Published in final edited form as:

ACS Chem Biol. 2018 March 16; 13(3): 782–792. doi:10.1021/acscchembio.7b00754.

Development of Activity-Based Chemical Probes for Human Sirtuins

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Abstract

Sirtuins consume stoichiometric amounts of nicotinamide adenine dinucleotide (NAD⁺) to remove an acetyl group from lysine residues. These enzymes have been implicated in regulating various cellular events and have also been suggested to mediate the beneficial effects of calorie restriction (CR). However, controversies on sirtuin biology also peaked during the past few years because of conflicting results from different research groups. This is partly because these enzymes have been discovered recently and the intricate interaction loops between sirtuins and other proteins make the characterization of them extremely difficult. Current molecular biology and proteomics techniques report protein abundance rather than active sirtuin content. Innovative chemical tools that can directly probe the functional state of sirtuins are desperately needed. We have obtained a set of powerful activity-based chemical probes that are capable of assessing the active content of sirtuins in model systems. These probes consist of a chemical “warhead” that binds to the active site of active enzyme and a handle that can be used for the visualization of these enzymes by fluorescence. In complex native proteome, the probes can selectively “highlight” the active sirtuin components. Furthermore, these probes were also able to probe the dynamic change of sirtuin activity in response to cellular stimuli. These chemical probes and the labeling strategies will provide transformative technology to allow the direct linking of sirtuin activity to distinct physiological processes. They will create new opportunities to investigate how sirtuins provide health benefits in adapting cells to environmental cues and provide critical information to dissect sirtuin regulatory networks.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.7b00754.

Photoaffinity labeling of recombinant human SIRT2 with probe 1, time-dependent labeling of SIRT2, labeling efficiency of probe 1, labeling of SIRT1 and SIRT3 with probe 1, labeling of individual sirtuin isoform in a mixture of partially purified recombinant human sirtuins, SIRT5 lysine desuccinylase activity, competitive inhibition of SIRT5 by probe 5, schematic representation of sirtuin labeling and enrichment, labeling of SIRT6 with different tripeptide probes, and synthesis of chemical probes (PDF)

Sirtuins are nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases that are found in 7 distinct sequences (SIRT1–7) in mammalian cells^{1,2} and are compartmentalized into nuclear, cytosolic, and mitochondrial loci.³ Sirtuin activities are thought to mediate the beneficial effects of calorie restriction (CR),⁴ which increases lifespan in a variety of organisms from yeast to mammals.^{5–7} This compelling idea has been reinforced by a growing body of evidence that sirtuins are involved in regulating numerous physiologic processes such as adipogenesis,⁸ fatty acid catabolism,⁹ mitochondrial biogenesis,¹⁰ and insulin secretion.^{11,12} In addition to being the “master regulator” of metabolic programming, sirtuins have also been implicated in various pathological conditions and have been shown to be forceful potentiators and sustainers of diseases such as cancer.^{13,14} The diverse effects of sirtuins have magnified the need to dissect the molecular mechanism underlying their biological functions.

The biological and biochemical properties of sirtuins in cells are subject to multiple forms of regulation. During CR or fasting, several human sirtuins demonstrate increased gene expression pattern and protein abundance.^{15,16} However, there is no direct correlation between these changes and sirtuin enzymatic activity. On the other hand, intracellular NAD⁺ level serves as another critical control point of sirtuin activity.^{17,18} For example, pharmacological inhibition of nicotinamide phosphoribosyltransferase (NAMPT), the rate limiting enzyme in the NAD⁺ biosynthetic pathway, led to the depletion of cellular NAD⁺ content.¹⁸ Subsequently the acetylation level of α -tubulin increased, presumably due to SIRT2 activity reduction. Interestingly, there is no apparent change of SIRT2 protein level upon NAD⁺ deprivation.¹⁸ Additionally, the functional state of sirtuins is dictated by protein–protein interactions.¹⁹ Deleted in Breast Cancer-1 (DBC1), an endogenous binding partner of SIRT1, downregulates SIRT1 activity without altering its protein content.²⁰ Furthermore, the post-translational modification (PTM) enzymes themselves are also subject to PTMs.^{21,22} Reversible modifications (phosphorylation, methylation, nitrosylation) at either the N- or the C-terminus can affect the functions of sirtuins by inducing structural rearrangements,^{23–25} and in most cases, these modifications have no influence on protein levels.²¹ It becomes clear that it is the enzymatic activity, rather than mere abundance, that eventually governs the cellular functions.

