N^{ε}-Methanesulfonyl-lysine as a non-hydrolyzable functional surrogate for N^{ε}-acetyl-lysine†

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Through parallel studies on peptides containing N^{ε}-methanesulfonyl-lysine or N^{ε}-acetyl-lysine, N^{ε}-methanesulfonyl-lysine as a replacement for N^{ε}-acetyl-lysine was shown i) not to compromise the binding affinity for a bromodomain, ii) to confer resistance to human HDAC8 and SIRT1 (two distinct protein deacetylases), and iii) to confer only weak inhibition against human HDAC8 and SIRT1. These results suggested N^{ε}-methanesulfonyl-lysine as a non-hydrolyzable functional surrogate for N^{ε}-acetyl-lysine.

Protein post-translational reversible lysine N^{ε}-acetylation and deacetylation has been recognized as an emerging intracellular signaling mechanism that plays critical roles in regulating gene transcription, cell-cycle progression, apoptosis, DNA repair, and cytoskeletal organization.[1,2] Acetyltransferase-catalyzed creation, deacetylase-catalyzed destruction, and bromodomain-mediated specific recognition of N^{ε}-acetyl-lysine on proteins define the central events of this signaling mechanism (Fig. 1).[1–3] Anti-cancer therapeutic potential can thus be expected by modulating these events. Indeed, several inhibitors for the classical (or Zn^{2+}-dependent) protein deacetylases are being evaluated in clinical trials for their anti-cancer potential.[4] However, further mechanistic details and therapeutic potentials of this signaling mechanism are to be defined and revealed. Due to the fact that N^{ε}-acetyl-lysine serves as the key recognition motif of this signaling mechanism, we reasoned that its analogs may help develop novel chemical modulating strategies and modulators that could provide potential therapeutics and chemical tools for a molecular dissection of this signaling mechanism. In this study, we developed the first non-hydrolyzable (or intracellular protein deacetylase-resistant) functional surrogate, *i.e.* N^{ε}-methanesulfonyl-lysine, for N^{ε}-acetyl-lysine regarding the bromodomain binding interaction (Fig. 2). Similar to the well-established applications of non-hydrolyzable analogs of phosphorylated tyrosine/serine/threonine,[5] the availability of N^{ε}-methanesulfonyl-lysine will promote the development of novel inhibitors of bromodomain–N^{ε}-acetyl-lysine recognition, whose chemical modulating strategies and modulators are still under-developed as compared to other events involved in this signaling mechanism.[6] Furthermore, the incorporation of N^{ε}-methanesulfonyl-lysine into proteins by protein engineering techniques such as unnatural amino acid mutagenesis[7] and expressed protein ligation[8] should provide the constitutive phenotype of protein acetylation, thus facilitating the functional examination of this type of important protein post-translational modification.

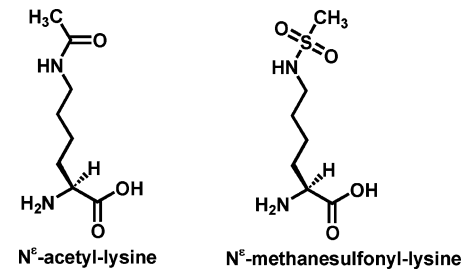

Fig. 2 Structural comparison of N^{ε}-acetyl-lysine and N^{ε}-methanesulfonyl-lysine.

Our design of N^{ε}-methanesulfonyl-lysine as a non-hydrolyzable functional surrogate for N^{ε}-acetyl-lysine was initially inspired by previous literature reports demonstrating the resistance to proteases and peptidases as a result of the sulfonamide replacement for a peptide bond.[9] For our design, we also paid particular

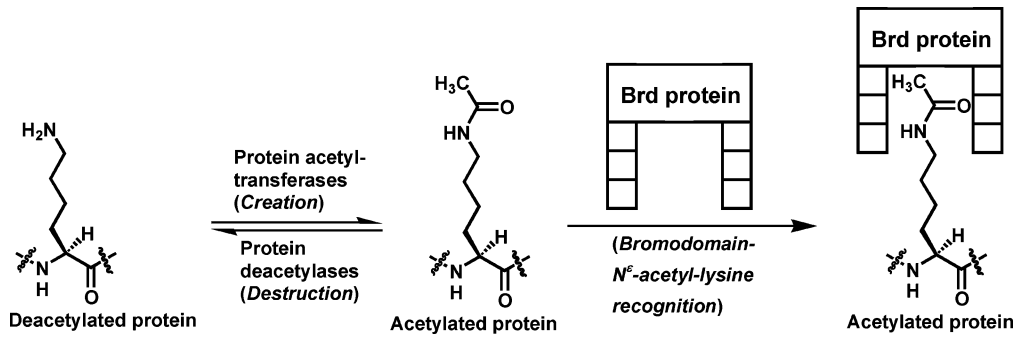

Fig. 1 Acetyltransferase-catalyzed creation, deacetylase-catalyzed destruction, and bromodomain-mediated specific recognition of N^{ε}-acetyl-lysine on proteins. Brd protein: bomodomain-containing protein.

