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Trichostatin A inhibits deacetylation of histone H3 and p53 by SIRT6

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ABSTRACT

SIRT6 is an epigenetic modification enzyme that regulates gene transcription through its deacetylase activity. In addition to histone protein, SIRT6 also modify other proteins and enzymes, some of which are central players in metabolic reprogramming and aging process. Therefore, SIRT6 has emerged as a therapeutic target for the treatment of metabolic disorder and age-related diseases. Here, we report that SIRT6 deacetylates lysine 382 of p53 in short synthetic peptide sequence and in full length p53. Further studies showed that the deacetylation of H3K9Ac and p53K382Ac are insensitive to nicotinamide inhibition, but are sensitive to trichostatin A (TSA) inhibition. Detailed kinetic analysis revealed that TSA competes with the peptide substrate for inhibition, and this inhibition is unique to SIRT6 in the sirtuin family. Taken together, this study not only suggests potential roles of SIRT6 in regulating apoptosis and stress resistance via direct deacetylation of p53, but also provides lead compound for the development of potent and selective SIRT6 inhibitors.

1. Introduction

The mammalian sirtuins are NAD$^+$-dependent protein deacetylases that regulate chromatin, transcription factors, co-transcription regulators, cytoskeletal proteins and metabolic enzymes via their catalytic activities. In human there are seven sirtuin isoforms (SIRT1-7) [1,2] located within distinct subcellular compartments, with SIRT1, SIRT6 and SIRT7 found predominantly in the nucleus, SIRT2 in the cytosol, and SIRT3, SIRT4 and SIRT5 in the mitochondrion [2]. They play critical roles in apoptosis [3,4], metabolism [5,6], mitochondrial biogenesis [7,8], DNA repair [9], insulin secretion [10], and neuroprotection [11,12]. Recent studies also imply that sirtuins are important cell adaptor proteins that respond to low calorie conditions with the alteration of cell physiology.

Among the “magnificent seven” human sirtuins, SIRT6 is of special interest. SIRT6 locates in the nuclear compartment, and is tightly associated with chromatin [13]. The *N*-terminus of SIRT6 is indispensable for its enzymatic activity and association with chromatin, while the *C*-terminus is imperative for its subcellular localization [13]. Recent studies have uncovered the critical role of SIRT6 in maintaining genomic integrity and regulating metabolic network [5,14–17]. SIRT6 knockout mice demonstrated premature aging phenotype due to dysregulated metabolism and genome instability [5,18]. Overexpression of SIRT6, however, extends the lifespan of male mice with improved metabolic profiles as compared to the control littermates [17]. Additionally SIRT6 has also been implied as a tumor suppressor [19,20]. At the molecular level, SIRT6 controls various cellular events through its deacetylation activity. For example, acetylated histone H3K9 was the first identified physiological substrate of SIRT6 [14]. *In vitro* screening of acetylated histone tail peptides revealed the specific deacetylation activity of SIRT6 against acetylated H3K9 [14]. In SIRT6-deficent cells hyperacetylation of H3K9 was detected at telomeres [14]. This modification regulates the interaction between telomeric chromatin and target genes [14]. SIRT6 depletion therefore compromises telomere integrity and drives cells entering premature senescence [14]. In addition to the deacetylase activity, SIRT6 also possesses mono-ADP ribosylase [21] and defatty-acylase activities [22,23]. Through these novel activities, SIRT6 promotes DNA repair [21], facilitates the secretion of tumor necrosis factor-α (TNF-α) [22], and regulates the membrane localization of R-Ras2 [24].