Currently, a direct method to assess the functional state of a specific sirtuin in complex biological samples is not available. Western blot or immunohistochemistry can probe individual sirtuins but require a distinct and relatively expensive antibody for analysis and fail to address the important question of whether the sirtuin being detected is in fact active to accomplish a signaling process attributed to it. Similarly, quantitative proteomics study also lacks the ability to directly report the functional content. Reliance on Western blots that

determine acetylation status of sirtuin substrate proteins can infer deacetylase activity (e.g., p53 AcK382 as a reporter for SIRT1),²⁶ and, while useful, is confounded by competing activities of acetyltransferases that acetylate the same target, as well as competing deacetylase activities.²⁶ The commonly employed *Fluor de Lys* assay provides readout of sirtuin activity,²⁷ but it is poor at distinguishing sirtuin isoform activities and is notoriously well-known to give false positive signals.^{28,29} Put simply, the current methods cannot identify if a specific sirtuin isoform is active or not under certain physiological conditions. Thus, it is impossible to determine if a distinct sirtuin isoform is switched on or off by a given stimulus. A strategy that can assay the active content of a specific sirtuin isoform in native proteomes is imperatively needed.

Herein, we report the development of activity-based small molecule probes that can directly confer the functional state of a specific sirtuin isoform in complex biological samples. We made use of a unique property of thioacetyllysine peptides to accomplish this goal.³⁰ These peptides bind to sirtuin active site and initiate chemistry analogous to the chemistry that occurs for their acetyllysine counterparts.^{31–33} Acetyllysine forms an imidate intermediate that can either reverse back to regenerate NAD⁺ through a process called “base-exchange” or proceed forward to achieve deacetylation (Scheme 1).³⁴ When the oxygen in the amide carbonyl group is replaced by a sulfur, the formation of the thioimide complex becomes irreversible (nicotinamide (NAM) cannot be base-exchanged into NAD⁺ with thioacetyllysine peptides, Scheme 1).³¹ Thioimide extremely slowly dethioacetylates, ultimately resulting in mechanism-based inhibition.^{35,31,33} The species has been crystallized by two different research groups on two distinct enzymes, *Thermotoga maritima* Sir2 and SIRT3.^{32,36} Interestingly, the thioacetyllysine peptides do not inhibit other classes of histone deacetylases (HDACs).³⁵ We envision that thioacetyllysine peptide could serve as the scaffold of our probes because they form stable complexes at the active site of active sirtuins. The thioimide-enzyme interaction can be further strengthened with the incorporation of a photoaffinity group. Benzophenone, diazirine and aromatic azide are well-known photoactivatable groups.³⁷ They can form covalent interactions with the protein in their vicinity upon irradiation.³⁸ In order to provide signal readout, a bioorthogonal functional group, such as terminal alkyne, will also be appended to the probe to allow the conjugation of the thioimide–enzyme complex to a reporter. In the presence of copper(I) catalyst, terminal alkynes can react with azides to form triazole derivatives via so-called “click chemistry”.³⁹ This reaction can tolerate a broad array of functional groups and has been widely used in interrogating biological events.^{40,41} Conjugation of the probe–enzyme complex with azido-dye or azido-biotin would enable the subsequent visualization or enrichment. The general labeling strategy is shown in Scheme 2. The strategy will selectively “highlight” the active sirtuin content in a complicated cellular context. Side-by-side comparison of functional sirtuin profiles under different physiological and pathological conditions, combined with proteomics analysis, should unwind the intricate interaction loops between human sirtuins and various cellular pathways and empower the better manipulation of these epigenetic enzymes for therapeutic purposes.

