Bioorganic & Medicinal Chemistry 17 (2009) 1381–1386

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Bisubstrate Inhibitors of the MYST HATs Esa1 and Tip60

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ARTICLE INFO

Article history:
Received 6 September 2008
Revised 30 November 2008
Accepted 7 December 2008
Available online 24 December 2008

Keywords:
Histone acetyltransferase
HAT
MYST
Esa1
Tip60
Bisubstrate
Inhibitor

ABSTRACT

Esa1 (essential Sas2-related acetyltransferase 1) and Tip60 (HIV-1 TAT-interactive protein, 60 kDa) are key members of the MYST family of histone acetyltransferases (HATs) and play important functions in many cellular processes. In this work, we designed, synthesized and evaluated a series of substrate-based analogs for the inhibition of Esa1 and Tip60. The structures of these analogs feature that coenzyme A is covalently linked to the side chain amino group of the acetyl lysine residues in the histone peptide substrates. These bisubstrate analogs exhibit stronger potency in the inhibition of Esa1 and Tip60 compared to the small molecules curcumin and anacardic acid. In particular, H4K16CoA was tested as one of the most potent inhibitors for both Esa1 and Tip60. These substrate-based analog inhibitors will be useful mechanistic tools for analyzing biochemical mechanisms of Esa1 and Tip60, defining their functional roles in particular biological pathways, and facilitating protein crystallization and structural determination.

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1. Introduction

The lysine acetylation of core histones in eukaryotic chromatin is catalyzed by histone acetyltransferases (HATs). The acetylation elicits nucleosomal remodeling and chromatin structural relaxation which modulate many chromatin-associated nuclear processes such as gene transcription and DNA repair.[1–4] HATs comprise of several distinct families based on sequence and structural homology,[5–9] which include Gcn5/PCAF, the MYST family (named after its founding members, MOZ, YBF2/SAS3, SAS2 and Tip60);[10] and the p300/CBP family. HAT proteins are not only essential to regulate gene expression in normal cellular processes (e.g., growth, differentiation, apoptosis), but malfunctioning of many HAT members, has also been observed in a range of disease states, especially cancer.[6,11–15] As such, chemical inhibitors or activators of HATs posses attractive pharmacological values in targeting the disease types that are regulated by dysfunctional acetylation. Indeed, recent years witnessed a surging effort of research to develop HAT inhibitors by various strategies.[16–20] Among these research endeavors, inhibitors of p300/CBP and PCAF/GCN5 have received considerable attention probably because they are considered robust HATs in mammalian cells and their enzymatic properties have been well characterized.[21–24] On the other hand, inhibitors of the MYST HATs are rarely reported. Anacardic acid, a weak and generic HAT inhibitor has been previously shown to inhibit the MYST HAT Tip60 and sensitize the tumor cells to the cytotoxic effects of ionizing radiation.[25] Given the essential roles of MYST HATs in normal cell function and pathogenesis, further effort (e.g., high-throughput screening or mechanism-based design) is needed to develop structurally diversified organic inhibitors to provide as selectable chemical tools for the mechanistic study of the HATs and as potential agents for pharmacological intervention. In this report, we present our work on designing bisubstrate analog inhibitors of Tip60 and its yeast orthologue Esa1.

Tip60 and Esa1 share 70% of sequence homology and the well conserved MYST domain in the two proteins is responsible for the enzymatic acetylation activity. Tip60 has been reported to be involved in gene activation by a variety of transcription factors, such as c-Myc,[26] E2F,[27] NF-Kβ,[28] and p53.[29–33] Tip60 participates in many important cellular processes, including apoptosis,[31,34] DNA repair,[4,34–38] developmental cell signaling,[39] and ribosomal gene transcription.[40] In particular, deregulation of Tip60 has been reported in certain human disease conditions,[13,15,32,38,41,42] including neurodegenerative disease,[43–45] viral infection,[46,47] and prostate cancer.[48,49] Therefore, new inhibitors with desirable potency and selectivity will be particularly useful in elucidating the functions of Tip60 and its role in disease.

