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Histone Deacetylase 1 Inhibition by Peptides Containing a DNA Damage-Induced, Nonenzymatic, Histone Covalent Modification

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Abstract

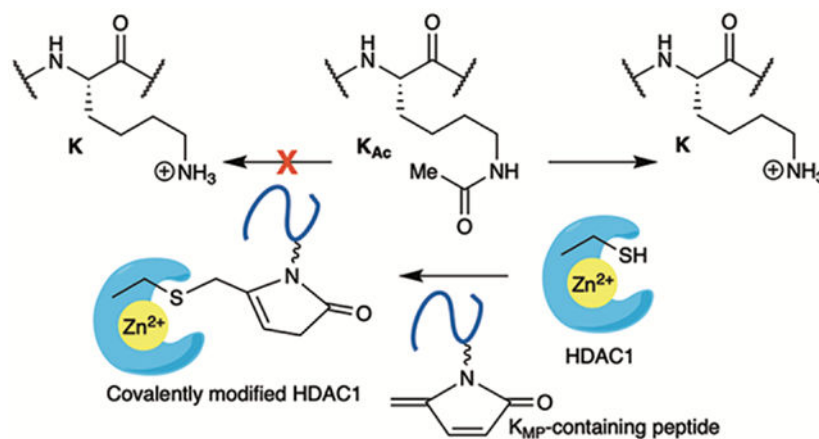
Treatment of HeLa cells with the DNA damaging agent, bleomycin, results in the formation of a nonenzymatic 5-methylene-2-pyrrolone histone covalent modification on lysine residues (K_{MP}). K_{MP} is much more electrophilic than other N-acyllysine covalent modification and post-translational modifications, including N-acetyllysine (K_{Ac}). Using histone peptides containing K_{MP} we show that this modification inhibits the class I histone deacetylase, HDAC1, by reacting with a conserved cysteine (C261) located near the active site. HDAC 1 is inhibited by histone peptides whose corresponding N-acetylated sequences are known deacetylation substrates, but not one containing a scrambled sequence. The HDAC1 inhibitor, trichostatin A, competes with covalent modification by the K_{MP} -containing peptides. HDAC1 is also covalently modified by a K_{MP} -containing peptide in a complex milieu. These data indicate that peptides containing K_{MP} are recognized by HDAC1 and are bound in the active site. The effects on HDAC1 indicate that K_{MP} formation in cells may contribute to the biological effects of DNA damaging agents, such as bleomycin that form this nonenzymatic covalent modification.

Graphical Abstract

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Supporting Information. Experimental details for all experiments. Scheme for HDAC1 activity assay, NMR spectra of compounds, LC-MS/MS data, peptide chromatograms. Supporting Information is available free of charge on the ACS Publications website.

Accession Codes. HDAC1 (UniProt entry: Q6IT96).



Introduction.

A variety of N-acyllysine post-translational modifications (PTMs) have been identified. Many are produced enzymatically (e.g. K_{Ac}, K_{Cr}), but some are formed nonenzymatically (e.g. K_{Fo}, K_{One}) (Scheme 1A) on histones and other proteins.¹ N-acetyllysine (K_{Ac}) is an enzymatic PTM that affects chromatin structure and plays important roles in regulating cellular processes, including transcription.^{2,3} Three families of proteins are involved in histone acetylation (Scheme 1B). Histone acetyl transferases (HATs) install K_{Ac}, which results in increased chromatin accessibility that is partly attributed to loss of positive charge, and a weakening of the interaction between DNA and histone proteins.^{2,4} K_{Ac} sites are read in chromatin by bromodomain containing proteins, which interact with protein complexes that control processes such as transcription. Acetyl removal from lysine by histone deacetylases (HDACs) turns off gene expression and favors chromatin compaction. Regulation of histone acetylation is important in human health. For instance, HDAC overexpression and mistargeting occur in a variety of cancers.⁵