In cells the biochemical activity of SIRT6 is subjected to multiple forms of regulation. During calorie restriction (CR) or fasting, SIRT6 demonstrates increased gene expression and protein abundance [25]. This induction triggers the metabolic reprogramming and physiologic adaptation to nutritional cues [25]. On the other hand, intracellular NAD$^+$ level serves as another critical control point of SIRT6 activity. Pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPRT), the rate limiting enzyme in NAD$^+$ biosynthetic pathway, led to the depletion of cellular NAD$^+$ content [26]. Subsequently the production of TNF-α decreased, presumably due to SIRT6 activity

Abbreviations: NAD$^+$, nicotinamide adenine dinucleotide; NAM, nicotinamide; AADPR, *O*-acetyl-ADP-ribose; TSA, trichostatin A; HDAC, histone deacetylase; TFA, trifluoroacetic acid; MMS, methyl methanesulfonate

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reduction [26]. Accumulating data suggests that targeted inhibition of SIRT6 could serve as a novel therapeutic approach for a broad spectrum of diseases. But to our knowledge so far only a few SIRT6 inhibitors have been identified [27–29]. They have demonstrated modest potency and limited isoform selectivity [27–29]. The need for novel scaffolds that can selectively inhibit SIRT6 becomes apparent.

Herein, we report the discovery of a pharmacological SIRT6 inhibitor, trichostatin A (TSA). TSA, a known inhibitor of the canonical Class I and Class II histone deacetylases (HDACs), reduces SIRT6 catalyzed deacetylation of synthetic peptide substrates as well as full length histone proteins isolated from HEK293 cells. This inhibition is very specific for SIRT6 as other mammalian sirtuins were not inhibited by TSA. Additionally, we have identified that SIRT6 deacetylates p53 at lysine 382 *in vitro*. This deacetylation can also be inhibited by TSA. Tumor suppressor p53 plays critical roles in various signaling pathways, many of which regulate cell cycle and stress-induced apoptosis. Previously, p53 has been identified as an endogenous substrate of SIRT1. SIRT1 physically interacts with p53 and deacetylates lysine 382 in a NAD^+-dependent fashion [30]. This modification attenuates p53 transcriptional activity and downregulates p53-mediated apoptosis during severe stress [30,31]. Recent study has demonstrated that p53 upregulates SIRT6 expression. Subsequently, the deacetylation of forkhead box protein O1 (FoxO1) by SIRT6 causes the nuclear exclusion of this transcription factor, leading to the down-regulation of several gluconeogenic genes [32]. It has long been postulated that SIRT6, another nuclear sirtuin, may also regulate p53 activity through direct deacetylation [33]. Our results shed important light on the cellular target, biological function and regulation of this intriguing enzyme.

2. Methods and materials

2.1. Reagents and instrument

All reagents were purchased from Aldrich or Fisher Scientific and were of the highest purity commercially available. UV spectra were obtained with a Varian Cary 300 Bio UV-visible spectrophotometer. HPLC was performed on a Dionex Ultimate 3000 HPLC system equipped with a diode array detector using Macherey-Nagel C18 reverse-phase column. Radiolabeled samples were counted in a Beckman LS6500 scintillation counter. HRMS was acquired with either a Q-Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Scientific) coupled to an EASY-nLC 1000 Liquid Chromatography (Thermo Scientific), or a LTQ Orbitrap Discovery (Thermo Scientific) Mass Spectrometer coupled to a Surveyor HPLC system (Thermo Scientific).

2.2. Synthetic peptides

Synthetic peptides H3K9Ac: ARTKQTAR(K-Ac)STGGKAPRKQLAS, p53K382Ac: KKGQSTSRHK(K-Ac)LMFKTEG, p53K381Ac: KKGQSTSRH(K-Ac)KLMFKTEG, p53K373Ac: K(K-Ac)GQSTSRHKKLMFKTEG, p53K372Ac: (K-Ac)KGQSTSRHKKLMFKTEG, p53K120Ac: LHSGTA(K-Ac)SVT were synthesized and purified by Genscript. The peptides were purified by HPLC to a purity > 95%.

2.3. Protein expression and purification

Plasmids of SIRT1 (full length), SIRT2 (38–356), SIRT3 (102–399), SIRT5 (34–302) and SIRT6 (full length) were the generous gifts from Dr. Hening Lin (Cornell University). The proteins were expressed and purified according to previously published protocols [34]. The identity of the protein was confirmed by tryptic digestion followed by LC-MS/MS analysis performed at Vermont Genetic Network (VGN) Proteomics Facility. Protein concentrations were determined by Bradford assay.