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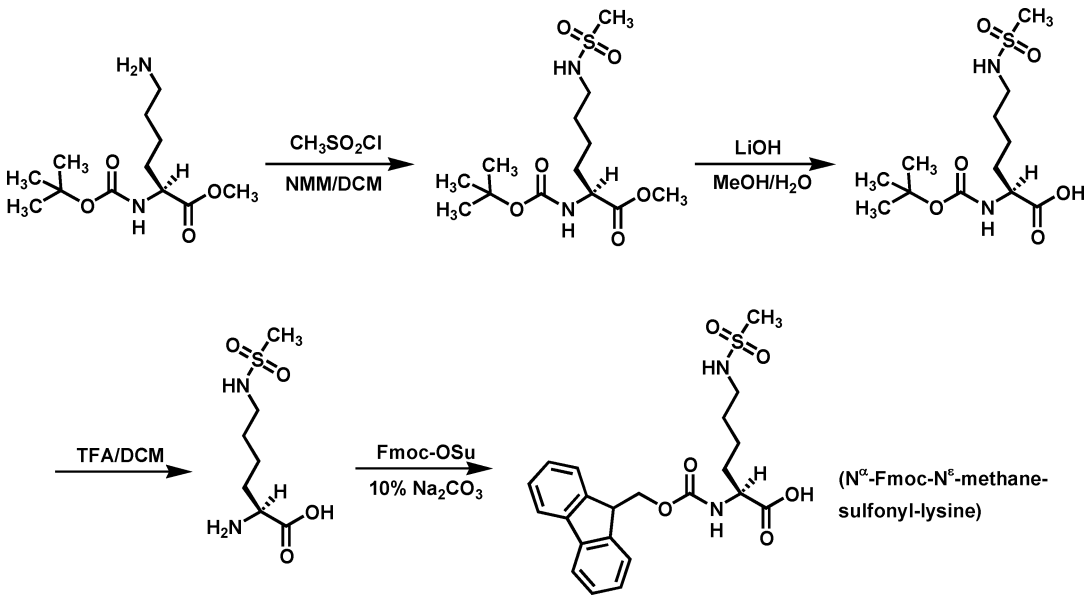

Scheme 1 Synthesis of N^{α}-Fmoc-N^{ε}-methanesulfonyl-lysine.

attention to the bromodomain–N^{ε}-acetyl-lysine interactions because bromodomain-mediated specific recognition of N^{ε}-acetyl-lysine serves as one major function for the creation of N^{ε}-acetyl-lysine on proteins. The bromodomain represents a family of N^{ε}-acetyl-lysine binding protein modules that contain ~110 amino acid residues and are present in many chromatin-associated proteins.[3] Bromodomains can bind to both lysine N^{ε}-acetylated histone and non-histone proteins such as the human p53 tumor suppressor protein.

The three-dimensional structures have been solved for four bromodomains either in complex with lysine N^{ε}-acetylated peptides or in apo form, including those of CBP, PCAF, and GCN5 proteins by NMR and those of GCN5 and $TAF_{II}250$ proteins by X-ray crystallography.[3c,3d,10] Together with biochemical studies, these structural studies demonstrated that specific recognition of N^{ε}-acetyl-lysine is a conserved function of all bromodomains found in different proteins, and revealed the molecular details of the binding interactions within the N^{ε}-acetyl-lysine binding pocket. Besides extensive hydrophobic interactions with the aliphatic portion of the N^{ε}-acetyl-lysine side chain, two hydrogen bonding interactions play a key role for the specific recognition of N^{ε}-acetyl-lysine by a bromodomain, *i.e.* those involving acetamide NH as the hydrogen bond donor and acetamide C=O as the hydrogen bond acceptor.[10c] By replacing acetamide with methanesulfonamide, these two key hydrogen bonding interactions should thus be maintained.

