**b**) and 250 μM of H3K14sme (**c**), which is consistent with the notion that CoA is the dominant factor for the observed enzyme inhibition. In addition, both CoASH and H3K14CoA (**a**) inhibited the activity of CAT-Tip60 at similar potency ($16.0 \pm 3.1 \mu\text{M}$ and $14.7 \pm 0.9 \mu\text{M}$) as that of FL-Tip60, which is agreeable with the established model that the CoA moiety binds to the active site of the MYST domain.^[18]

To further improve the inhibition potency of H3K14CoA inhibitor, we incorporated K9me3 or K4me1 marks to the H3 peptide sequence, so that H3K9me3K14CoA (**d**) and H3K4me1K14CoA (**f**) were synthesized. First, we tested the inhibitory effect of H3K9me3K14CoA (**d**) on the acetyltransferase activity of Tip60. As expected, the potency of H3K9me3K14CoA for FL-Tip60 increased by 8-folds ($IC_{50} = 1.2 \pm 0.1 \mu\text{M}$) over the parent inhibitor (**a**). This clearly demonstrates that the trimethyl mark on K9 enhances the ligand-enzyme binding. However, H3K9me3K14CoA (**d**) did not show a significant difference for the inhibition of FL-Tip60 versus CAT-Tip60 ($IC_{50} = 1.6 \pm 0.2 \mu\text{M}$), which indicates that the K9me3-recognition site in Tip60 is located in the MYST domain, but not in the chromodomain (Figure 3). The effect of the K9me3 mark is also clear from the comparative inhibitory data of H3K9me3K14sme (**e**) and H3K14sme (**c**). H3K9me3K14sme (**e**) showed a stronger inhibition than H3K14sme (**c**), indicating that K9me3 creates a binding niche for Tip60 and thus enhances the binding affinity between the ligand and enzyme. The little difference in IC_{50} between FL-Tip60 and CAT-Tip60 inhibition by H3K9me3K14sme (**e**) again supports the notion that the binding site of K9me3 in Tip60 is located in the MYST domain region, not in the chromodomain.

Similarly, we tested the effect of monomethyl K4 (K4me1) on the potency of the H3-CoA inhibitor. Compared with H3K14CoA (**a**), the potency of H3K4me1K14CoA (**f**) increased by about 4-fold ($IC_{50} = 2.1 \pm 0.4 \mu\text{M}$). This result supports that the K4me1 mark increases the interaction of H3 peptide with Tip60 as well. However, the increasing effect did not seem to be as strong as that of the K9me3 mark. For example, the presence of K4me1 did not apparently increase the inhibition activity of H3K4me1K14Br (**g**) in comparison with H3K14Br (**b**). Again, we did not observe any significant difference between FL-Tip60 inhibition and CAT-Tip60 inhibition (less than one-fold difference) by H3K4me1K14CoA (**f**). Thus, the K4me1 binding site in Tip60 should also be located in the MYST domain (Figure 3).

Since both K9me3 and K4me1 marks increased the potency of the parent H3K14CoA (**a**) inhibitor, we also synthesized and tested the anti-Tip60 effect of a multivalent inhibitor containing both K4me1 and K9me3 marks, namely H3K4me1K9me3K14CoA (**h**). Interestingly, in comparison to the inhibitors (**d**) and (**f**) that contain a single K9me3 or K4me1 mark, H3K4me1K9me3K14CoA (**h**) exhibited similar IC_{50} values: $3.8 \pm 0.2 \mu\text{M}$ for FL-Tip60 and $2.5 \pm 0.1 \mu\text{M}$ for CAT-Tip60. This result suggests that the effect of K4me1

and K9me3 are not synergistic with each other. Possibly, the MYST domain of Tip60 does not recognize the two modification marks simultaneously.