The use of bisubstrate strategy to design enzyme inhibitors has been applied in different systems, for example, protein kinases[50] and GNAT (general control nonrepressed factor 5-related *N*-acetyltransferase) acetyltransferases.[51] One of the very first examples of bisubstrate analogues is the proposition of a carnitine-CoA adduct that was involved in the self-catalyzed inactivation of carnitine acetyltransferase.[52] Gentamicin-acetylCoA is another early

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doi:10.1016/j.bmc.2008.12.014

example of bisubstrate analogue that inhibits the gentamicin *N*-acetyltransferase with nanomolar affinity.[53] Early in the 1980s, several polyamine-CoA conjugates were found to block HAT activity present in cellular extracts.[54,55] Cole and colleagues reported several indole-CoA bisubstrate analogues containing different linkers that bind to serotonin *N*-acetyltransferase with nanomolar to submicromolar affinity.[56,57] The same group also synthesized bisubstrate HAT inhibitors, such as lysine-CoA for p300/CBP and histone H3 peptide-CoA analogues for PCAF/Gcn5.[16,58–60] More recently, AuClair et al.[61–63] designed and synthesized a series of 6′-*N*-acetylCoA bisubstrate analogues with nanomolar to micromolar potencies for the *Enterococcus faecium* aminoglycoside 6′-*N*-acetyltransferase type Ii (AAC(6′)-Ii). Some of these inhibitors have been applied in the kinetic and structural studies of AAC(6′)-Ii and AAC(6′)-Iy.[61,64] A priori, the rational design of bisubstrate inhibitors is better warranted if the target bireactant enzyme utilizes a sequential, ternary complex mechanism with either random or ordered substrate binding.[51] Although the enzymatic mechanism of Esa1/Tip60 is still not yet completely resolved,[65,66] a recent study indeed showed features of ordered BiBi mechanism for the catalysis of Esa1.[65] This points toward the possibility of developing bisubstrate analogs as Esa1/Tip60 inhibitors.

2. Results

2.1. Design and synthesis of the bisubstrate analog inhibitors

Esa1 and Tip60 exhibit similar substrate specificity on histones. Recombinant Esa1 and Tip60 acetylate the amino-terminal tails of histones H2A, H3, and H4, but not H2B.[67,68] On chromatin, only H2A and H4 are acetylated by Tip60.[33] We reasoned that by covalently linking the coenzyme A substrate analog to the peptide substrate at the site of respective acetylated lysine residues, we may obtain bisubstrate analogs as highly potent HAT inhibitors (Fig. 1). Recombinant Esa1 and Tip60 were reported to acetylate up to six lysine residues on histones, that is, K5 of H2A; K14 of H3; and K5, 8, 12, 16 of H4.[68,69] Based on such sequence and site specificity, we synthesized a series of bisubstrate analogs with CoASH covalently attached on each of the individual acetyl lysine residues in the histone peptides. The general synthetic route followed solid-phase peptide synthesis first and then solution-phase coupling of CoASH to the peptides (Fig. 2). To link CoASH with the side-chain of the lysine residue on the peptide, a bromoacetyl linker was introduced. All the compounds were purified by reverse-phased HPLC on a semi-preparative C18 column to a purity of higher than 95%. The sequences and mass spectrometric data are listed in Table 1. Prior to the inhibition evaluation, the bisubstrate compounds were dissolved in water and sodium hydroxide was used to adjust pH to the neutral range. The concentration of each sample was determined based on the absorption of the CoA motif at 260 nm.

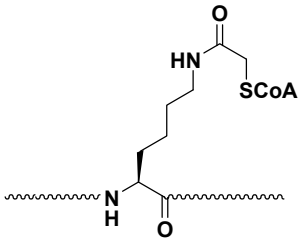

Figure 1. General structure of the bisubstrate HAT inhibitors. The curly lines stand for amino acid sequence.

2.2. Inhibition of Esa1 and Tip60 by the bisubstrate analogs

With those bisubstrate compounds in hand, we studied their inhibitory effect on the Esa1- and Tip60-catalyzed HAT reaction. The HAT reaction was performed using recombinant Esa1 and Tip60 enzymes expressed from *Escherichia coli*. ^{14}C-labeled acetyl-CoA and a peptide corresponding to the amino-terminal 20 amino acids of histone H4 (H4-20) were used as HAT substrates. Reactions were initiated with each enzyme after the other components were incubated at 30 °C for 5 min. The reaction was quenched by loading the mixtures onto the surface of a P81 filter paper disc. After washing and drying the paper, liquid scintillation was used to quantify the amount of product.