By employing the C-terminal peptide (amino acid residue 372–389: H_2N-KKGQSTSRHK-(K^{382})LMFKTEG-COOH) of the human p53 tumor suppressor protein as the template, N^{ε}-acetyl-lysine and N^{ε}-methanesulfonyl-lysine were respectively substituted for K^{382}, the N^{ε}-acetylation of which has been shown to recruit the bromodomain of CBP, thus enhancing the transcriptional activity of the p53 protein.[3d] Scheme 1 shows the synthesis of N^{α}-Fmoc-N^{ε}-methanesulfonyl-lysine that was the building block for incorporating N^{ε}-methanesulfonyl-lysine into a peptide sequence by the Fmoc chemistry-based solid phase peptide synthesis (SPPS).[11]

The resulting two peptides (*i.e.* the N^{ε}-acetyl-lysine-containing and the N^{ε}-methanesulfonyl-lysine-containing p53 peptides (peptides **2** and **3**, respectively)) were first evaluated, together with the negative control peptide (*i.e.* the K^{382}-containing p53 peptide (peptide **1**)), for their relative binding affinities for the CBP bromodomain in an *in vitro* GST pull-down assay.[3d] Briefly, the immobilized GST-bromodomain (onto the glutathione-agarose beads) was incubated with different test peptides, and the peptides retained on the immobilized GST-bromodomain after washing were detected and quantified by reversed phase high pressure liquid chromatography (RP-HPLC). Fig. 3 shows the three representative HPLC chromatograms from one of the three independent experiments. Although it is clear from Fig. 3 that only a modest retention of peptides was observed, and, for the three independent experiments, we obtained different HPLC peak areas for each of the three test peptides (*i.e.* peptides **1**, **2**, and **3** in Fig. 3) due to the use of different batches of GST-bromodomain with different degrees of immobilization onto the glutathione-agarose beads, nearly identical peptide retention ratios were obtained in each of these three experiments, with the average peptide retention ratio being 1.0 : (1.53 ± 0.07) : (3.25 ± 0.08) for peptides **1**, **2**, and **3** (mean ± standard deviation, based on the integrated peak areas from the three independent experiments). This result should reliably demonstrate that peptides **2** and **3** showed greater binding to the CBP bromodomain than peptide **1**. This result further suggested that N^{ε}-methanesulfonyl-lysine can be employed to replace N^{ε}-acetyl-lysine without a loss of binding affinity for a bromodomain. It should be noted that peptides **1** and **2** were used by us[12] and others[13] previously for protein deacetylase assays and/or X-ray crystallographical studies.

Peptides **1**, **2**, and **3** were further evaluated in protein deacetylase assays to assess the resistance of peptide **3** to protein deacetylases (Fig. 4). Peptide **1** was used as the synthetic authentic deacetylation peptide product, whereas peptide **2** was employed as the positive control. As described previously,[12] human HDAC8 (histone deacetylase 8 named after its first discovered protein