HDAC1 is a member of the Class I family of histone deacetylases, which utilize Zn²⁺ to activate water for nucleophilic attack on N-acetyllysine (K_{Ac}).⁶ The list of N-acyllysine PTMs that are substrates for HDAC1 and other deacetylases is growing.⁷⁻⁹ The sirtuins, such as Sirt2, preferentially deacetylate N-acetyllysine in the histone H4 “basic patch” at H4K16. To a lesser extent, it also deacetylates H3K9Ac. SIRT2 displays a relatively broad N-acyllysine substrate-scope. For example, The 4-ketoamide histone adducts formed under oxidative stress during inflammation, such as K_{One} are substrates of Sirt2.^{10,11} Sirt2 is also shown to remove benzoyl groups in histone benzol-lysine (K_{Bz}).¹² Others such as the nonenzymatic covalent modification formylation (K_{Fo}), which is formed predominantly via initial reaction with formaldehyde is not reported to be an excellent substrate for HDACs.^{8,13} However, K_{Fo} could affect chromatin structure and gene expression. We recently reported HeLa cell treatment with bleomycin (BLM) results in formation of a DNA damage-induced, nonenzymatic covalent modification (NECM) of histones that contains an electrophilic 5-methylene-2-pyrrolone (K_{MP}, Scheme 1A,C).¹⁴ Herein, we characterize the interaction between K_{MP}-containing histone peptides and HDAC1. Our data suggest that

K_{MP}-containing histones could potentially covalently modify HDAC1 in cells and affect cellular processes.

K_{MP} forms concomitantly with a single-strand DNA break via histone lysine attack on the oxidized abasic site (C4-AP, Scheme 1C).^{15, 16} The half-life for the formation of K_{MP} in nucleosome core particles is as short as ~15 min. C4-AP is produced by ionizing radiation and several antitumor agents that oxidatively damage DNA, including bleomycin.^{17–20} K_{MP} was detected via chemoproteomic analysis at 17 of 57 core histone lysine residues in HeLa cells treated with bleomycin.¹⁴ The sites at which the NECM are determined by C4-AP location, and it is likely that K_{MP} distribution is even broader following treatment with more promiscuous DNA damaging agents, such as ionizing radiation. Of the 17 positions at which K_{MP} was detected, 15 are also lysine acetylation sites.²¹ Various deacetylases act on the group of acetylated lysines that overlap with sites at which K_{MP} is formed.⁶ We sought to address whether K_{MP} affects HDAC1 activity.

Results and Discussion.

Peptide design and preparation.

Histone H3K56 is a position at which K_{MP} is formed within the globular region of the octameric core upon treatment of HeLa cell with bleomycin.¹⁴ In vivo acetylation at H3K56 (H3K56Ac) affects chromatin structure and regulates transcription.^{22, 23} Cellular levels of H3K56Ac are regulated by HDAC1.²⁴ Similarly, histone H4K16Ac is an important acetylation site within the corresponding histone tail. H4K16Ac inhibits chromatin compaction and is also a substrate for HDAC1.^{25, 26} Consequently, we examined the interaction of histone peptides containing H3K56MP (**1a**) and H4K16MP (**2a**) with HDAC1 (Chart 1). A peptide comprised of the same amino acid composition as **2a**, but with scrambled sequence (**3a**) was also prepared. Peptides containing K_{MP} were synthesized via a method that distinguishes between multiple lysines using orthogonal ϵ -amine protecting groups in which the electrophilic 5-methylene-2-pyrrolone is introduced after peptide cleavage from the solid-phase support.¹⁴

HDAC1 inhibition by K_{MP}-containing histone peptides.

The possibility that K_{MP}-containing histone peptides are recognized by HDAC1 was explored using a commercially available fluorescence assay kit (Scheme S1) that takes advantage of trypsin cleavage of deacylated lysine (Figure 1). HDAC1 activity was measured following preincubation with peptides **1a** (Figure 1A) and **2a** (Figure 1B). The peptides exhibited comparable levels of inhibition between 0.5 and 50 μ M. HDAC1 activity was reduced by ~50% following preincubation with either peptide at the maximum concentration employed (50 μ M). However, the corresponding peptides (50 μ M) containing either lysine (**4a**, **6a**) or N-acetyllysine (**5a**, **7a**) had no effect on HDAC1 activity when incubated with the enzyme. Importantly, inhibition by the scrambled version of **2a** (**3**, Figure 1C, S4) was clearly evident only at 25 μ M of **3**. There also was no statistical difference in the extent of inhibition in the presence of 25 or 50 μ M of **3**, and the level of activity retained was considerably higher than when HDAC1 was preincubated with **1a** or **1b** at these same concentrations. The smaller effect observed utilizing the scrambled sequence

(3) supports the hypothesis that K_{MP} -containing peptides based on the H3K56 and H4K16 histone modifications are not due to the promiscuity of the electrophile. It also eliminates inactivation of trypsin, which is employed in the assay, as the source of the observed HDAC1 inhibition.

