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Identification of a cell-active non-peptide sirtuin inhibitor containing *N*-thioacetyl lysine

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ABSTRACT

To identify cell-active sirtuin inhibitors containing *N*-thioacetyl lysine, we synthesized compound **1**, which was designed based on the structure of the reported *N*-ethoxycarbonylacetyl lysine-based sirtuin inhibitor NCS-12k. Compound **1** selectively inhibited SIRT1 in enzyme assays. Compound **1** also caused a dose-dependent increase in p53 acetylation in human colon cancer HCT116 cells, indicating the inhibition of SIRT1 in these cells.

Reversible protein acetylation is an important posttranslational modification that regulates the function of histones and many non-histone proteins.[1–3] Sirtuins are enzymes that catalyze the deacetylation of acetylated lysine residues of proteins using NAD^+ as a cofactor, and are involved in a diversity of cellular functions, such as genome maintenance, longevity, and metabolism.[4–6] Humans have seven distinct sirtuin gene products (SIRT1–7). Among these, SIRT1 and SIRT2 are reportedly associated with certain disease states such as cancer and neurodegenerative disorders.[7–9] Therefore, SIRT inhibitors are of interest not only as tools for elucidating the detailed biological functions of the enzyme, but also as potential therapeutic agents.[10,11]

To date, several classes of sirtuin inhibitors have been identified by us and other groups.[12–16] Among them, thioacetyl lysine-containing peptides (Fig. 1) have been reported as mechanism-based inhibitors of sirtuins.[17–19] Mechanism-based enzyme inhibitors are relatively inert until they are processed by the target enzyme, which unmasks a chemically reactive warhead that leads to the covalent modification of residues or cofactors in the active site. This class of enzyme inhibitor has the advantage of a high specificity. In the case of thioacetyl sirtuin inhibitors, the sulfur of the thioamide nucleophilically attacks NAD^+ at the active site of sirtuins to enable a stable conjugation with ADP-ribose, leading to the inhibition of enzyme activity.[18] Although thioacetyl lysine-containing peptides show potent inhibitory activities in enzyme assays, these peptides are difficult to use in cellular studies because of the poor membrane permeability resulting from their peptide structure. This consideration led us to design a non-peptide sirtuin inhibitor containing *N*-thioacetyl lysine. Herein, we report the SIRT-inhibitory activity, inhibitory mechanism, and cellular activity of a non-peptide thioacetyl lysine analogue.

Recently, we reported the *N*-ethoxycarbonylacetyl lysine analogue NCS-12k (Fig. 1) as another mechanism-based inhibitor of sirtuin.[15] The enol form of NCS-12k nucleophilically attacks NAD^+ in the active site of SIRTs to enable the creation of a stable NCS-12k-ADP-ribose conjugate, which inhibits SIRTs. Based on these findings, we designed a novel *N*-thioacetyl lysine analogue **1** (Fig. 1) in which the anilino group and the benzyloxycarbonyl group are attached to the carbonyl and amino groups of lysine, respectively. These substituents are recognized by amino acid residues at the entrance to the N-acetylated lysine binding channel of

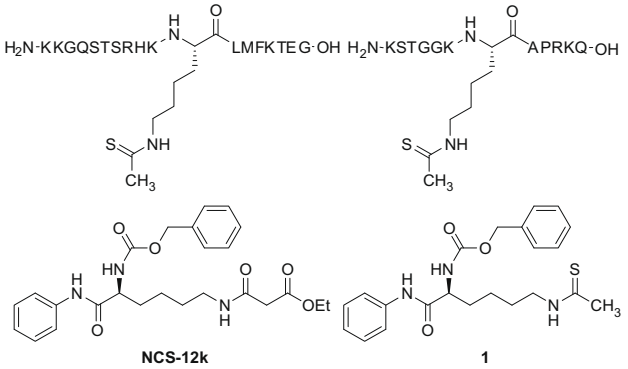

Figure 1. Structures of thioacetyl lysine peptides, NCS-12 k and compound **1**.

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SIRTs, similar to NCS-12k, leading to the potent inhibition of SIRTs.[15] In addition, the attachments of these non-peptide small groups could overcome the membrane permeability problem of peptides.