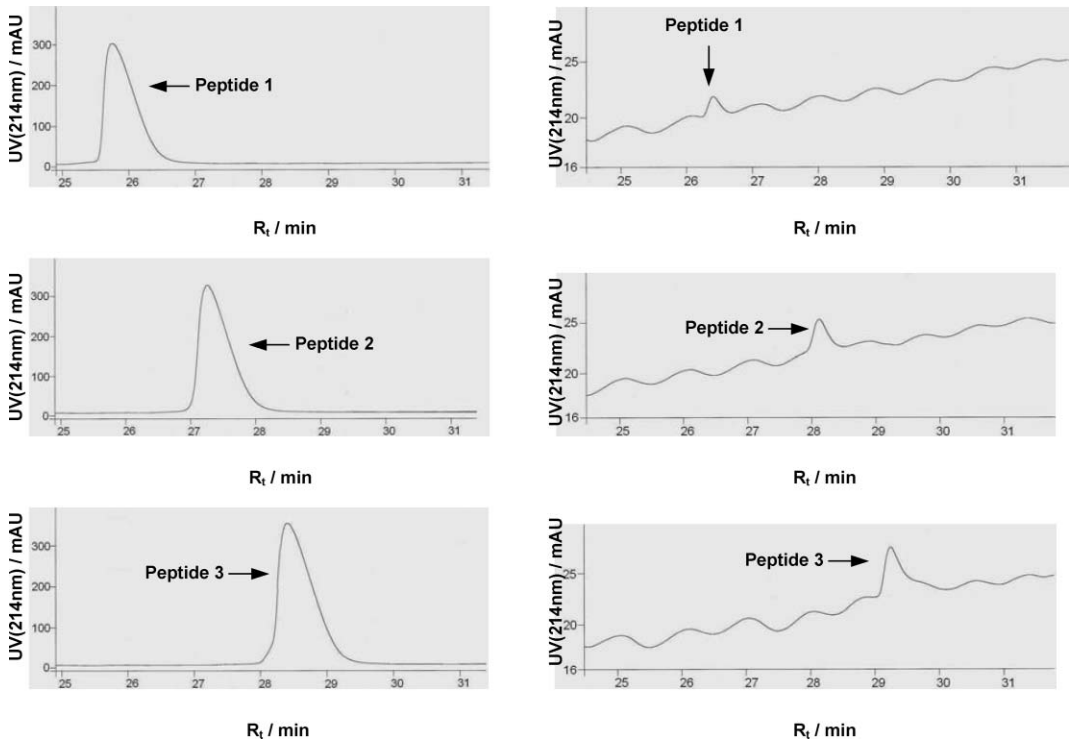

Fig. 3 Analytical RP-HPLC analysis of the GST pull-down assay to evaluate the relative binding affinities of peptides **1**, **2**, and **3** for the CBP bromodomain. Left: three chromatograms denote the purified peptides **1**, **2**, and **3** (100 μM each with essentially the same observed integrated peak areas). The same amount of input was used for peptides **1**, **2**, and **3** in the GST pull-down assay. Peptide sequences are H_2N-KKGQSTSRHK**X**LMFKTEG-COOH with **X** = Lys (peptide **1**), N^{ε}-acetyl-lysine (peptide **2**), and N^{ε}-methanesulfonyl-lysine (peptide **3**). Right: three chromatograms denote peptides **1**, **2**, and **3** that were retained on the immobilized GST-bromodomain after washing. Retained peptides were detected by comparison with authentic samples and the unbound peptides from the same incubation experiments. Peptide peak areas were obtained by integration with the Interactive Graphics software of Varian Inc.[17] Noise contributions were subtracted.

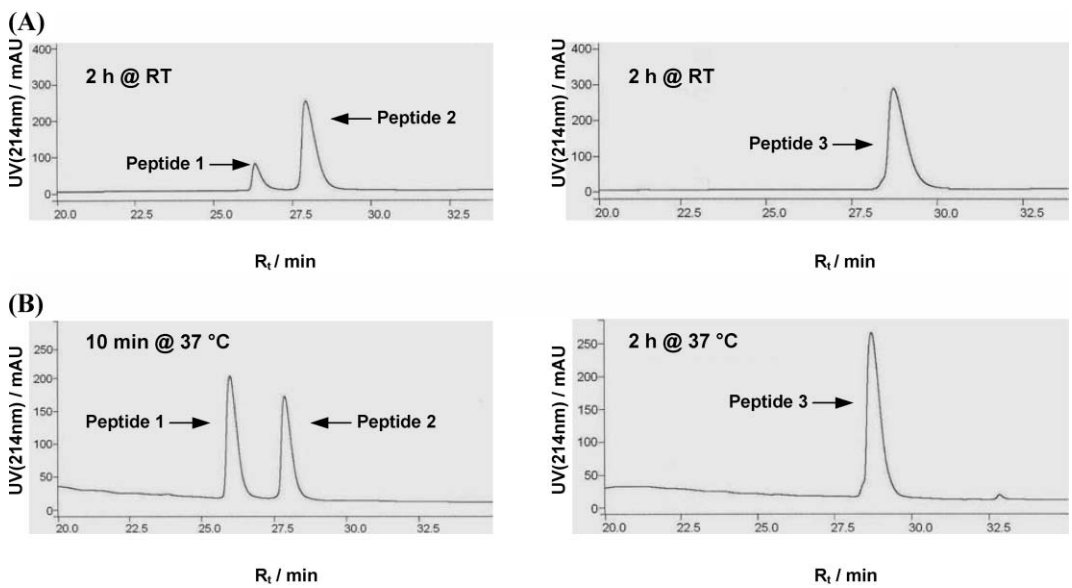

Fig. 4 Protein deacetylase assays. A) Representative HPLC chromatograms from a HDAC8 assay; B) representative HPLC chromatograms from a SIRT1 assay. All assays were performed in duplicate and essentially the same HPLC chromatograms were obtained for duplicates.

substrate histone) and human SIRT1 (Sirtuin type 1) were chosen as representative members respectively from the Zn^{2+}-dependent and the NAD^+-dependent subfamilies of protein deacetylase enzymes.[2c–2e] Both peptides **2** and **3** were incubated for 2 h at room temperature in the HDAC8 assays, but no product peptide (*i.e.* peptide **1**) formation from peptide **3** was detected (Fig. 4A). For the SIRT1 assay, a 10 min incubation at 37 °C already resulted in over 50% substrate turnover from peptide **2**. However, no product peptide **1** formation from peptide **3** was detected even after a 2 h incubation at 37 °C (Fig. 4B). These results indicated that the N^{ε}-methanesulfonyl-lysine replacement for N^{ε}-acetyl-lysine conferred resistance to both HDAC8 and SIRT1, and suggested that this replacement could confer resistance to both the Zn^{2+}-dependent and the NAD^+-dependent protein deacetylase enzymes because the catalytic domains are highly conserved among members within each of these two subfamilies of deacetylase enzymes.[2c–2e]