Covalent modification of HDAC1 by K_{MP} -containing histone peptides.

Electrophoretic analysis under denaturing conditions using fluorescently labeled versions of **1a** and **2a** (**1b**, **2b**) revealed that the peptides covalently modify HDAC1 (Figure 2A, S1). In contrast, the corresponding peptides containing free lysine (**4b**, **6b**) or N-acetyllysine (**5b**, **7b**) do not. Although HDAC1 contains 9 cysteine residues (Figure 2B), none play a direct role in catalysis. However, C261 and C273, which are conserved throughout the class I HDACs, are less than 10 Å from the zinc ion that activates water for nucleophilic attack on N-acetyllysine (Figure 2C).²⁷ These residues, which are modified by electrophilic small molecules seemed like good candidates for reaction with K_{MP} in histone peptides.²⁷ Following incubation of HDAC1 separately with **1a** (Figure 2D, Table S1) or **2a** (Figure S2), the protein was digested with trypsin and analyzed by LC-MS/MS. Following incubation with the histone peptide H3K56MP (**1a**), we identified a modified peptide encompassing HDAC1 residues V248-R270 that contains C261. The expected fragment containing a modification on C261 that consists of the portion of the K_{MP} -containing peptide that remains after trypsin treatment (y11, Figure 2D) was detected. Fragments that lack C261 (e.g. y9 and b13) are unmodified, consistent with incorporation of the modification at this amino acid position. Similarly, the same modified peptide that includes C261 within the HDAC1 residues V248-R270 was detected following incubation with H4K16MP (**2a**) (Figure S2). MS2 spectra identified fragment ions establishing the modification on C261 (y10-y19 and b15, Figure S2). Fragment ions lacking C261 (e.g. y3-y6 and b2-b11) further support the assignment of the modification at this position. In contrast, no peptides containing a modification at C273 were detected following incubation with **1a** or **2a**; nor was modification of any of the other 7 cysteine residues, some of which are surface exposed, observed.

Covalent modification of C261, which is proximal to the HDAC1 active site supports the hypothesis that peptides **1a** and **2a** are bound at or near this region of the enzyme.

Furthermore, the lack of detection of modification of any of the other 8 cysteine residues suggest that covalent modification is not due to nonselective, promiscuous reactions of the electrophilic K_{MP} -containing histone peptides with the deacetylase.

An HDAC1 competitive inhibitor prevents covalent modification by a K_{MP} -containing histone peptide.

LC-MS/MS analyses of trypsin digests are consistent with recognition of the K_{MP} -containing histone peptides in a comparable manner as the corresponding N-acetyllysine molecules, and that K_{MP} is positioned within or near the active site. This hypothesis was explored further by examining the ability of trichostatin A (TSA) to inhibit HDAC1 modification by the K_{MP} -containing histone peptides. TSA is a small molecule competitive inhibitor of HDAC1 that binds in the N-acetyllysine binding site. Although **1b** and **2b**

covalently bind HDAC1, we anticipated that the small molecule could decrease covalent modification by the K_{MP} -containing histone peptides that interact with the active site due to its tight binding ($K_D = 12$ nM).²⁸ HDAC1 (0.1 μ M) was preincubated (10 min) with 0.5 or 5 μ M TSA prior to adding a K_{MP} -containing histone peptide. The yield of covalently modified HDAC1 with the fluorescently labeled H3K56MP (**1b**, Figure 2E) or H4K16MP (**2b**, Figure S3) peptide decreased by similar amounts when preincubated with TSA and the decrease was dependent on TSA concentration. Preincubation with 5 μ M TSA reduced covalent modification by ~90%. These data are consistent with peptide binding in the N-acetyllysine binding site. Importantly, the observation that only ~10% of the covalent modification remains after preincubation with TSA is consistent with non-promiscuous HDAC1 modification by the K_{MP} -containing histone peptides. Although, we are unable to measure the binding constant for HDAC1 recognition of K_{MP} -containing peptides, the fact that HDAC1 cross-linking persists even in the presence of TSA present at almost 1,000-times its K_D suggests that peptides containing the modification are bound reasonably strongly by the enzyme. It also suggests that proteins containing K_{MP} will also be effective at covalently modifying HDAC1 in cells.