To quantitatively analyze the inhibition, we conducted the HAT reaction at a range of concentrations of individual inhibitors. A typical inhibition curve is shown in Figure 3. The data were fitted to the Langmuir isotherm equation (Eq. 1) to obtain the IC_{50} values of the inhibitors. The inhibition data of the tested compounds are summarized in Table 2.

From the inhibition experiments, it is clear that different bisubstrate analogs inhibit Esa1 and Tip60 with varied magnitudes of potency. The IC_{50} values of the bisubstrate analogs are in the range of 4.8–20 μM for Esa1 and 17.3–143 μM for Tip60. The difference in the inhibitory potency of these bisubstrate analogs for either Esa1 or Tip60 indicates that the amino acid sequences flanking the CoA-attaching site can impact the binding affinity of individual compounds to the HAT enzyme. Interestingly, the potency of the analog inhibitors is not restrictedly in line with the substrate and

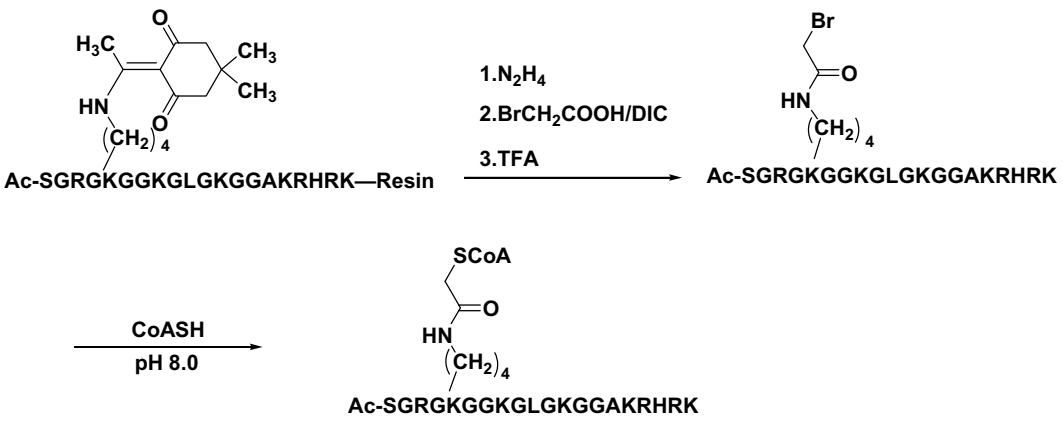

Figure 2. Synthetic scheme of a bisubstrate analog, H4K5CoA (**1**).

Table 1
Sequences of the bisubstrate HAT inhibitors

Name	Sequence	Expected mass (Da)	Observed mass (Da)
H4K5CoA (**1**)	Ac-SGRGK(CoA)GGKGLGKGGAKRHRK	2840.9	2841.3
H4K8CoA (**2**)	Ac-SGRGKGGK(CoA)GLGKGGAKRHRK	2840.9	2841.2
H4K12CoA (**3**)	Ac-SGRGKGGKGLGK(CoA)GGAKRHRK	2840.9	2841.5
H4K16CoA (**4**)	Ac-SGRGKGGKGLGKGGAK(CoA)RHRK	2840.9	2841.3
H2AK5CoA (**5**)	Ac-SGRGK(CoA)QGGKARAKAKTRSSRA	3006.0	3007.3
H3K14CoA (**6**)	Ac-ARTKQTARKSTGGK(CoA)APRKQL	3036.4	3034.4
Lys-CoA (**7**)	Ac-Lys(CoA)-NH_2	998.2	995.3

