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Design, Synthesis, and Kinetic Characterization of Protein N-Terminal Acetyltransferase Inhibitors

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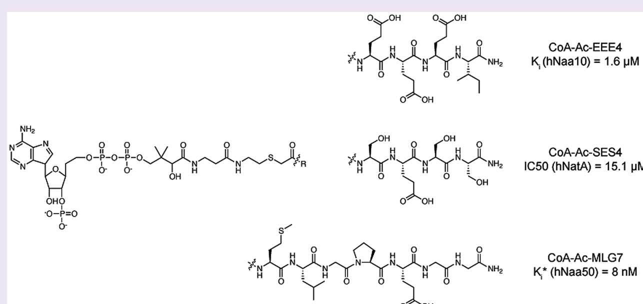
Supporting Information

ABSTRACT: The N-termini of 80−90% of human proteins are acetylated by the N-terminal acetyltransferases (NATs), NatA−NatF. The major NAT complex, NatA, and particularly the catalytic subunit hNaa10 (ARD1) has been implicated in cancer development. For example, knockdown of hNaa10 results in cancer cell death and the arrest of cell proliferation. It also sensitized cancer cells to drug-induced cytotoxicity. Human NatE has a distinct substrate specificity and is essential for normal chromosome segregation. Thus, NAT inhibitors may potentially be valuable anticancer therapeutics, either directly or as adjuvants. Herein, we report the design and synthesis of the first inhibitors targeting these enzymes. Using the substrate specificity of the enzymes as a guide, we synthesized three bisubstrate analogues that potently and selectively inhibit the NatA complex (CoA-Ac-SES4; IC_{50} = 15.1 μM), hNaa10, the catalytic subunit of NatA (CoA-Ac-EEE4; K_i = 1.6 μM), and NatE/hNaa50 (CoA-Ac-MLG7; K_i* = 8 nM); CoA-Ac-EEE4 is a reversible competitive inhibitor of hNaa10, and CoA-Ac-MLG7 is a slow tight binding inhibitor of hNaa50. Our demonstration that it is possible to develop NAT selective inhibitors should assist future efforts to develop NAT inhibitors with more drug-like properties that can be used to chemically interrogate *in vivo* NAT function.



Protein acetylation has received significant attention in the past decade due to its important roles in regulating eukaryotic cell signaling. Of the various residues known to be acetylated *in vivo*, lysine acetylation has received the most attention due to its critical role in controlling gene transcription and how dysregulation of this process contributes to the onset and progression of disease. While the importance of lysine acetylation is clear, it is now evident that N-terminal (Nt)-acetylation, the second major type of protein acetylation, also plays a critical role in eukaryotic cell signaling. This is the case because the majority of eukaryotic proteins are Nt-acetylated, mostly cotranslationally,[1] and the functional consequences of this modification are quite diverse,[2] including (i) degradation of Nt-acetylated proteins by a novel branch of the N-end rule pathway;[3] (ii) inhibition of post-translational ER-translocation;[4] (iii) protein complex formation;[5] and (iv) protein targeting to membranes.[6,7]

Nt-acetylation occurs when the acetyl moiety of acetyl coenzyme A (Ac-CoA) is transferred to the α-amino group of a polypeptide by one of the six N-terminal acetyltransferases (NATs), i.e., NatA−NatF. Each NAT is composed of one or more distinct subunits, and these enzymes acetylate specific subsets of proteins that are largely defined by their N-terminal amino acid sequence.[1] For example, the highly conserved NatA complex, which is composed of the catalytic subunit hNaa10 (Ard1) and the nonenzymatic auxiliary subunit Naa15 (Nat1/NATH),[8−11] acetylates N-termini starting with Ser, Ala, Thr, Gly, Val, and Cys; NatA acts on these N-termini after the initiator Met (iMet) has been removed by methionine aminopeptidases.[11,12] Interestingly, the substrate specificity of hNaa10 shifts when tested independently of hNaa15, such that on its own, hNaa10 acetylates acidic actin N-termini *in vitro*.[13] Although hNaa50 (Nat5/San), the catalytic subunit of NatE, interacts with the NatA complex,[14,15] it is considered a separate NAT because it acetylates a distinct group of substrates starting with the iMet.[13,16]