We synthesized compound **1** using the route outlined in Scheme 1. 2-Benzyloxycarbonylamino-6-*tert*-butoxycarbonylaminohexanoic acid **2** was allowed to react with aniline in the presence of EDCI and HOBt to yield the corresponding anilide **3**. The removal of the Boc group of anilide **3** produced amine **4**. Amine **4** was then reacted with ethyldithioacetate in the presence of Na_2CO_3 in MeOH to yield the desired compound **1**.[20]

Compound **1** was initially tested in an in vitro SIRT inhibition assay using human recombinant SIRT1, SIRT2, and SIRT3.[21] As shown in Table 1, compound **1** inhibited SIRT1 with an IC_{50} of 2.7 μM, and exhibited selectivity for SIRT1 over SIRT2 and SIRT3 (IC_{50} for SIRT2 = 23 μM; IC_{50} for SIRT3 >100 μM); this finding contrasts that for nicotinamide,[22] a known non-selective sirtuin inhibitor.

To examine the mechanism of SIRT1 inhibition by compound **1**, we initially determined whether inhibition by compound **1** was time-dependent. The time course of product formation was monitored in the absence and presence of compound **1**. As shown in Figure 2, compound **1** was found to be a time-dependent inhibitor of SIRT1, exhibiting nonlinear progress curves and reaching a plateau value. These data suggest that compound **1** is an irreversible inhibitor.

To gain further insight into the mechanism of SIRT1 inhibition by compound **1**, a mass spectroscopic analysis of a mixture of SIRT1 incubated with compound **1** was performed.[23] If compound **1** reacts with NAD^+ as expected, compound **1**-ADP-ribose conjugate **5** should be generated (Scheme 2). As depicted in Figure 3, while the peak of NAD^+ was observed at *m/z* 662.6, a significant peak at *m/z* 953.8 was also observed. This peak corresponds to the predicted molecular weight of compound **1**-ADP-ribose conjugate **5**. The peak was dependent on the presence of SIRT1 and compound **1**; that is, it was not detected in the absence of SIRT1 or compound **1** (data not shown). These results indicate that adduct **5** was generated as a result of the SIRT1-catalyzed reaction of compound **1** with NAD^+. The data from the mass spectroscopic analysis supported the idea that compound **1** reacts with NAD^+ in the active site of SIRT1, yielding compound **1**-ADP-ribose, which causes SIRT1 inhibition (Scheme 2).

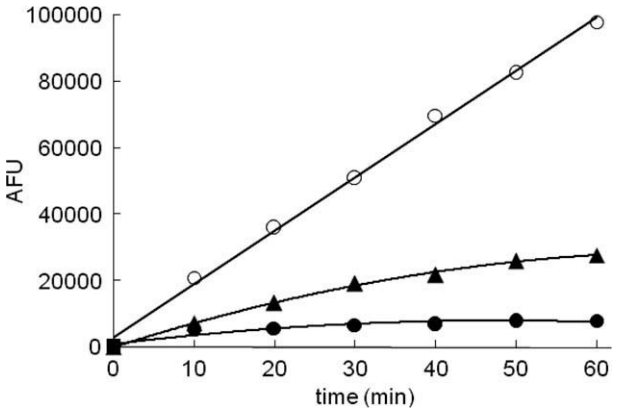
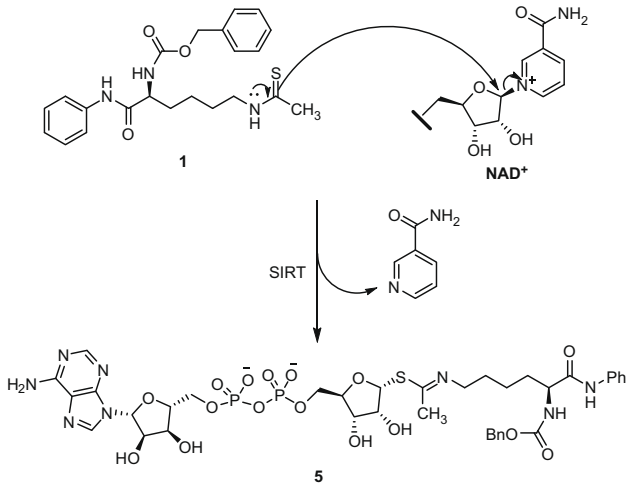
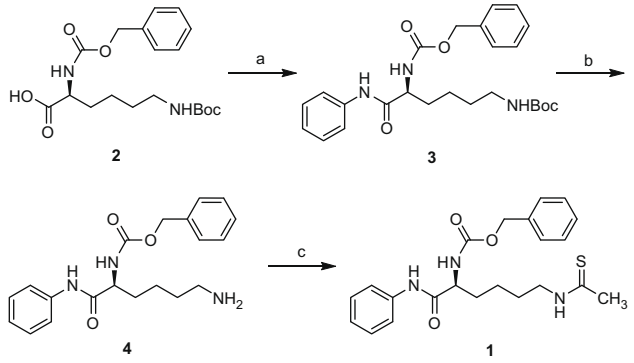


Figure 2. Plots of product formation versus time in the absence (○) and presence of 100 (▲) and 300 (●) μM of compound **1**. AFU stands for arbitrary fluorescence unit.