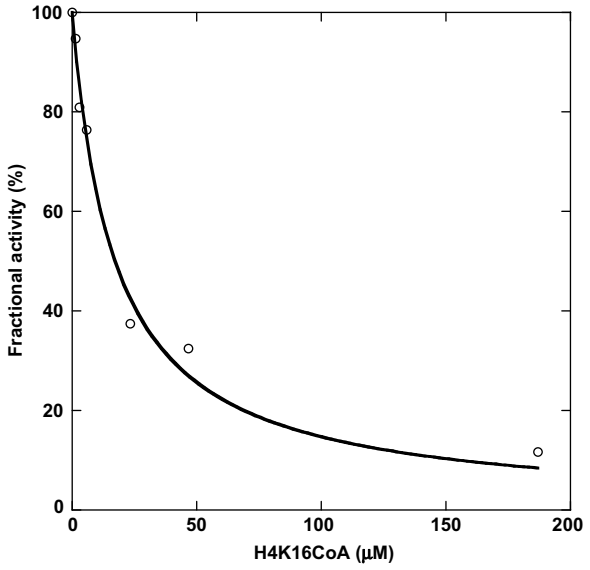

Figure 3. Inhibition of Tip60 by H4K16CoA (**4**). The HAT reaction solution contains 100 nM of Tip60, 10 μM of ^{14}AcCoA, and 200 μM of H4-20.

site specificity of Esa1 and Tip60. For example, both Esa1 and Tip60 were reported to favorably acetylate H4K5 and H4K8 and H2AK5;[68,69] nevertheless, H4K5CoA (**1**) and H4K8CoA (**2**) and H2AK5CoA (**5**) are shown here not to be the strongest inhibitors. Instead, the compound H4K16CoA (**4**) is shown to be a potent inhibitor for both Esa1 and Tip60, with IC_{50} value of 5.5 and 17.3 μM, respectively (in addition, Esa1 can also be inhibited effectively by H3K14CoA (**6**) with an IC_{50} of 4.8 μM). These data suggest that H4-K16 may also be a quite favorable acetylation site by Esa1 and Tip60. Further biochemical study is needed to address this hypothesis. As seen from Table 2, the IC_{50} values of the tested analogs for Tip60 are generally higher than for Esa1, the reason of which is not clear at this stage but may be associated with certain conformational differences in the free recombinant forms of the two enzymes.

We also compared the inhibitory potency of the bisubstrate analogs with curcumin and anacardic acid, two previously reported small-molecule generic HAT inhibitors. In particular, anacardic acid has been previously shown to inhibit the HAT activity of Tip60.[25] As shown in Table 2, all the tested bisubstrate analogs are much more potent HAT inhibitors than the two small molecules. Notably, H4K16CoA (**4**) is 20-fold more potent than anacardic acid in inhibiting the HAT activity of Tip60 and 50-fold more potent in inhibiting Esa1. It is worthwhile to point out that the measured IC_{50} value of anacardic acid here (347 μM) for Tip60 is much higher than the literature value (9 μM).[25] The difference is likely because in our experiments the purified recombinant enzymes and radioactive assay were used, but an ELISA assay on immunoprecipitated Tip60 from HeLa cell extracts was used in the previous study. It is known that IC_{50} is a relative parameter in evaluating potency of enzyme inhibitors, and it largely depends on practical experimental conditions such as assay protocol, type of signal readouts, enzyme resources, substrate concentrations, temperature. In our experiment, curcumin is also a weaker HAT inhibitor than the bisubstrate compounds, but due to its poor aqueous solubility—less than 1 mM in 10% DMSO/water, the IC_{50} data cannot be accurately assessed. The much stronger potency of the bisubstrate analogs compared to the small molecule HAT inhibitors strongly highlights the effectiveness of the bisubstrate strategy in designing HAT inhibitors.

2.3. Inhibition pattern of the bisubstrate analog H4K16CoA (4)

We further tested whether the bisubstrate compounds are competitive or noncompetitive inhibitors versus the HAT substrates. This information will be useful to calculate the K_i of the designed HAT inhibitors and may also provide mechanistic insight into the enzymatic mechanisms of the HATs. Since H4K16CoA (**4**) exhibited a high potency for both Esa1 and Tip60, we examined its inhibition

Table 2
IC_{50} of the compounds tested for the inhibition of Esa1, Tip60, p300 and PCAF