RESULTS AND DISCUSSION

Development of First Generation of Activity-Based Probe

The first generation of probe bearing a benzophenone photoaffinity group was synthesized (probe 1, Figure 1A). The scaffold was based on a known sirtuin inhibitor, probe 2 (Figure 1A).³¹ This remarkably simple peptide is a potent inhibitor (competitive with peptide substrates, noncompetitive with NAD⁺) of human SIRT1, SIRT2, and SIRT3 (IC_{50} = 6.5 ± 1.0 , 13.1 ± 0.6 , and $272 \pm 80.3 \mu\text{M}$, respectively) but less potent against SIRT5 and SIRT6. The photoaffinity probe 1 inherited the Lys-Ala-Ala tripeptide with thioacetyl modification on the epsilon amino group of the lysine residue and methyl ester at the C-terminal alanine residue. A photoactivatable benzophenone moiety was attached to the N-terminus with a propargyl ether appended to it as the “clickable” tag. This probe also preserved the inhibitory effect against sirtuins. The IC_{50} values for SIRT1, SIRT2, and SIRT3 were 39 ± 2.8 , 7 ± 0.5 , and $166 \pm 23 \mu\text{M}$ (Table 1), respectively.

The cell permeability and bioactivity of probe 1 were also assessed. HEK293 cells were treated with various combinations of HDAC inhibitors at indicated concentrations for 6 h (Figure 1B). Whole cell lysate was then collected and probed for acetylated p53 lysine 382. Treatment with a combination of Trichostatin A (TSA, a Class I/II HDAC inhibitor) and NAM (the physiological sirtuin inhibitor)^{42,43} resulted in increased acetylation level of p53, consistent with the notion that different pathways leading to the deacetylation of p53 were inhibited. Similarly, when combined with TSA, probe 1 can also elevate the acetylation level of p53 in a dose-dependent manner, presumably through the inhibition of SIRT1 (Figure 1B).

Because probe 1 demonstrated improved selectivity for SIRT2, recombinant human SIRT2 was used for the initial labeling experiments. SIRT2 was incubated with NAD⁺ and probe 1, followed by UV irradiation, Cu(I) catalyzed click conjugation to fluorescent dye and then in-gel fluorescence analysis. Several negative controls were carried out at the same time. The samples with all the necessary ingredients showed robust labeling of SIRT2 (Figure S1). This labeling was concentration-dependent. Labeling was detected even at the lowest probe concentration ($3 \mu\text{M}$, Figure 2A). Time dependence of the labeling was also examined. SIRT2 was incubated with $25 \mu\text{M}$ probe 1 and $500 \mu\text{M}$ NAD⁺. The samples were irradiated at 365 nm for various times (Figure S2). The labeling reached plateau between 45 and 60 min. Thus, 1 h was picked as the irradiation time for the labeling experiments. The labeling efficiency was investigated via a pulldown experiment (Figure S3). Recombinant SIRT2 was incubated with $500 \mu\text{M}$ NAD⁺ and $50 \mu\text{M}$ probe 1 at 37°C for 10 min. After irradiation, the sample was click conjugated to cleavable diazo biotin-azide. The biotinylated protein was then captured by high-capacity streptavidin beads. The captured protein can be eluted by incubation with $25 \text{ mM Na}_2\text{S}_2\text{O}_4$, $250 \text{ mM NH}_4\text{HCO}_3$, and 0.05% SDS. Uncaptured protein and the protein eluted off the beads were then analyzed with SDS-PAGE gel. Clearly, at $50 \mu\text{M}$ concentration, probe 1 was able to label close to 100% of the recombinant protein (Figure S3). To clarify whether the probe was directed at sirtuin or just a random nonspecific labeling, a competition labeling experiment was also performed (Figure 2B). Probe 2 (Figure

1A), a nonclickable thioacetyllysine tripeptide, was found to out-compete the labeling by probe 1 in a concentration-dependent manner, indicating the on-target activity of the probe.