Scheme 2. Proposed mechanism of SIRT inhibition by compound **1**.



Scheme 1. Reagents and conditions: (a) aniline, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), 1-hydroxybenzotriazole hydrate (HOBt·H_2O), DMF, rt, 94%; (b) HCl, AcOEt, rt, 99%; (c) ethyldithioacetate, Na_2CO_3, H_2O, EtOH, rt, 85%.

Table 1
In vitro SIRT1-, SIRT2- and SIRT3-inhibitory activities of compound **1** and nicotinamide[a]

Compound	IC_{50} (μM)			Selectivity	
	SIRT1	SIRT2	SIRT3	SIRT2/SIRT1	SIRT3/SIRT1
1	2.7 ± 0.11	23 ± 3.1	>100	8.5	>37
Nicotinamide	100 ± 18	11 ± 1.7	14 ± 2.3	0.11	0.14

[a] Values are the means of at least three experiments.

Unlike peptide inhibitors, compound **1** is a small-molecule SIRT inhibitor that might be active in cellular assays. To test whether this is the case, we performed a cellular assay using western blot analysis. Since SIRT1 is known to catalyze the deacetylation of p53 in cells with DNA damage,[24] the acetylation level of p53 in human colon cancer HCT116 cells after etoposide-induced DNA damage was analyzed.[25] As can be seen in Figure 4, the level of acetylated p53 was elevated depending on the dosage of compound **1**. These results suggest that compound **1** inhibits SIRT1 in cells and can be used as a tool for probing the biological role of SIRT1.

In summary, we have designed and synthesized a non-peptide *N*-thioacetyl lysine analogue **1**, which acts as a sirtuin inhibitor. Compound **1** showed a potent and selective SIRT1-inhibitory activity. A kinetic analysis and mass spectroscopic analysis suggested that the inhibitory mechanism involves the SIRT1-catalyzed synthesis of compound **1**-ADP-ribose conjugate **5** from thioacetyl lysine analogue **1** and NAD^+. Compound **1** was confirmed to inhibit SIRT1 in a cellular study.

Thus, we have identified a novel lead structure, compound **1**, from which it should be possible to develop potent and isoform-selective sirtuin inhibitors by modifying the anilino and benzyloxycarbonyl groups. This should be helpful in the development

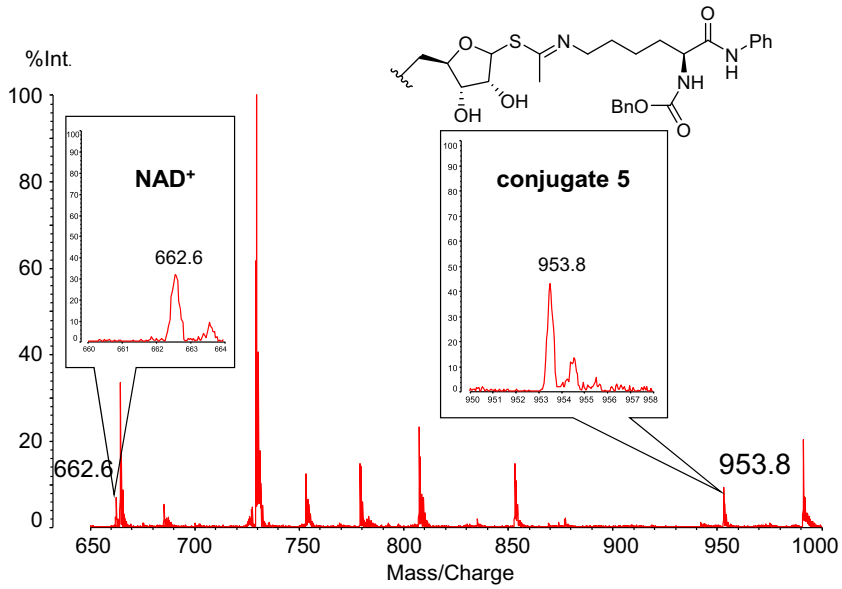


Figure 3. Mass spectrometric detection of compound **1**-ADP-ribose conjugate **5**.