Covalent modification of HDAC1 by a K_{MP} -containing histone peptide in nuclear lysate.

The ability of biotinylated **1** (**1c**) to covalently modify HDAC1 in a complex milieu was examined using HeLa nuclear lysate. Lysate was incubated with **1c** or the corresponding biotinylated free lysine (**4c**) or N-acetyllysine (**5c**) peptides (10 μ M). After pulling down the biotinylated substrates using Neutravidin resin, vigorous washing and release, the eluted materials were interrogated via Western blot analysis using anti-HDAC1 antibody (Figure 2F). Consistent with the above experiments, HDAC1 was detected when lysate was incubated with K_{MP} -containing histone peptide (**1c**) but not the unmodified (**4c**) or N-acetyllysine peptides (**5c**). Hence, covalent modification of HDAC1 in this complex milieu is consistent with a selective and strong interaction with the K_{MP} modified peptide (**1c**).

Summary

K_{MP} is one of a growing family of NECMs.^{29–33} Notably, K_{MP} is part of a subset of NECMs that are sufficiently electrophilic to react further with nucleophilic amino acid residues. Covalent modification of HDAC1 by K_{MP} -containing peptides reflects its greater electrophilicity than other N-acylated lysine histone modifications. Although such a reaction (Scheme 1C) is reversible in the presence high thiol concentration (20 mM), the adducts were stable under physiological conditions in our hands.³⁴ The recalcitrance of K_{Cr} to react with thiols is reflected by the covalent capture of proteins containing it by phosphine-containing nucleophilic probes.³⁵ However, an engineered Sirtuin containing an unnatural thiol amino acid does covalently capture K_{Cr} .³⁶ N-formyllysine (K_{Fo}), which is refractory to repair by HDAC1, also does not react with cysteine residues in proteins.^{9, 13} Covalent modification of HDAC1 by K_{MP} -modified histone proteins in cells could result in aberrant gene expression. K_{MP} formation is a secondary effect in cells treated with bleomycin, a cytotoxic DNA damaging agent.¹⁴ To our knowledge, it is not known whether bleomycin treatment affects cellular HDAC1 activity or expression level. However, pulmonary fibrosis

is a side reaction of bleomycin. Increased levels of HDAC2 (Class I histone deacetylase) and HDAC4 have been detected in mouse lung fibroblasts treated with bleomycin.³⁷ Although it is not known whether K_{MP} contributes to this effect, the observations described above indicate that DNA damaging agents that produce C4-AP could have downstream effects in cells that warrant investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