	Esa1 (μM)	Tip60 (μM)	p300 (μM)	PCAF (μM)
H4K5CoA (**1**)	18.33 ± 1.07	143.35 ± 21.70	2.88 ± 0.46	65.93 ± 6.41
H4K8CoA (**2**)	13.94 ± 2.36	111.70 ± 19.24	8.15 ± 0.70	124.30 ± 13.61
H4K12CoA (**3**)	20.30 ± 2.70	25.87 ± 8.09	4.35 ± 0.39	53.57 ± 9.83
H4K16CoA (**4**)	5.51 ± 0.98	17.59±2.40	6.62 ± 0.56	58.47 ± 4.22
H2AK5CoA (**5**)	12.09 ± 0.30	20.91 ± 2.48	17.35 ± 1.39	60.54 ± 2.96
H3K14CoA (**6**)	4.78 ± 1.05	79.62 ± 17.22	7.54 ± 1.15	2.27 ± 0.14
Lys-CoA (**7**)	7.00 ± 1.18	29.75 ± 2.88	0.98 ± 0.01	108.30 ± 6.73
CoASH	68.58 ± 8.07	82.27 ± 16.25	45.94 ± 6.92	41.91 ± 4.20
Anacardic acid	297.23 ± 96.08	347.59 ± 55.39	>1000	667.05 ± 349.51
Curcumin	>150	>200	>40	—

For the Esa1 assay, the reaction contains 60 μM of H4-20, 10 μM of ^{14}AcCoA and 50 nM of Esa1. For the Tip60 assay, the reaction contains 200 μM of H4-20, 10 μM of ^{14}AcCoA and 100 nM of Tip60. For the p300 assay, the reaction contains 60 μM of H4-20, 10 μM of ^{14}AcCoA and 5 nM of p300; for the PCAF assay, the reaction contains 60 μM of H3-31, 10 μM of ^{14}AcCoA and 5 nM of PCAF.

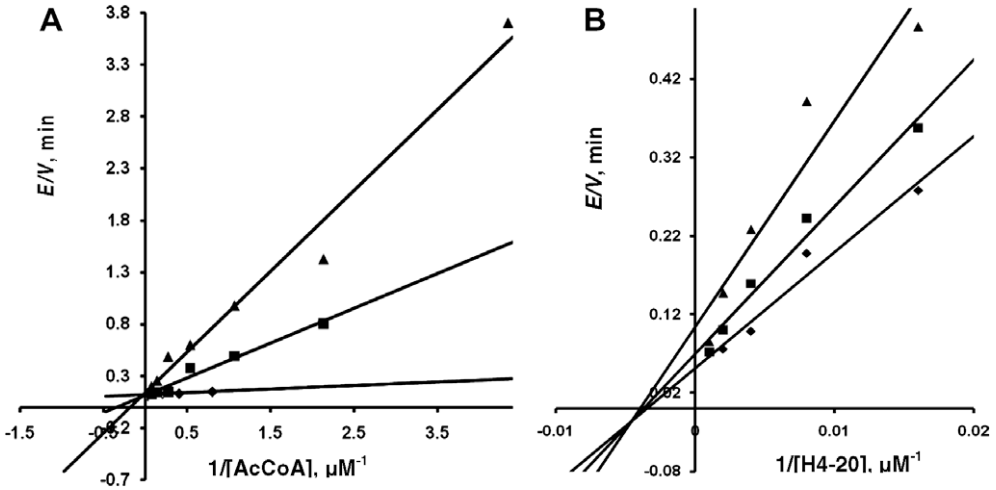

Figure 4. Steady-state kinetic analysis of H4K16CoA in the inhibition of Esa1. A. *E/V* versus 1/[acetyl-CoA] at fixed H4-20 peptide substrate concentration (200 μM) and varying H4K16CoA: 0 μM (♦), 5 μM (■), 20 μM (▲). B, *E/V* versus 1/[H4-20] at fixed acetyl-CoA concentration (10 μM) and varying H4K16CoA: 0 μM (♦), 5 μM (■), 10 μM (▲).