H3K14CoA was originally designed by the Cole group as a bisubstrate inhibitor for PCAF.^[19] As a curiosity, we tested whether the introduction of K4me1 and K9me3 influences the potency of H3K14CoA (**a**) blocking the HAT activity of PCAF. The IC₅₀ of these compounds for PCAF was determined using the similar radiometric assay procedure as shown for Tip60. Compared to the IC₅₀ of H3K14CoA ($0.35 \pm 0.05 \mu\text{M}$), the potencies of inhibitors containing K9me3 or K4me1 (i.e. compound (**d**) and (**f**) are essentially in the same range, with IC₅₀ of $0.28 \pm 0.06 \mu\text{M}$ for H3K9me3K14CoA (**d**) and $1.1 \pm 0.1 \mu\text{M}$ for H3K4me1K14CoA (**f**), indicating that K9me3 and K4me1 have minor effects on ligand-PCAF binding. The subtle difference between potencies of (**d**) and (**f**) may suggest that K9me3 mark has a slightly more favourable effect than K4me1. Moreover, the doubly methylated inhibitor, H3K4me1K9Me3K14CoA (**h**), showed an inhibition close to that of (**d**), with an IC₅₀ at $0.33 \pm 0.06 \mu\text{M}$, a fact supporting that the role of K9me3 mark is slightly more important than K4me1.

Put together, this work was aimed at producing more potent substrate-based inhibitors for HAT Tip60 by introducing additional binding modalities. Previously, we showed that histone H3 and H4 peptide-CoA bisubstrate inhibitors display desired inhibitory activity towards Tip60.^[16] It was recently reported that the chromodomain of Tip60 binds specific methylated lysine residues in H3 peptides, e.g. H3K4me1 and H3K9me3.^[17] To take advantage of the free energy of chromodomain binding with H3K4me1 or H3K9me3, we attempted to build multivalent inhibitors to further improve the inhibition potency of H3-CoA bisubstrate inhibitors. Thus, a series of H3K14CoA derivative compounds with or without K4me1 and/or K9me3 were synthesized and analyzed. As expected, by introducing K4me1 or K9me3 to the H3K14CoA conjugate, we are able to increase the inhibition potency of the resultant inhibitors (**d**) and (**f**) by 8 and 4 folds, in reference to the parent inhibitor H3K14CoA (**a**). However, we did not observe any significant difference in the IC₅₀ value between the FL-Tip60 and CAT-Tip60 enzyme forms, indicating that the chromodomain of FL-Tip60 is not engaged in the interaction of Tip60 with the K4me1 or K9me3 group. Instead, the data imply that the methyl-binding site(s) of Tip60 is located in the catalytic domain (Figure 3). Also, we found that the inhibition potency of these inhibitors for the HAT domain of PCAF was slightly increased when K9me3 was incorporated to H3K14CoA (**a**). Thus, K9me3 binding site in PCAF is also located within the catalytic domain. Clearly these data demonstrate that the interaction of the HAT with its substrate can be tailored by simple chemical modifications. It should be stated that these experimental results are not necessarily paradox with the previous reports showing that Tip60 chromodomain binds to K4me1 and K9me3 marks. One possible explanation is that, the interaction of these H3-CoA inhibitors with Tip60 is dominated by the CoA moiety so that the significance of chromodomain is minimized. In addition, the confined spatial relationship between CoA and the methyl marks in the H3-CoA inhibitors may hinder Tip60's ability to concurrently bind CoA and the methyl mark(s) via two separate domain regions. Nevertheless, our experimental results offer an interesting finding that the MYST domain has a capacity of interacting with the methyl marks in the histone H3 substrate,

which accounts for the increased potency of the designed multivalent inhibitors. These compounds containing multiple interactive motifs are potential chemical probes for determining the substrate recognition mechanism of HATs. Future structural studies will be needed to elucidate how K4me1 and K9me3 marks enhance the potency of the H3-CoA compounds in HAT inhibition. Moreover, conjugation of these compounds with the TAT transduction domain would promote their application to live cellular systems.^[20]

Experimental Section

Chemicals and reagents

Escherichia coli BL21(DE3) competent cells were purchased from Stratagene. Fmoc-protected amino acids and preloaded Wang resin were purchased from NovaBiochem. Reagents for organic synthesis were purchased from Sigma-Aldrich and used without further purification. [¹⁴C]-acetyl CoA was purchased from Perkin Elmer.