Not surprisingly, human SIRT1 and SIRT3 can also be labeled (Figure S4). These results prompted us to further compare the relative labeling ability of different sirtuins. Partially purified recombinant SIRT1, SIRT2, SIRT3, SIRT5, and SIRT6 were mixed together and then subjected to the cross-linking–conjugation process. SIRT2 and SIRT3 were preferentially labeled. With increasing concentration of probe 1, SIRT1 can be labeled too. These labelings can be competed out by probe 2 (Figure S5).

Furthermore, probe 1 showed encouraging results in labeling of SIRT2 in cell lysate. HEK293 cells were transfected with pcDNA3.1-SIRT2. The whole cell lysate was subjected to the labeling protocol with or without probe 1. Only when probe 1 was present could the overexpressed SIRT2 be selectively labeled (Figure 3A). Next, endogenous proteome labeling was carried out using HEK293 cells as well (Figure 3B). At 10 μ M concentration probe 1 was able to label several proteins. The most intensively labeled protein was confirmed to be SIRT2 by Western blot (Figure 3B). The labeling was outcompeted with excess nonclickable thioacetyllysine tripeptide probe 2, consistent with the previous results. Overall, probe 1 not only showed robust labeling of individual recombinant sirtuin but also demonstrated promising results on sirtuin profiling from a complex mixture.

Design and Development of SIRT5 and SIRT6 Selective Probes

Probe 1 was able to label SIRT1, SIRT2, and SIRT3 but not SIRT5 and SIRT6. A different scaffold will increase the possibility of identifying SIRT5 and SIRT6 selective probes. Recently, these two sirtuins have been found to possess unique enzymatic activities other than deacetylation. SIRT5 can efficiently remove succinyl, malonyl, or glutaryl groups from lysine residue,^{44–46} while SIRT6 is a long chain deacylase.^{47–49} SIRT5 indeed was able to desuccinylate a synthetic peptide sequence that is identical to the N-terminus of histone H3 bearing succinylated lysine 9 (H3K9Suc). HPLC-based assay was employed to analyze the enzymatic reactions. Incubation of SIRT5 with H3K9Suc and NAD⁺ led to the formation of a new peak (Figure S6A). This product was identified as desuccinylated H3K9 by MS analysis (Figure S6B).

To investigate SIRT6 deacylation mechanism, two probes bearing octanoyl (probe 3) or myristoyl group (probe 4) on the lysine residue were synthesized (Figures 4A and 5B). It is well established that sirtuin catalyzed deacetylation involves an ADP-ribosyl-peptidyl imidate intermediate.^{50–54} The subsequent collapse of the imidate intermediate leads to the formation of deacetylated lysine product, and at the same time NAD⁺ is converted to a novel metabolite, *O*-acetyl-ADP-ribose (AADPR). Similarly, when octanoyllysine probe 3 was incubated with NAD⁺ and SIRT6, the formation of *O*-octanoyl-ADP-ribose (OADPR) was detected (Figure 4B), consistent with a previous report.⁴⁸

To further confirm that deacylation also requires a similar imidate intermediate as deacetylation, SIRT6 catalyzed base-exchange was performed using synthetic probe 4 as the substrate (Figure 5A). The rate of incorporation of exogenous ¹⁴C-NAM into unlabeled NAD⁺ in the presence of probe 4 and SIRT6 was measured (Figure 5C). As expected, the

formation of $^{14}\text{C-NAD}^+$ increased with increasing concentration of NAM (Figure 5D), supporting the notion that reverse base-exchange competes with forward deacylation for the same imidate intermediate. The K_m value was determined to be $528\ \mu\text{M}$.