pattern. First, the kinetic parameters of Esa1 were measured and are as follows: k_{cat} = 44.1 ± 2.6 min^{-1}, $K_{H4\text{-}20}$ = 220.2 ± 25.4 μM and K_{AcCoA} = 3.51 ± 1.80 μM. For inhibition pattern study, we measured the initial velocity of Esa1 against either acetyl-CoA or H4-20 at a fixed, unsaturating concentration of the other substrate and several selected concentrations of H4K16CoA (**4**). The primary double reciprocal plots with *E*/*v* versus 1/acetyl-CoA] and *E*/*v* versus 1/[H4-20] identify the intersecting points on and to the left of the ordinate, respectively (Fig. 4). Such a pattern characterizes that H4K16CoA (**4**) is a competitive inhibitor as to acetyl-CoA, and a noncompetitive inhibitor as to the H4 peptide substrate. The inhibition kinetic data points were also fitted to the nonlinear competitive or noncompetitive inhibition equations (Eqs. 2 and 3).[70] The apparent K_i determined from the competitive pattern versus AcCoA is 0.74 ± 0.13 μM, and the noncompetitive inhibition versus H4-20 yields K_{is} of 12.02 ± 4.16 μM and K_{ii} of 9.27 ± 3.20 μM. Our study of Esa1 inhibition by H4K16CoA (**4**) most likely suggests an ordered substrate binding with acetyl-CoA binding to Esa1 first, followed by the peptide substrate binding.[51] This agrees well with the reported models of Esa1 catalysis.[65,66] Previously, it was shown that H3CoA20 (referred to as compound **6**, H3K14CoA here) is competitive versus acetyl-CoA and noncompetitive versus the histone substrates in the analysis of PCAF.[24] Hence, it is likely a general feature that the bisubstrate inhibitors of HATs are competitive versus the acetyl-CoA, and noncompetitive versus protein substrates.

3. Discussion

The use of bisubstrate strategy to design enzyme inhibitors has been well documented in the literature. Cole and co-workers are among the first to design HAT inhibitors by using the bisubstrates strategy.[58] From their work, two substrate-based analogs, H3K14CoA (**6**) and Lys-CoA (**7**) were found to be potent and selective inhibitors for PCAF and p300, respectively, and have been used widely as technical tools in studying functions and structures of HATs in different biological contexts.[16] Our experiments confirmed some of the previously reported features of H3K14CoA (**6**) and Lys-CoA (**7**). For example, Lys-CoA (**7**), amongst all the tested compounds, is particularly selective for p300 when compared to Esa1, Tip60, and PCAF. The compound H3K14CoA (**6**) is most potent for PCAF, which is likely due to the fact that H3K14 (**6**) is a favorable acetylation site for PCAF.[71] A minor notion is that H3K14CoA (**6**) can also block Esa1 activity with a fairly low value of IC_{50} (4.8 μM). Thus, if one is to use H3K14CoA (**6**) to study GCN5 function in yeast, caution need be taken due to its impact on the activity of Esa1. Similarly, although H4K16CoA (**4**) presents nice inhibition of the activities of Esa1 and Tip60, its inhibitory effect on p300 should not be neglected.

Prior to this report, it has remained an unknown question whether bisubstrate inhibitors can be developed for the MYST family of HATs. The bisubstrate strategy is presumably feasible when the enzyme of study follows a ternary complex model in catalysis. This feature is particularly true for the GNAT superfamily acetyltransferases, for which many members have been reported to be effectively inhibited by covalent bisubstrate analogues.[51] Structural homology analysis suggests that Esa1 may also belong to the GNAT superfamily,[5] and a recent kinetic study offers evidence of ternary complex mechanism for the Esa1 catalysis.[65] Our data successfully demonstrate that bisubstrate analogs can be made for the inhibition of Esa1/Tip60 with a much higher potency than the previously reported small molecule HAT inhibitors. Notably, the inhibition of Tip60 by H4K16CoA (**4**) is 20-fold more potent than anacardic acid (Table 2). In addition, we showed that the inhibition of Esa1 by H4K16CoA (**4**) is linearly competitive against acetyl-CoA and noncompetitive against the peptide substrate, which support an ordered substrate binding mechanism.