Protein expression and purification

The His6x-tagged full-length Tip60 (FL-Tip60), Tip60 catalytic domain (CAT-Tip60) or PCAF HAT domain was expressed using *Escherichia coli* and purified on Ni-NTA Beads. Each DNA plasmid pET-21a(+)-FL-Tip60 (1–512), pET21a(+)-CAT-Tip60 (221–512) or pET28a-PCAF (493–658) was transformed into BL21(DE3) competent cells through the heat shock method, respectively. The cells containing pET-21a(+)-FL-Tip60/CAT-Tip60 or pET28a-PCAF were spread on ampicillin or kanamycin treated agar plates, respectively, and incubated at 37 °C. Colonies were then harvested and grown in 8 mL then in 2 L cultures containing LB media and ampicillin or kanamycin at 37 °C. Protein expression was induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C for 20 h. Cells were harvested by centrifugation, suspended in lysis buffer (25 mM Na-HEPES pH 8, 150 mM NaCl, 1 mM MgSO₄, 5% glycerol, 5% ethylene glycol, and 1 mM PMSF) and then French pressed. The protein supernatant was purified on the Ni-NTA resin (Novagen). Before protein loading, Ni-NTA beads were equilibrated with column buffer (25 mM Na-HEPES pH 8, 500 mM NaCl, 30 mM imidazole, 10% glycerol and 1 mM PMSF). After protein loading, the column was washed thoroughly with washing buffer (25 mM Na-HEPES pH 8, 300 mM NaCl, 70 mM imidazole, 10% glycerol, and 1 mM PMSF) and the protein was eluted with elution buffer (25 mM Na-HEPES pH 7, 300 mM NaCl, 100 mM EDTA, 200 mM imidazole, 10% glycerol, and 1 mM PMSF). The elution fractions were individually checked on 12% SDS-PAGE to ensure the desired protein was present. The elution fractions were combined and dialyzed against dialysis buffer (25 mM Na-HEPES pH 7, 300 mM NaCl, 1 mM EDTA, 10% glycerol and 1 mM DTT), followed by concentration using Millipore centrifugal filters. Protein concentration was determined using Bradford assay. Final proteins were aliquoted and stored at –80 °C for future use.

Synthesis of inhibitors

Solid phase peptide synthesis (SPPS) was carried out on a PS3 peptide synthesizer using the Fmoc [N-(9-fluorenyl) methoxycarbonyl] strategy. A series of peptide inhibitors based on the H3–20, the first 20 amino acids of histone H3 (ac-ARTKQTARKSTGGKAPRKQL), were synthesized. Pre-loaded Leu Wang resins were used as solid phase. The amino acids

and coupling reagent HCTU [O-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] were weighed out with an equivalence ratio four times greater than the amount of resins. The removal of Fmoc group was achieved by using 20% V/V piperidine/DMF. The N-terminal of the peptide was capped with acetyl group using acetic anhydride. After the synthesis of peptide, the Dde group (dimethyldioxycyclohexylidene) on lysine 14 was cleaved with 2% hydrazine monohydrate in DMF for 2 h. The resins was treated with 10 equiv. of bromoacetic acid and 10 equiv. of DIC (N, N'-Diisopropylcarbodiimide) in DMF for 4 h, followed by washing and drying under vacuum. The bromo-containing peptide was then cleaved from the resin by treatment with 95% TFA, 2.5% triisopropylsilane and 2.5% H₂O for 5 h. The crude product was precipitated with cold ethyl ether, purified using reverse-phased HPLC and characterized by MALDI-MS. Conjugation of CoASH with bromo peptide was accomplished by mixing 1 equiv. of bromo-peptide with 2 equiv. of CoASH in a minimum amount of sodium phosphate buffer (100 mM, pH 8). The mixture was kept in darkness with shaking for 16 h. The compound containing Sme moiety was synthesized in the similar manner. 1 equiv. of the purified bromo-peptide was mixed with 1.5 equiv. of sodium thiomethoxide in the sodium phosphate buffer (100 mM, pH 8). The mixture was kept in darkness with shaking for 16 h.

The reaction mixtures were respectively subjected to reverse-phased-HPLC (C18, Varian) on a Varian Prostar HPLC system using linear gradient of H₂O/0.05% TFA (solvent A) versus acetonitrile/0.05% TFA (solvent B). UV detection wavelength was fixed at 260 nm. The purified compounds were dissolved in water and neutralized with NaOH. The concentrations of the compounds containing CoA were determined by UV absorption at 260 nm (extinction coefficient = 16,045 M⁻¹cm⁻¹). Concentrations of compounds without CoA moiety were determined based on the weight of the pure compound.