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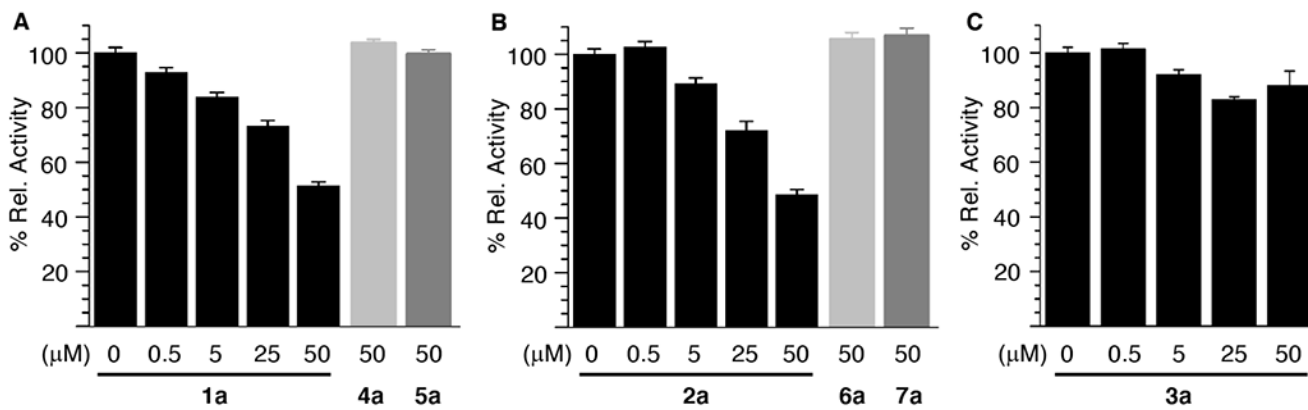