A growing number of studies report the upregulated expression, at both the mRNA and protein level, of the two main components of the NatA complex (i.e., hNaa10 and hNaa15) in several different cancer types, suggesting that

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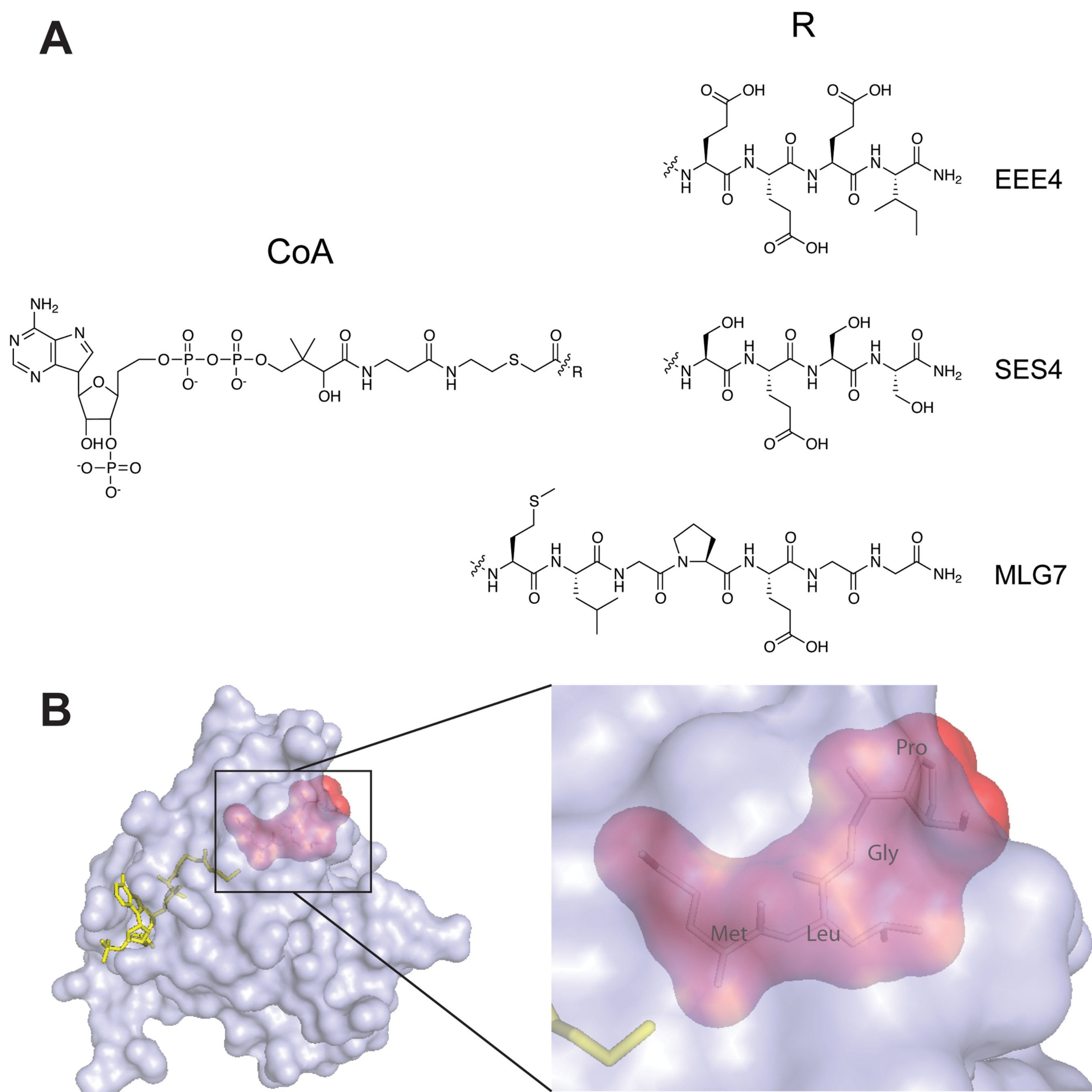

Figure 1. Inhibitor design. (A) Structures of bisubstrate analogue-based inhibitors targeting hNaa10, the hNatA complex, and hNaa50. CoA is coupled to a peptide through an acetamide linker. The peptides were based on known substrates of the NAT enzymes, γ-actin (EEEI) for hNaa10, high mobility group protein A1 (SESS) for hNatA, and hnRNP F (MLGPEGG) for hNaa50/hNatE. (B) Crystal structure of the CoA·hNaa50·peptide complex in which hNaa50 is shown in gray, CoA in yellow, and substrate peptide in red. The first four N-terminal residues of a substrate peptide (MLGP) are bound to the enzyme and thought to be the main contributors to binding. This figure was prepared using PDB ID 3TFY.