Peptide **3** was further evaluated as a potential inhibitor for SIRT1- and HDAC8-catalyzed deacetylation of peptide **2** to determine if the N^{ε}-methanesulfonyl-lysine replacement for N^{ε}-acetyl-lysine could confer inhibition against protein deacetylase enzymes. In our opinion, a weak inhibition could be advantageous in furnishing a useful non-hydrolyzable functional surrogate for N^{ε}-acetyl-lysine with minimal side effects arising from the inhibition of protein deacetylase enzymes because these enzymes accept a plethora of intracellular proteins as their substrates.[2c–2l] Peptide **3** was found to inhibit SIRT1 and HDAC8 with IC_{50} values being ~1000 μM and ~450 μM, respectively. These IC_{50} values indicated that peptide **3** only weakly inhibited SIRT1 and HDAC8, suggesting that the N^{ε}-methanesulfonyl-lysine replacement for N^{ε}-acetyl-lysine could have perturbed the binding at the SIRT1 active site (of note, the K_M of peptide **2** for SIRT1 was measured to be 48 μM, Fatkins and Zheng, unpublished results) and did not furnish a transition state analog inhibitor for HDAC8. This latter suggestion is also consistent with the previously reported inability of sulfonamide and tetrahedral phosphorus-containing derivatives of suberoyl anilide hydroxamic acid (SAHA) to serve as transition state analog inhibitors for the Zn^{2+}-dependent protein deacetylase-catalyzed deacetylation reactions.[14] These results further suggested that the N^{ε}-methanesulfonyl-lysine replacement for N^{ε}-acetyl-lysine could only confer weak inhibition against both the Zn^{2+}-dependent and the NAD^+-dependent protein deacetylase enzymes because, as stated above, the catalytic domains are highly conserved among members within each of these two subfamilies of deacetylase enzymes.[2c–2e]

Taken together, the results in this study suggested that the N^{ε}-methanesulfonyl-lysine replacement for N^{ε}-acetyl-lysine i) did not compromise the binding affinity for the bromodomain, ii) conferred resistance to protein deacetylases, and iii) conferred only weak inhibition against protein deacetylases. Furthermore, under physiological conditions, the side chains of N^{ε}-methanesulfonyl-lysine and N^{ε}-acetyl-lysine should both be present predominantly as the neutral species because the acetamide and the methanesulfonamide have pK_a values of ~15–16 and ~11–12, respectively, for their ionizable NH's.[15] Therefore, we have identified N^{ε}-methanesulfonyl-lysine as the first non-hydrolyzable (or intracellular protein deacetylase-resistant) functional surrogate for N^{ε}-acetyl-lysine. Future work will address the exciting applications that the availability of this non-hydrolyzable analog offers, especially in the following two specific areas.

i) The availability of this non-hydrolyzable analog will promote the development of novel inhibitors of the bromodomain–N^{ε}-acetyl-lysine recognition. The currently available biochemical and structural studies for bromodomains demonstrated not only that specific recognition of N^{ε}-acetyl-lysine is a conserved function of all bromodomains, but also that individual bromodomains in different proteins maintain selective recognition of their cognate acetylated target sequences *via* specific recognition of those amino acid residues surrounding N^{ε}-acetyl-lysine.[3c,3d,10] Rationally designed peptides that incorporate N^{ε}-methanesulfonyl-lysine will thus be expected to be selective competitive inhibitors of the bromodomain–N^{ε}-acetyl-lysine recognition suitable for cellular studies. The availability of various types of protein transduction domain (PTD) peptides[16] should promote the cellular applications of peptide inhibitors by carrying them through cellular membranes. This should be complementary to the approach through structure-based chemical library screening that was employed in the only two currently documented reports of the inhibitors for bromodomain–N^{ε}-acetyl-lysine recognition, *i.e.* those for inhibiting PCAF and CBP bromodomains.[6] ii) The incorporation of this non-hydrolyzable analog into proteins by protein engineering techniques such as unnatural amino acid mutagenesis[7] and expressed protein ligation[8] should provide the constitutive phenotype of protein acetylation, thus facilitating the functional examination of this type of important protein post-translational modification.

Acknowledgements

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