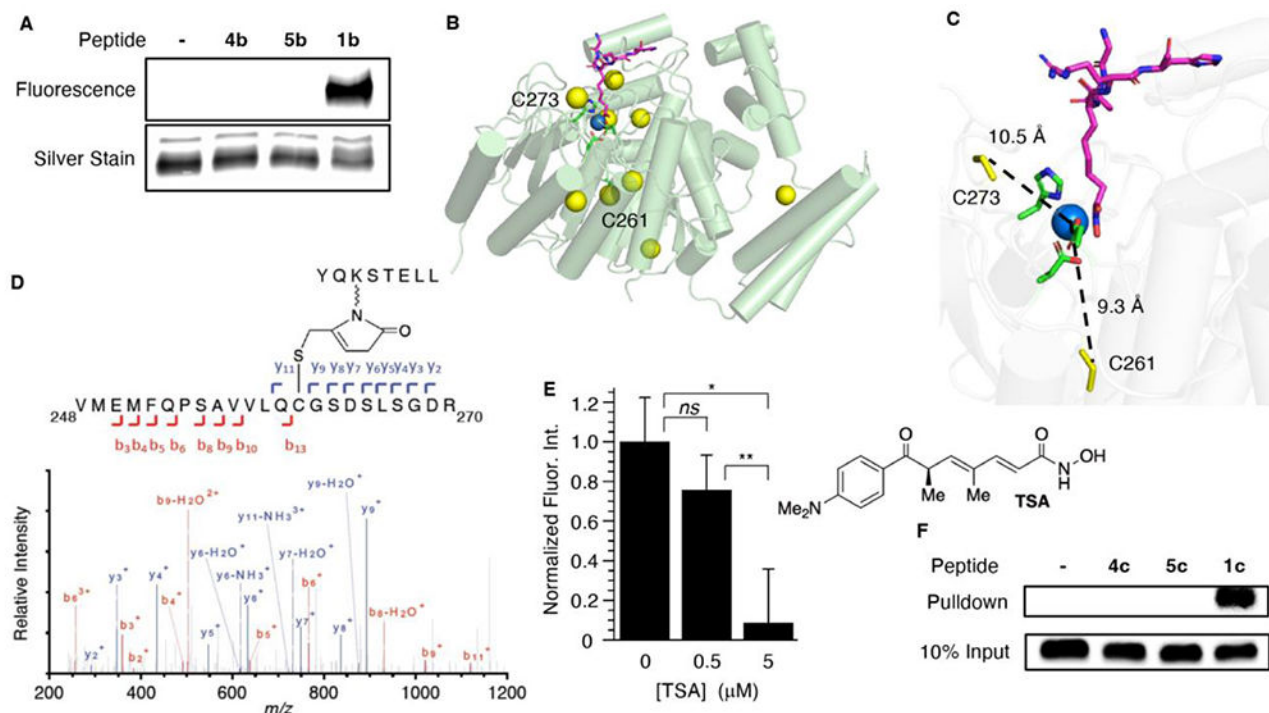
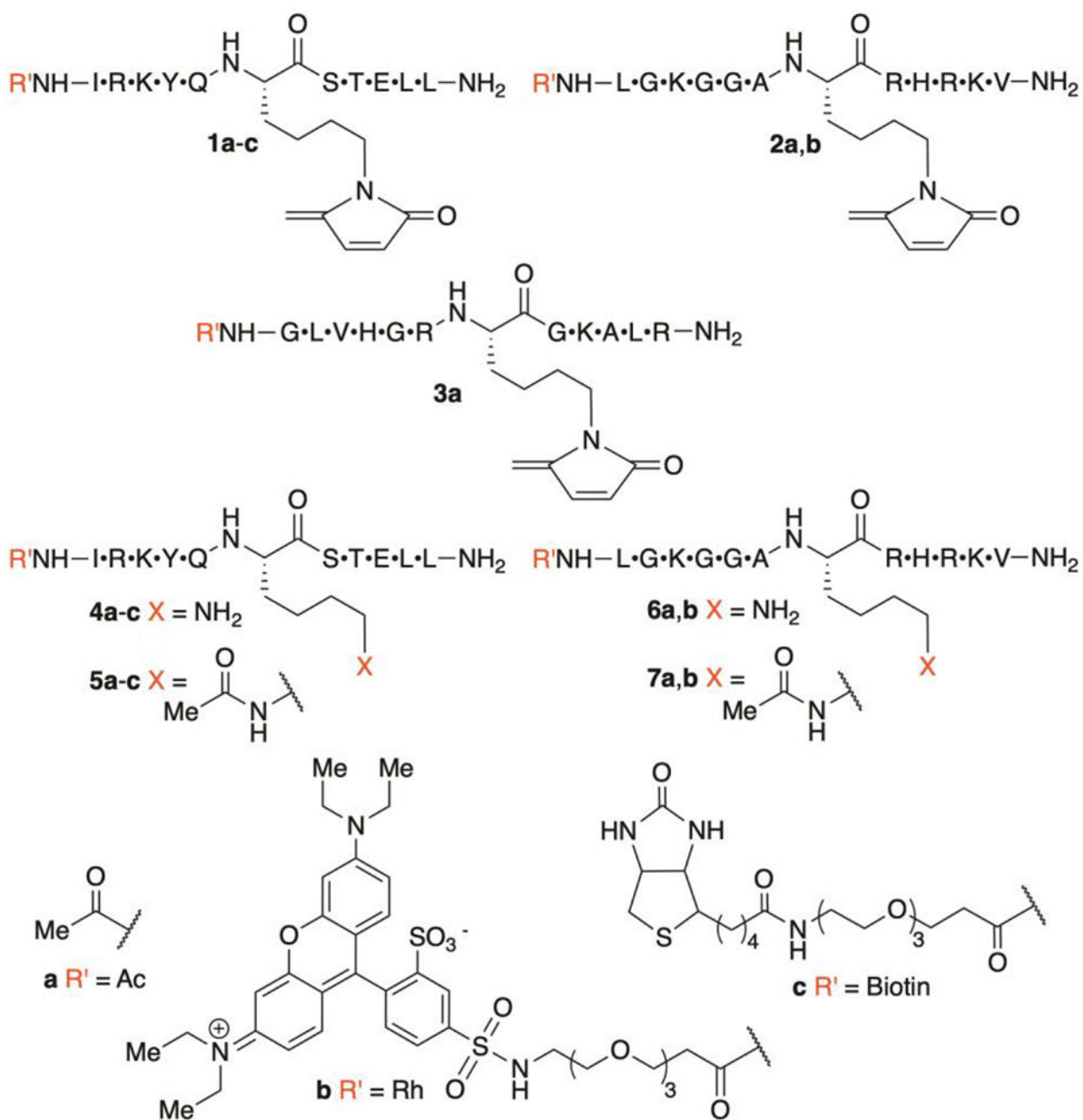