hNatA is associated with cancer development, proliferation, and survival.[17] Despite the fact that several hNatA regulated pathways have been revealed,[18−23] the exact mechanisms linking hNatA to tumorigenesis remain elusive. Nevertheless, the data suggests that hNatA is a potential therapeutic target as knockdown of hNaa10 in a variety of thyroid cancer cell lines inhibited cell proliferation and increased sensitivity to drug-induced cytotoxicity.[24] Additionally, knockdown studies have shown that the entire NAT machinery (i.e., NatA−NatF) are important for cell proliferation and normal cell cycle progression.[17] Naa50, for example, is essential for normal sister chromatid cohesion and chromosome condensation.[25−27]

Given these results, the NATs, particularly NatA, are potential therapeutic targets; however, there are no known inhibitors for these enzymes. Therefore, we developed the first NAT specific inhibitors targeting NatA and NatE, which, based on the knockdown data (see above), represent interesting therapeutic targets. Furthermore, they are well suited to demonstrate proof of concept because they both acetylate well-defined, but distinct substrate N-termini.[13] Herein, we

Table 1. IC_{50} Values

short name	structure	target NAT	IC_{50} (μM) NatA	hNaa10	hNaa50/NatE
CoA-Ac-EE2	CoA-Ac-Glu-Glu	Naa10		63.4 ± 8.0	
CoA-Ac-EEE4	CoA-Ac-Glu-Glu-Glu-Ile	Naa10	420 ± 25	10.1 ± 1.4	50.5 ± 4.5
CoA-Ac-EEE7	CoA-Ac-Glu-Glu-Glu-Ile-Ala-Ala-Leu	Naa10	160 ± 50	16.3 ± 2.0	97.8 ± 7.0
Pant-Ac-EEE7	Pantetheine-Ac-Glu-Glu-Glu-Ile-Ala-Ala-Leu	Naa10		>1000	
CoA-Ac-SES4	CoA-Ac-Ser-Glu-Ser-Ser	NatA	15.1 ± 1.7	>1000	360 ± 35
CoA-Ac-SES7	CoA-Ac-Ser-Glu-Ser-Ser-Ser-Lys-Ser	NatA	16.3 ± 0.6	240 ± 10	685 ± 100
CoA-Ac-MLG3	CoA-Ac-Met-Leu-Gly	NatE			140 ± 10
CoA-Ac-MLG4	CoA-Ac-Met-Leu-Gly-Pro	NatE	>1000	830 ± 375	2.46 ± 0.24
CoA-Ac-MLG7	CoA-Ac-Met-Leu-Gly-Pro-Glu-Gly-Gly	NatE	>1000	975 ± 290	1.29 ± 0.11
Pant-Ac-MLG7	Pantetheine-Ac-Met-Leu-Gly-Pro-Glu-Gly-Gly	NatE			310 ± 25
CoA-Ac-MLG12	CoA-Ac-Met-Leu-Gly-Pro-Glu-Gly-Gly-Arg-Trp-Gly-Arg-Pro	NatE			5.03 ± 0.20
Desulfo-CoA	Desulfo-CoA		>1000	610 ± 50	>1000