2.4. Deacetylation assay

The K_m and k_{cat} of SIRT6 were measured for both NAD^+ and synthetic peptide substrate. A typical reaction was performed in 100 mM phosphate buffer pH 7.5 in a total volume of 50 μL. For NAD^+ parameter measurement, the reactions contained various concentrations of NAD^+, 1 mM H3K9Ac or p53K382Ac. For synthetic peptide substrate measurement, the reactions contained various concentrations of H3K9Ac or p53K382Ac, 800 μM NAD^+. Reactions were initiated by the addition of 10 μM of SIRT6 and were incubated at 37 °C for 2 h before being quenched by 8 μL of 10% TFA. The samples were then injected on an HPLC fitted to a Macherey-Nagel Nucleosil C18 column. Co-substrate NAD^+, products nicotinamide (NAM) and *O*-acetyl-ADP-ribose (AADPR) peaks were resolved using a gradient of 0–20% methanol in 20 mM ammonium acetate. Chromatograms were analyzed at 260 nm. Reactions are quantified by integrating areas of peaks corresponding to NAD^+ and deacetylation product AADPR. Rates were plotted as a function of substrate concentration and best fits of points to the Michaelis-Menten equation were performed by Kaleidagraph®.

2.5. Nicotinamide inhibition assay

To determine nicotinamide inhibition, reactions were performed in 100 mM phosphate buffer pH 7.5 containing 800 μM NAD^+, 500 μM H3K9Ac or 800 μM p53K382Ac, and various concentrations of NAM. The reactions were initiated by the addition of 10 μM of SIRT6 and were incubated at 37 °C for 2 h before being quenched by 8 μL of 10% TFA. The samples were then injected on an HPLC fitted to a Macherey-Nagel Nucleosil C18 column. Acetylated and deacetylated peptides were resolved using a gradient of 10%–40% acetonitrile in 0.1% TFA. Chromatograms were analyzed at 215 nm. Reactions were quantified by integrating area of peaks corresponding to acetylated and deacetylated peptides. Rates were plotted as a function of NAM concentration, and points were fitted to the equation:

$$\nu = \nu_0 - \nu_{inh}\left(\frac{[I]}{K_i + [I]}\right)$$

where ν is the rate observed for a given concentration of NAM, ν_0 is the uninhibited rate, ν_{inh} is the maximal inhibition, K_i is the apparent inhibition constant, and $[I]$ is the concentration of NAM.

2.6. ^{14}C-nicotinamide base exchange assay

The reactions were carried out in 100 mM phosphate buffer pH 7.5 containing 800 μM NAD^+, 1 mM H3K9Ac or p53K382Ac, 300,000 cpm [carbonyl- ^{14}C]-nicotinamide (^{14}C-NAM, American Radiolabeled Chemicals Inc.), and various concentrations of NAM. The reactions were initiated by the addition of 10 μM of SIRT6 and were incubated at 37 °C for 2 h before being quenched by 8 μL of 10% TFA. The samples were then injected on an HPLC fitted to a Macherey-Nagel Nucleosil C18 column. NAD^+ and NAM were resolved using a gradient of 0–20% methanol in 0.1% TFA. Fractions containing NAM and NAD^+ were collected and the radioactivity determined by scintillation counting. Rates were expressed as cpm/s incorporated into NAD^+, and converted to turnover rate (s^{-1}) after adjustment for specific radioactivity and enzyme concentration. Rates were plotted as a function of NAM concentration and best fits of points to the Michaelis-Menten equation were performed by Kaleidagraph®.

2.7. Sirtuin inhibition assay

A typical reaction contained 800 μM NAD^+, 500 μM peptide substrate (H3K9Ac for SIRT2, SIRT3 and SIRT6, p53K382Ac for SIRT1 and SIRT5), varying concentrations of trichostatin A (TSA) in 100 mM phosphate buffer pH 7.5. The reactions were initiated by the addition of