Figure 1.

Effect of K_{MP} -containing histone peptides on HDAC1 activity. A) Histone peptides containing H3K56 (**1a**, **4a**, **5a**) B) Peptides containing H4K16 (**2a**, **6a**, **7a**) C) Histone peptides containing H3K16 with sequence scrambled (**3**) [HDAC1] = 10 nM. 100% Relative activity was determined by comparing enzymatic activity with and without peptide. Data are presented as the ave. \pm std. dev. (n=3). For indicators of statistical significance see Figure S4.

**Figure 2.**

Covalent modification of HDAC1 by a K_{MP} -containing peptide (H3K56MP). A) Covalent modification of HDAC1 (0.1 μ M) by fluorescently labeled K_{MP} -containing histone peptide (**1b**, 50 μ M). B) X-ray crystal structure of HDAC1 bound with a peptide inhibitor (PDB: 5ICN), showing active site zinc (blue) and 9 cysteine residues (yellow). C) Active site showing the distance between Zn^{2+} and C261, C273. D) LC-MS/MS analysis of HDAC1 trypsin fragment V₂₄₈-R₂₇₀ that is covalently modified by a histone peptide containing H3K56MP (**1a**). E) Trichostatin A (TSA) competition for covalent modification of HDAC1 (100 nM) by K_{MP} -containing histone peptide H3K56MP (**1b**, 5 μ M). Average fluorescence intensity of reaction in the absence of TSA is normalized to 1.0. Data are presented as the ave. \pm std. dev. (n=3). (ns) indicates not significantly different, (*) indicates $p < 0.0005$ and (**) indicates $p < 0.005$. F) Covalent modification of HDAC1 by histone peptide containing H3K56MP (**1c**, 10 μ M) in HeLa cell nuclear lysate. Detection is carried out using anti-HDAC1 antibody. HDAC1 migrates at \sim 60 kDa on the gel (Figure S1).



Scheme 1.
N-Acyllysine histone modifications.

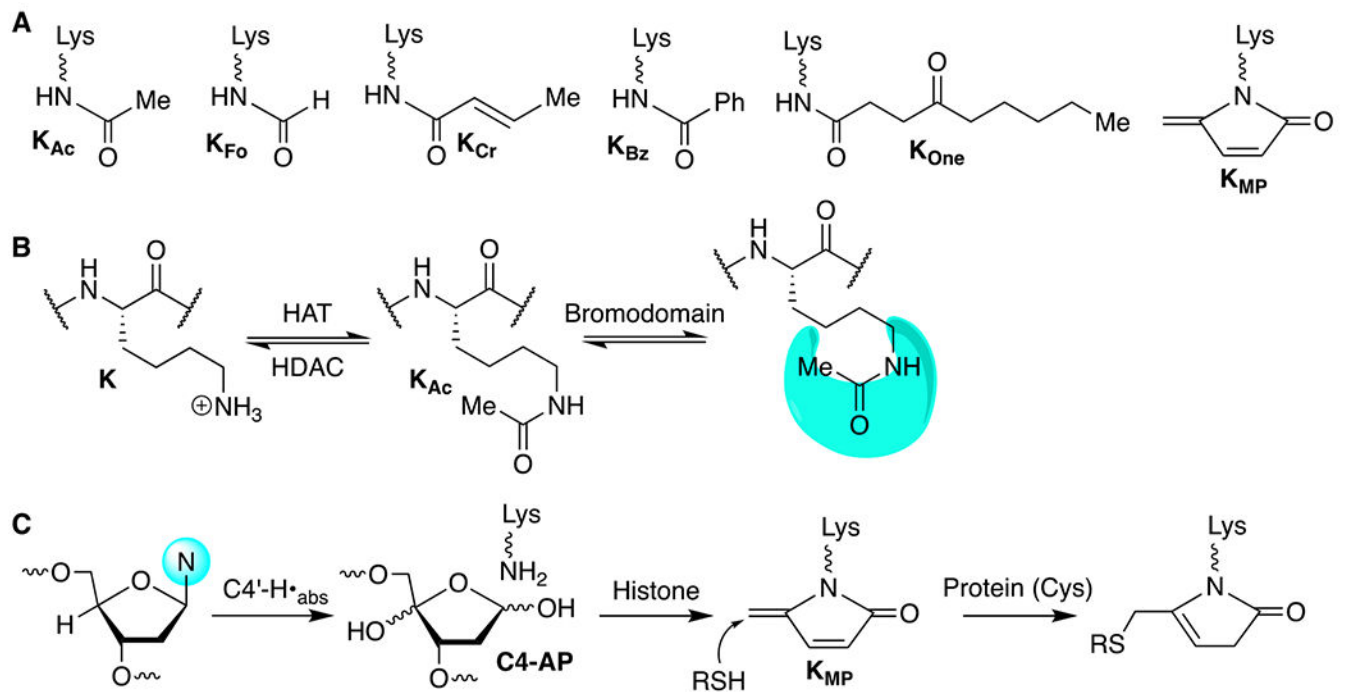


Chart 1.
Histone peptides used in these experiments.