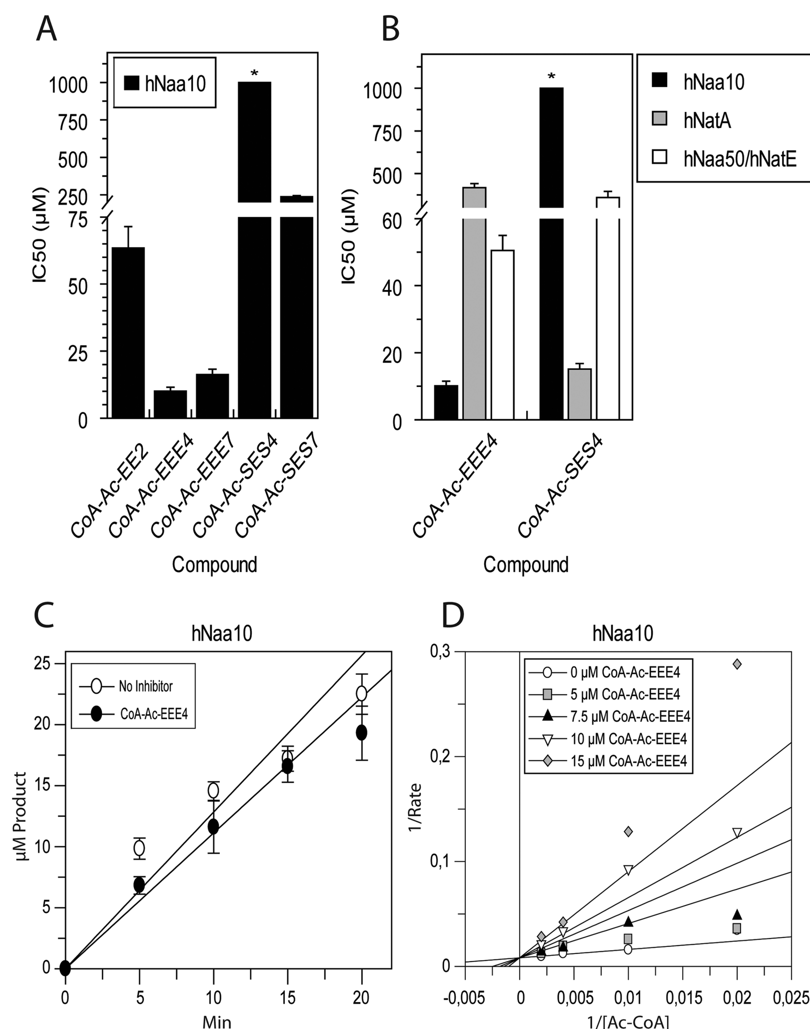

Figure 2. hNaa10 and hNatA inhibition assays. (A) IC_{50} values of CoA-Ac-EEE and CoA-Ac-SES inhibitors with various peptide lengths toward hNaa10. * IC_{50} value determined to be >1000 μM. (B) Comparison of IC_{50} values of CoA-Ac-EEE4 and CoA-Ac-SES4 toward hNaa10, hNatA, and hNaa50. (C) Recovery activity of hNaa10 after preformation of hNaa10·CoA-Ac-EEE4 complex. (D) Steady-state analysis of Ac-CoA in the presence of various concentration of CoA-Ac-EEE4 is consistent with competitive inhibition.

report, the design and synthesis of three bisubstrate analogues that potently and selectively inhibit the NatA complex (CoA-Ac-SES4; IC_{50} = 15.1 μM), hNaa10, the catalytic subunit of NatA (CoA-Ac-EEE4; K_i = 1.6 μM), and NatE/hNaa50 (CoA-Ac-MLG7; K_i^* = 8 nM); CoA-Ac-EEE4 is a reversible competitive inhibitor of hNaa10, and CoA-Ac-MLG7 is a slow tight binding inhibitor of hNaa50. With these inhibitors, we demonstrate that it is possible to selectively inhibit the different NAT complexes, which should drive future efforts to develop NAT inhibitors with more drug-like properties (e.g., cell-penetrating) that can be used to chemically interrogate *in vivo* NAT function.