Radiometric HAT assay

The radioactive assay was carried out at 30 °C in the buffer containing 50 mM HEPES, pH 8.0, 0.1 mM EDTA and 1 mM DTT. The final reaction volume is 30 µL. In the assay, [¹⁴C]-acetyl CoA was used as the acetyl donor and the peptide H4–20 for Tip60 or H3–20 for PCAF containing the first 20 amino acids sequence of histone H4 or H3 was used as the substrate, respectively. For the inhibition assay, the inhibitors at a series of concentrations were incubated with [¹⁴C]-acetyl CoA and H4–20 for 5 min, followed by addition of HAT enzyme to initiate to the reaction. The reactions were quenched by spreading the reaction mixture on Whatman P81 filter disc. The filter discs were washed with 50 mM sodium bicarbonate (NaHCO₃), pH 9.0 and air dried. The amounts of radioisotope labelled products were quantified by liquid scintillation. The data were fitted to the Langmuir isotherm equation (Equation 1) to obtain the IC₅₀ value. Each assay for every inhibitor was repeated at least two times.

$$\text{Fractional activity} = 1 / (1 + [I] / IC_{50}) \quad (1)$$

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Figure 1.

The initial idea of designing multi-module inhibitors of Tip60. CD denotes chromodomain and CAT denotes the catalytic MYST domain of Tip60.

Figure 2.

The general scheme for the synthesis of the peptide-CoA inhibitors.

Figure 3.
Proposed binding model of the multivalent inhibitors for HAT Tip60 based on the experimental data.

Scheme 1.

Table 1

List of synthesized inhibitors.

Name	Sequence	Expected mass (Da)	Observed mass (Da)
H3K14CoA (a)	Ac-ARTKQTARKSTGGK(CoA)APRKQL	3034.8	3034.2
H3K14Br (b)	Ac-ARTKQTARKSTGGK(Br)APRKQL	2348.5	2348.4
H3K14sme (c)	Ac-ARTKQTARKSTGGK(Sme)APRKQL	2313.6	2314.1
H3K9me3K14CoA (d)	Ac-ARTKQTARK(me)3STGGK(CoA)APRKQL	3074.6	3075.3
H3K9me3K14sme (e)	Ac-ARTKQTARK(me)3STGGK(Sme)APRKQL	2355.2	2355.4
H3K4me1K14CoA (f)	Ac-ARTK(me)QTARKSTGGK(CoA)APRKQL	3048.1	3047.3
H3K4me1K14Br (g)	Ac-ARTK(me)QTARKSTGGK(Br)APRKQL	2361.6	2361.4
H3K4me1K9me3K14CoA (h)	Ac-ARTK(me)QTARK(me)3STGGK(CoA)APRKQL	3089.2	3089.4

Table 2

IC₅₀ of the compounds tested for FL-Tip60, CAT-Tip60 and PCAF.

Inhibitor	FL-Tip60 (μM)	CAT-Tip60 (μM)	PCAF (μM)
CoASH	9.1 ± 1.0	16.0 ± 3.1	8.4 ± 0.4
H3K14CoA (a)	9.0 ± 1.1	14.7 ± 0.9	0.35 ± 0.05
H3K14Br (b)	***	***	N/A
H3K14sme (c)	**	**	N/A
H3K9me3K14CoA (d)	1.2 ± 0.1	1.6 ± 0.2	0.28 ± 0.06
H3K9me3K14sme (e)	34.5 ± 2.0	27.4 ± 2.6	N/A
H3K4me1K14CoA (f)	2.1 ± 0.4	3.5 ± 0.1	1.1 ± 0.1
H3K4me1K14Br (g)	***	***	N/A
H3K4me1K9me3K14CoA (h)	3.8 ± 0.2	2.5 ± 0.1	0.33 ± 0.06

For the FL- or CAT-Tip60 catalyzed reaction: 1 μM of [¹⁴C]-AcCoA, 100 μM of H4-20 were used. For PCAF catalyzed reaction: 1 μM of [¹⁴C]-AcCoA, 100 μM of H3-20 were used.

*** : no inhibition was observed at 500 μM.

** : no inhibition was observed at 250 μM.

N/A: not tested.