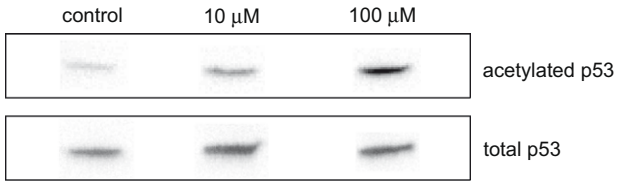


Figure 4. Western blot detection of acetylated p53 levels in HCT116 cells after 8 h of incubation with 20 μM of etoposide and 10 or 100 μM of compound **1**.

of therapeutic agents for various diseases, as well as tools for studying the biological roles of sirtuins.

Acknowledgements

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20. *The analytical data of compound* **1**: mp 128–129 °C; ^{1}H NMR (DMSO-d_6, 500 MHz, δ; ppm) 10.01 (1H, s), 9.96 (1H, m), 7.58 (2H, d, J = 6.1 Hz), 7.57 (1H, d, J = 7.9 Hz), 7.37–7.29 (7H, m), 7.05 (1H, t, J = 7.5 Hz), 5.04 (2H, s), 4.13 (1H,q, J = 8.7 Hz), 3.46 (2H, m), 2.36 (3H, s), 1.60 (2H, m), 1.41–1.34 (4H, m); ^{13}C NMR (DMSO-d_6, 500 MHz, δ; ppm) 198.80, 171.04, 156.09, 138.91, 137.00, 128.70, 128.34, 127.79, 127.70, 123.29, 119.24, 65.43, 55.36, 45.21, 32.80, 31.55, 26.92, 23.20; MS (FAB) *m/z*: 414 (M^+); Anal. Calcd for $C_{22}H_{27}N_3O_3S$: C, 63.90; H, 6.58; N, 10.16. Found: C, 63.71; H, 6.68; N, 10.25.
21. The SIRT activity assay was performed using SIRT fluorimetric drug discovery kits (AK-555, AK-556, and AK-557; BIOMOL Research Laboratories), according to the supplier's protocol. SIRT (human, recombinant) (15 μL/well), NAD^+ (25 μM), and various concentrations of the samples were incubated at 37 °C for 60 min. Fluor de Lys-SIRT substrate (25 μM) was then added to the mixture. Reactions were stopped after 60 min by adding Fluor de Lys™ Developer II with nicotinamide, which stopped further deacetylation. Then, 45 min after the addition of this developer, the fluorescence of the wells was measured using a fluorometric reader with excitation set at 360 nm and emission detection set at 460 nm. The percent-inhibition (%-inhibition) was calculated from the fluorescence readings of the inhibited wells relative to those of the control wells. The compound concentration resulting in 50% inhibition was determined by plotting log [Inh] versus the logit function of%-inhibition. IC_{50} values were determined using a regression analysis of the concentration/inhibition data.
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23. Reactions were conducted in 20 μL volumes containing 1 mM of DTT, 600 μM of compound **1**, 500 μM of NAD^+, 4 μM of SIRT1 (human recombinant) (BIOMOL Research Laboratories), and 20 μM of pyridine buffer adjusted to pH 7 with formic acid for 60 s at 25 °C. Controls were run without the compound or the enzyme. The reaction mixtures were subjected to mass spectral analysis utilizing an AXIMA CFR-Plus matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) instrument (SHIMADZU/KRATOS) in linear negative mode with 20 kV of acceleration. α-Cyano-4-hydroxycinnamic acid was used as the matrix.
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25. HCT-116 cells (5×10^5) were treated for 8 h with 20 μM of etoposide and compound **1** at the indicated concentrations in 10% FBS-supplemented McCoy's 5A medium, then collected and extracted with SDS buffer. The protein concentrations of the lysates were determined using a Bradford protein assay kit (Bio-Rad Laboratories); equivalent amounts of proteins from each lysate were resolved in 15% SDS–polyacrylamide gel and were then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). After having been blocked for 30 min with Tris-buffered saline (TBS) containing 3% skim milk, the transblotted membrane was incubated overnight at 4 °C with acetylated p53 antibody (cell signaling; 1:1000 dilution) or p53 antibody (calbiochem; 1:1000 dilution) in TBS containing 3% skim milk. The membrane was probed with the primary antibody, then washed twice with water, incubated with goat anti-mouse IgG-horseradish peroxidase conjugates (diluted 1:2500) for 2 h at room temperature, and again washed twice with water. The immunoblots were visualized using enhanced chemiluminescence.