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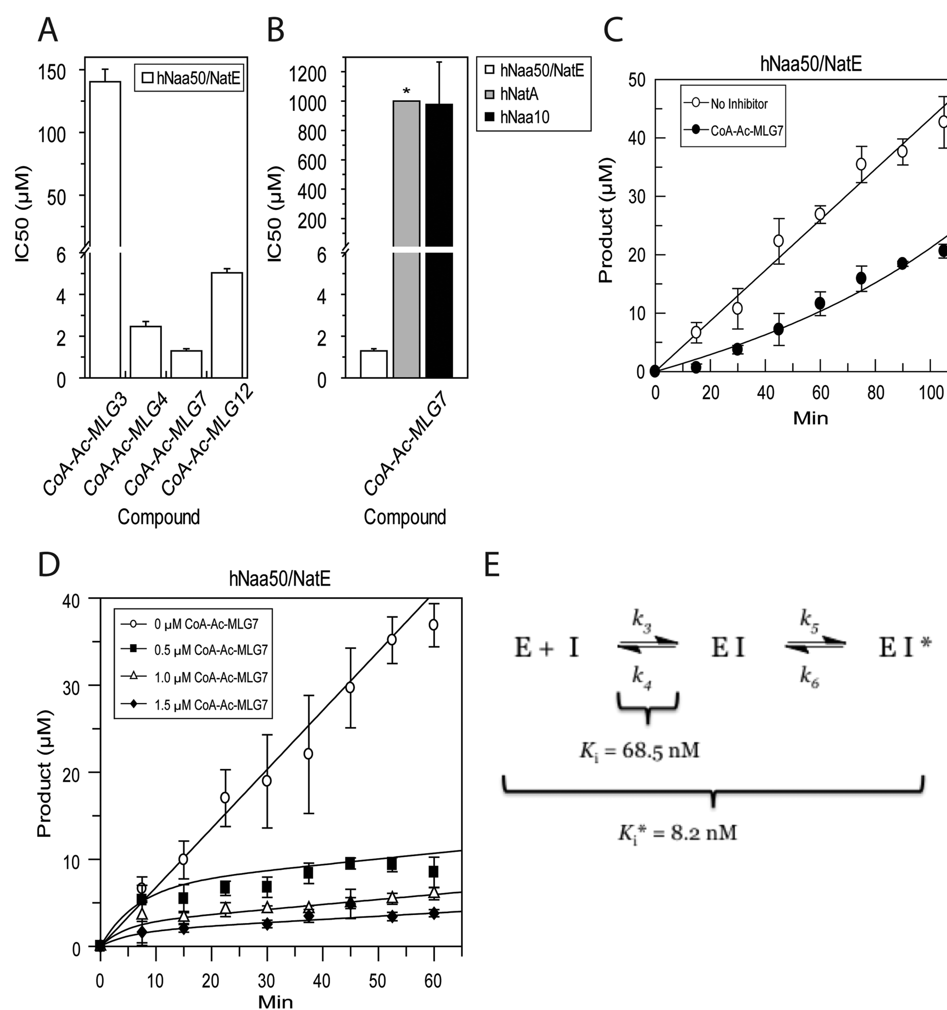

Figure 3. hNaa50 inhibition assays. (A) IC_{50} values of CoA-Ac-MLG inhibitors with various peptide lengths toward hNaa50. (B) IC_{50} values of CoA-Ac-MLG7 toward hNaa50, hNatA, and hNaa10. (C) Recovery of hNaa50 activity after preformation of the hNaa50·CoA-Ac-MLG7 complex. (D) Progress curves obtained in the presence of various concentrations of CoA-Ac-MLG7. (E) Inhibition mechanism of CoA-Ac-MLG7 to hNaa50.

Given that the NATs use a ternary complex mechanism,[1,28] we initially focused on developing bisubstrate analogues in which CoA is coupled to a peptide substrate through an acetamide linker. Figure 1A shows our general inhibition strategy, while Supplementary Table S1 lists all of the synthesized compounds. To gain selectivity between the different isozymes, i.e., NatA, hNaa10, and hNaa50, we used our understanding of the substrate specificity of the NATs to guide our selection of the specific sequences and lengths of the peptide portion of the inhibitors. Below, we describe our efforts to identify inhibitors targeting NatA, hNaa10, the catalytic subunit of NatA, and NatE/hNaa50. The compounds were synthesized analogously to methods established by Cole and colleagues for the lysine acetyltransferases.[29]

Since the NatA complex prefers substrates with serine as the initial amino acid, we based the peptide portion of the NatA inhibitor on the N-terminus of high mobility group protein A1 (i.e., SESSSKS), which was previously found to be completely Nt-acetylated *in vivo*.[11] Using the crystal structure of an hNaa50•peptide complex as a guide (Figure 1B), we focused on generating a four amino acid containing compound, as the first four amino acids appear to play the major role in hNaa50 substrate binding to the enzyme;[30] structures of hNaa10 bound to its substrates are not available. In parallel, we also synthesized an inhibitor targeting the catalytic subunit hNaa10. Here, the sequence of the peptide portion is based on the first four amino acids of mature γ-actin (i.e., EEEIAAL), as in the absence of the auxiliary subunit hNaa15, the substrate specificity of hNaa10 shifts toward acidic substrates.[13] The initial two compounds targeting NatA and hNaa10 are denoted CoA-Ac-SES4 and CoA-Ac-EEE4, respectively (see Table 1).