It is well known that chemical inhibitors and activators are powerful mechanistic tools for investigating mechanisms and functions of enzymes in vitro and in vivo.[50,72] In particular, bisubstrate analogues can be applied as diagnostic probes to investigate enzyme kinetic mechanisms.[51] Also, bisubstrate analogs have been demonstrated to be binding ligands for the structural characterization of target enzymes to examine amino acid residues involved in substrate recognition and catalysis.[60,64,73] Although the bisubstrate compounds shown here might be limited to in vitro use and presumably are not cell-permeable owing to the anionic nature of the CoA motif, the limit can be overcome by applying different strategies, for example, by linking with a cell-permeable sequence such as the TAT transduction domain.[74,75] Together, the analogs reported here will be useful biochemical probes to define the kinetic mechanisms of HATs, dissect the relative roles of MYST HATs in protein acetylation and transcription, and serve as affinity ligands for protein crystallization and structural determination.

4. Conclusion

We designed and synthesized a series of bisubstrate analogs for the inhibition of MYST HATs Esa1 and Tip60. Enzymatic assays

showed that these bisubstrate analogs are much more potent than small molecules curcumin and anacardic acid. In particular, H3K16CoA (**4**) was tested as one of the most potent inhibitors for both Esa1 and Tip60, and the inhibition is competive versus acetyl-CoA and noncompetitive versus the histone H4 substrate. It is expected that these new bisubstrate inhibitors will find use in the mechanistic and structural studies of Esa1, Tip60 and other MYST HATs.

5. Experimental

5.1. Synthesis of the bisubstrate inhibitors

Solid phase peptide synthesis (SPPS) was performed on a PS3 synthesizer using the Fmoc [*N*-(9-fluorenyl) methoxycarbonyl] strategy. Pre-loaded Wang resins were used as the solid phase. For the coupling of each amino acid (AA), HBTU [2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and HOBt (*N*-Hydroxybenzotriazole) were used as activating reagents. Removal of Fmoc group was performed with 20% v/v piperidine/DMF. The N-terminal amino group was acetylated with acetic anhydride. After all the amino acids were coupled to the solid phase, the Dde group (dimethyldioxocyclohexylidene) was removed with 2% hydrazine in DMF for 2 h. The resin was then treated with 10 equiv of $BrCH_2COOH$ and 10 equiv of DIC (*N*,*N*-diisopropylcarbodiimide) in DMF for 4 h. After washing and drying in vacuum, the bromo-labeled peptides were cleaved from the resin by treatment with 95% TFA, 2.5% H_2O and 2.5% triisopropylsilane for 4 h. Crude products were precipitated with cold ether, and then purified with reverse-phased HPLC. All the bromopeptide products were characterized with analytical HPLC and MALDI-MS before the next-step reaction. To linking CoASH to the peptide, a mixture of 1 equiv bromopeptide and 2 equiv CoASH was dissolved in a minimum amount of sodium phosphate buffer (100 mM, pH 8). The mixture was allowed to stand in the dark for 16 h. The final bisubstrate products were purified with reverse-phased (RP) HPLC (C18, Varian) on a Varian Prostar HPLC system using linear gradients of H_2O/0.05% TFA (solvent A) versus acetonitrile/0.05% TFA (solvent B). Analytical HPLC and MALDI-MS were used for characterization. The purified inhibitors were dissolved in ddH_2O and adjusted to neural pH using NaOH. The concentrations of the inhibitor solutions were determined by UV absorption at 260 nm (extinction coefficient = 16,045 $M^{-1}cm^{-1}$).