Given that an expression system for the NatA complex is not yet available, we initially evaluated the ability of CoA-Ac-SES4 and CoA-Ac-EEE4 to inhibit hNaa10 by determining IC_{50} values (Figure 2A and Table 1). The results of these studies revealed that CoA-Ac-EEE4 is a highly potent hNaa10 inhibitor (IC_{50} = 10.1 ± 1.4 μM). In contrast, CoA-Ac-SES4 shows only minimal inhibition of this enzyme (IC_{50} > 1000 μM). Although these results are consistent with the known substrate specificities of hNaa10 and the NatA complex, we considered the possibility that the decreased potency of CoA-Ac-SES4 may be due to the lack of sufficient contacts between the peptide portion of the inhibitor and hNaa10. Therefore, we synthesized CoA-Ac-SES7 (IC_{50} = 240 ± 10 μM), which contains an additional three residues. As a control, we also synthesized CoA-Ac-EEE7 (IC_{50} = 16.3 ± 0.6 μM). IC_{50} values for the two compounds indicate that in both cases the additional three amino acids do not have a major impact on inhibitor potency.

To confirm that a four amino acid peptide fragment is the ideal length, we also synthesized the CoA-Ac-EE2 peptide. As depicted in Figure 2A and Table 1, the potency of this inhibitor is decreased by 6.3-fold relative to CoA-Ac-EEE4, suggesting that a four amino acid peptide segment is ideal, consistent with structural studies.

Given that the presence of hNaa15 in the NatA complex is known to influence the substrate specificity of hNaa10, we considered that the lack of potency observed for CoA-Ac-SES4 with hNaa10 may be due to the absence of hNaa15 in our recombinant enzyme preparations. To address this possibility, NatA was immunoprecipitated from A-431 cells and used in subsequent inhibition assays. Consistent with our prediction, the observed IC_{50} values were reversed from those obtained with hNaa10, that is, CoA-Ac-SES4 is a highly potent inhibitor of the NatA complex (IC_{50} = 14.9 ± 0.9 μM) and CoA-Ac-EEE4 only weakly inhibits this complex (IC_{50} = 420 ± 25 μM). In parallel, we also determined whether CoA-Ac-EEE4 or CoA-Ac-SES4 inhibited hNaa50. The results of these studies are again consistent with those predicted by the substrate specificity studies in that both compounds are quite poor inhibitors of this enzyme (IC_{50} = 50.5 ± 4.5 μM for CoA-Ac-EEE4 and IC_{50} = 360 ± 35 μM for CoA-Ac-SES4; Figure 2B and Table 1).

Having identified Co-Ac-EEE4 as a highly potent and selective hNaa10 inhibitor, we further characterized the inhibitory properties of this compound. First, we examined the reversibility of inhibition by preforming the hNaa10:CoA-Ac-EEE4 complex and then rapidly diluting the complex into assay buffer. The recovery of activity was then monitored as a function of time. As is typical of reversible inhibitors, product formation was linear with no apparent lag in the recovery of enzyme activity (Figure 2C). The mechanism of inhibition was then elucidated by determing the kinetic parameters for acetyl CoA in the absence and presence of various CoA-Ac-EEE4 concentrations. The results of this analysis revealed that, with respect to Ac-CoA, CoA-Ac-EEE4 is a competitive inhibitor with a K_i of 1.6 μM (Figure 2D).

In total, these data indicate that the presence of the hNaa15 complex influences not only the substrate specificity of hNaa10 but also its response to inhibitors. Given that it is possible to selectively inhibit both hNaa10 and the NatA complex, our data indicates that it will be possible to chemically interogate and target the cellular roles of both hNaa10 on its own and when complexed with its auxiliary subunit hNaa15.

To develop inhibitors targeting NatE/hNaa50, we again were guided by the substrate specificity of this enzyme. Since hNaa50 preferentially modifies N-termini beginning with methionine followed by a hydrophobic residue,[13,16] we based the peptide portion of the inhibitor on the N-terminal residues of the NatE substrate hnRNP F (i.e., MLGPEGG).[13] Although the crystal structure suggested that a 4-mer peptide would be ideal, we also synthesized a series of bisubstrate analogues containing peptides of variable length, including the first 3, 4, 7, or 12 amino acids of hnRNP (Table 1). Like the hNaa10 inhibitors, peptide length proved important for inhibition as there was a 50-fold increase in potency from CoA-Ac-MLG3 to CoA-Ac-MLG4 (Figure 3A). Additionally, CoA-Ac-MLG7 and Co-Ac-MLG12 showed only minor changes in potency when compared to CoA-Ac-MLG4, similar to the results with hNaa10 and CoA-Ac-EEE4 and CoA-Ac-EEE7.

Since CoA-Ac-MLG7 showed the highest potency, further analyses focused on this compound. To examine the selectivity of Co-Ac-MLG7, we evaluated its ability to inhibit hNaa10 and the NatA complex. The results of these studies indicate that CoA-Ac-MLG7 is a highly potent and selective hNaa50 inhibitor; the IC_{50} is at least 750-fold lower than the values obtained for hNaa10 and NatA (Table 1 and Figure 3B). As described above, CoA-Ac-EEE4 and CoA-Ac-SES4 were quite poor hNaa50 inhibitors (Table 1 and Figure 2B).

As with hNaa10, we examined the mechanism of inhibition of hNaa50 by first preforming the hNaa50:CoA-Ac-MLG7 complex, and then performing a rapid dilution assay. The results of these experiments revealed a lag in the recovery of enzyme activity, consistent with CoA-Ac-MLG7 being a slow binding inhibitor (Figure 3C). Slow binding inhibition is typically thought to occur in a multistep process that first involves the formation of an initial encounter complex, EI (Figure 3E). A conformational change in the enzyme or the inhibitor ensues to generate the EI* complex. Although formation of the EI complex occurs rapidly, the formation of the EI* complex is slower, likely due to a high thermodynamic barrier. In addition to demonstrating that CoA-Ac-MLG7 is a slow binding inhibitor, it is possible to extract kinetic data from the progress curves depicted in Figure 3D. Specifically, the recovery in activity by hNaa50 fits well to a first order recovery process, which allowed us to determine the off rate (k_{off}) for the compound (k_{off} = 0.026 min^{-1}) and its half-life ($t_{1/2}$ = 27 min) (Figure 3D). To provide additional information on the affinity of the compound, we determined the dissociation constant for the EI complex, K_i, as well as the overall dissociation constant that incorporates the slow binding characteristics of the compound, K_i*. For this analysis, progress curves were generated in the absence and presence of increasing concentrations of CoA-Ac-MLG7. As is characteristic of slow binding inhibitors, the progress curves are biphasic with an initial phase and a nonzero steady-state phase that occurs upon establishing the equilibrium between EI and EI*. From these plots, we determined the initial and steady-state velocities, i.e., v_i and v_s, for the two observable phases of the progress curves. Dixon plots (Supplementary Figure S1) were then used to calculate values for K_i (68.5 ± 27.8 nM) and K_i* (8.2 ± 0.2 nM) (Figure 3E).

To gain insights into the functional groups that are important for the high affinities of CoA-Ac-EEE4 and CoA-Ac-MLG7 for hNaa10 and hNaa50, we developed a limited structure−activity relationship. With respect to hNaa10, we determined IC_{50} values for desulfo-CoA (IC_{50} = 610 ± 50 μM) and Pant-Ac-EEE7 (IC_{50} >1000 μM), a CoA-Ac-EEE7 derivative that lacks the adenosine portion of CoA (Table 1). The results of these studies are quite intriguing in that they demonstrate that the adenosine ring is required for the high affinity of CoA-Ac-EEE4 but that, on its own, desulfo-CoA is an extremely poor hNaa10 inhibitor; it is 60-fold less potent. Similar results were obtained for hNaa50. For example, the IC_{50} for Pant-Ac-MLG7 (IC_{50} = 310 ± 25 μM) is ∼250-fold higher than the parent compound and desulfo-CoA shows almost no inhibition (Table 1). In total, these results suggested that the adenosine ring induces a conformational change that is transmitted the length of the pantetheine arm to the protein substrate binding pocket and that the added peptide further promotes complex stabilization.