5.2. Expression of HAT enzymes

Recombinant p300 protein was a gift from Dr. Philip Cole, and all the other HATs were expressed in our lab. His6x-tagged recombinant Esa1 (105-445) and full-length Tip60 alpha were expressed in *E. coli* and purified on metal affinity beads. In a typical procedure, the Esa1 (105-445)-pRSETB plasmid was transformed into *E. coli* BL21codon plus (DE3)-RIPL (Stratagene), the Tip60 (1-513)-pET15b plasmid was transformed into BL21(DE3), and PCAF HAT domain (493-658) in a pET28a vector was transformed into BL21(DE3) codon plus cells, using heat shock method. Protein expression was induced with 0.6-mM IPTG at 16 °C for 20 h. After protein expression, cells were pelleted by centrifuge; suspended in the lysis buffer (25 mM HEPES pH 8.0, 500 mM NaCl, 1 mM PMSF, 1 mM $MgSO_4$, 10% glycerol) and lysed by sonification or French Press. The protein supernatant was purified on the Ni-charged His6x-tag binding resin (Novagen). Before protein loading, the beads were equilibrated with a column buffer (25 mM Na-Hepes, pH 8.0, 300 mM NaCl, 1 mM PMSF, and 30 mM imidazole). After protein loading, the beads were washed thoroughly with the column buffer, and then the protein was eluted with the elution buffer (25 mM Na-HEPES, pH 7.0, 300 mM NaCl, 1 mM PMSF, 100mM EDTA and 200 mM imidazole). Different elution fractions were checked on SDS–PAGE. Then, protein fractions were combined and dialyzed against the storage buffer (25 mM Na-HEPES, pH 7.0, 500 mM NaCl, 1 mM EDTA, 10 mM DTT and 10% glycerol) at 4 °C. After dialysis, the protein solution was concentrated using Millipore centrifugal filters. The final protein samples were aliquoted, flash-frozen, and stored in a −80 °C freezer.

5.3. Enzymatic HAT assay

Radioisotope-labeled HAT assay was carried out at 30 °C in a reaction volume of 30 μL. The reaction buffer contained 50 mM HEPES at pH 8.0, 0.1 mM EDTA, BSA 50 μg/mL and 1 mM dithiothreitol. Typically, ^{14}C-labeled acetyl-CoA (GE Healthcare) was used as the acetyl donor and a peptide containing the N-terminal 20-amino acid sequence of histone H4 (H4-20) was used as the HAT substrate. To determine the IC_{50} values, a range of at least seven inhibitor concentrations varied at least 20-fold around the IC_{50} were used. The reaction was initiated with the HAT enzyme after the other components (acetyl-CoA, H4-20, and the inhibitor) were equilibrated at 30 °C for 5 min. Rate measurements were based on initial conditions (generally less than 10% consumption of the limiting substrate). After the reaction, the mixture was loaded onto a Waterman P81 filter paper and then washed with 50 mM of sodium bicarbonate (pH 9.0) for three times. The paper was air dried and the amount of radioactivity incorporated into the peptide substrate was quantified by liquid scintillation. In all cases, background acetylation (in the absence of enzyme) was subtracted from the total signal. The IC_{50} was obtained from the plot of fractional HAT activity versus inhibitor concentration using the Langmuir isotherm equation (1). All the assays were performed at least twice, and duplicates generally agreed within 20%.

$$\text{Fractional activity} = \frac{1}{1 + \frac{[I]}{IC_{50}}} \tag{1}$$

The inhibition pattern of the bisubstrate inhibitor H4K16CoA (**4**) was determined by measuring initial velocities of Esa1 at different concentration of one substrate, a fixed concentration of the second substrate, and selected concentrations of H4K16CoA (**4**). The data were fitted to Eqs. 2 and 3 which describe linear competitive and noncompetitive inhibition, respectively.

$$v = V[A]/[K_a(1 + [I_{A\text{-}B}]/K_{is}) + [A]] \tag{2}$$

$$v = V[A]/[K_a(1 + [I_{A\text{-}B}]/K_{is}) + [A](1 + [I_{A\text{-}B}]/K_{ii})] \tag{3}$$

where v is the measured initial reaction velocity, V is the maximal velocity (in the absence of inhibitor), $[A]$ is the concentration of the varied substrate, K_a is the corresponding apparent Michaelis–Menten constant, $[I_{A-B}]$ is the concentration of the bisubstrate inhibitor, and K_{is} and K_{ii} are the slope and intercept inhibition constants, respectively.

Acknowledgments

This work is supported by a Georgia Cancer Coalition Distinguished Cancer Scholar Award and a GSU Research Initiation Grant. We are thankful to Dr. Philip Cole for the p300 protein, Dr. Ronen Marmorstein for the Esa1 plasmid, and Dr. Jacques Côté for the Tip60 plasmid. The authors also thank the reviewers for their insightful suggestions and helpful comments during the reviewing process.

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