In summary, CoA-Ac-EEE4 (K_i = 1.6 μM), CoA-Ac-SES4 (IC_{50} = 14.9 μM), and the slow tight binding inhibitor CoA-Ac-MLG7 (K_i* = 8.2 nM) represent the first NAT inhibitors that selectively target hNaa10, the NatA complex, and hNaa50. In addition to demonstrating that a substrate peptide can be used

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to confer specificity within the NAT family of enzymes, our data provide three pieces of information that will be critical for future efforts to develop more drug-like (e.g., cell-penetrating) inhibitors targeting this family of enzymes. First, the inhibition profiles of hNaa10 and the NatA complex are vastly different, thereby indicating that the auxiliary subunit hNaa15 plays an important role in both substrate and inhibitor recognition. Given this fact, future efforts to develop inhibitors targeting the NatA complex will need to focus on screening the intact complex. Second, the fact that it is possible to generate inhibitors targeting hNaa10 suggests that it will be possible to use hNaa10 directed inhibitors to identify and characterize its hNaa15 independent functions, and third, the fact that the bisubstrate analogues lacking the adenosine ring, i.e., Pant-Ac-EEE7 (IC_{50} > 1000 μM) and Pant-Ac-MLG7 (IC_{50} = 310 ± 25 μM), are extremely poor inhibitors of both hNaa10 and hNaa50, comparable to desulfo-CoA on its own, indicates that the adenosine ring induces a conformational change that synergistically promotes the binding of the pantetheine-peptide portion of the inhibitor. This result suggests that inhibitors identified from screens performed in the absence and presence of AcCoA, or a CoA analogue, will yield vastly different chemotypes. In total, these data demonstrate that it is possible to develop isozyme specific NAT inhibitors. Future efforts are focused on developing cell permeable variants that can be used to assess the physiological roles of the various NAT complexes.

METHODS

Protein Expression, Purification, and Immunoprecipitation of hNatA. Plasmids used for expression of fusion proteins MBP-hNaa10 and GST-hNaa50 were pETM-41-hNAA10 and pETM-30-hNAA50, respectively. These plasmids, as well as protein expression, purification, and isolation of hNat from A-431 cells were previously described.[13]

Synthetic Substrate Peptides. The substrate peptides EEEIAAL, MLGPEGG, and SESSSKS were custom-made by Biogenes to a purity of 80−95%. Both oligopeptides contain seven unique amino acids at their N-termini, as these are the major determinants influencing Nt-acetylation.[1] The next 17 amino acids are essentially identical to the adrenocortictropic hormone peptide sequence (RWGRPVGRRRRPVRVYP), except the lysines were replaced by arginines to minimize any potential interference due to N^{ε}-acylation.

***In Vitro* Acetylation Assay.** A previously described acetylation assay[31] was adapted for use with hNaa10 and hNaa50. Briefly, the thiol present in the enzymatic product, CoA, readily cleaves 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB) and produces 2-nitro-5-thiobenzoate (NTB^-), which is readily quantified by monitoring the absorbance at 412 nm. Purified MBP-hNaa10 or GST-hNaa50 was mixed with substrate peptide and Ac-CoA in acetylation buffer (50 mM HEPES pH 7.4, 0.2 mM EDTA). Reactions were quenched with quenching buffer (3.2 M guanidinium-HCl, 100 mM sodium phosphate dibasic pH 6.8). To measure CoA production, DTNB (2 mM final, dissolved in 100 mM sodium phosphate dibasic pH 6.8 and 10 mM EDTA) was added to the quenched reaction and the absorbance at 412 nm was measured. Thiophenolate production was quantified assuming ε = 13.7 × 103 M^{-1} cm^{-1}. Background absorbances were determined and subtracted from the absorbance determined for each individual reaction. For immunoprecipitated hNatA, enzyme was mixed with substrate peptide and Ac-CoA in NatA acetylation buffer (50 mM Tris-HCl pH 8.5, 10% glycerol, 1 mM EDTA). Reactions were stopped by adding TFA to a final concentration of 1%. Acetylated peptide was subsequently quantified by RP-HPLC analysis as described previously.[13] Assays were performed in triplicate at 20 °C for hNaa10 and at 37 °C for hNaa50 and hNatA. Enzyme activity was linear for 30 min for hNaa10 and 60 min for hNatA and hNaa50, and turnover for the limiting substrate did not exceed 10%. Please see the Supporting Information for the methods used for inhibition assays.

ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, experimental details, kinetic methods, and supplemental figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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