The novel deacylase activities of SIRT5 and SIRT6 not only imply yet unknown physiological relevance but also provide new opportunities to develop inhibitors specific for these two enzyme isotypes. Results from other groups and our own lab indicate that thiosuccinyllysine peptides inhibit SIRT5⁵⁵ and thiomyristoyllysine peptides inhibit SIRT6.⁵⁶ Based on the prototype probe 1, additional probes with thiosuccinyllysine and thiomyristoyllysine warheads were synthesized to target SIRT5 and SIRT6 (probe 5 and 6, Figure 6A). Indeed, probe 5 with the thiosuccinyl warhead selectively inhibits SIRT5 with an IC_{50} of $3.2 \pm 0.4\ \mu\text{M}$ (Table 1). The type of inhibition was then characterized. Double-reciprocal plot showed that probe 5 is competitive with the acetylated peptide substrate as revealed by a series of lines that intersect at the y -axis (Figure S7). This probe demonstrated robust labeling of recombinant SIRT5 (Figure 6B). This labeling is very specific to SIRT5 because no appreciable labeling was detected for other sirtuin isoforms at up to $100\ \mu\text{M}$ probe 5. When applied to sirtuin mixtures, probe 5 demonstrated selective labeling of SIRT5 in the complex protein mixture (Figure 6C).

In the initial inhibition screening, probe 6 was determined as a SIRT2 and SIRT6 inhibitor with IC_{50} values in the low micromolar range (Table 1). Recent studies indicated that de-fatty-acylation is not unique to SIRT6.^{57,49} SIRT2, with a large hydrophobic binding site, can efficiently remove fatty acyl groups from lysine targets.⁵⁷ Labeling of both SIRT2 and SIRT6 by probe 6 was anticipated. However, to our surprise, although probe 6 exhibited dose-dependent labeling of SIRT6 (Figure 6D), no such labeling was observed for SIRT2. More strikingly, when probe 6 was applied to the mixture of sirtuins, SIRT6 was the only one that could be highlighted (Figure 6E). The identity of the protein being labeled in the sirtuin mixture was further confirmed with MS analysis. Briefly, after the photo-cross-linking was completed, the sample was divided into two equal parts. One half was used for click conjugation to fluorescent dye (Figure S8, top). The other half was click conjugated to cleavable diazo biotin-azide for enrichment (Figure S8, bottom). The biotinylated protein was then enriched with streptavidin and eluted off the beads. The eluent was concentrated by lyophilization. The captured protein was subjected to trypsin digestion, followed by LC-MS/MS analysis. The enrichment-MS experiment further confirmed that the protein being labeled by probe 6 in sirtuin mixture was SIRT6. Currently, we are investigating the mechanism of selective labeling by probe 6. Our working hypothesis is that the thiomyristoyl warhead of probe 6 can be accommodated by SIRT2, but the photoaffinity group is positioned out of the range of target protein, leading to negligible labeling.

The above-mentioned activity-based chemical probes structurally mimic acylated lysine substrates. The subsequent formation of a stalled thioimide intermediate and photo-cross-linking convert the transient interaction between probe and enzyme into a stable and permanent one. The thioacyl warhead is essential for the successful labeling of the target proteins. Indeed, intermediate 11, with the same tripeptide backbone as probe 6 but unmodified lysine residue, failed to label SIRT6 (Figure S9). On the other hand, probe 4, which harbors the regular myristoyl group on the epsilon-amino group of the lysine residue,

did label SIRT6 in a dose-dependent fashion (Figure S9). However, the labeling was significantly weaker compared to the labeling by probe 6. This is likely caused by the fast turnover of the myristoyllysine peptide.

***In Situ* Labeling Using Activity-Based Chemical Probe**

A critical aspect of our study is to establish active sirtuin profiles in native proteomes. Emerging evidence indicates that it is the functional state rather than the protein level that dictates its biological functions. The ability to probe specifically the catalytic function of sirtuins will capture the dynamic changes of enzyme activity resulting from transient cellular events such as post-translational modifications or protein–protein/protein–inhibitor interactions. This strategy can also be complemented with perturbations that cause gain or loss of sirtuin function for a better understanding of its role in signaling pathways. This is particularly important because it will accelerate the identification of biomarkers, suggest possible regulatory circuitry, and even lead to the discovery of unique sirtuin forms in cells.

For a proof-of-concept test, HEK293 cells were transiently transfected with pcDNA3.1-SIRT2 and then subjected to treatment known to alter cellular NAD⁺ level. As mentioned before, FK866 is a potent and specific inhibitor of NAD⁺ biosynthetic enzyme NAMPT (Figure 7A), causing reduction of cellular NAD⁺ concentration.¹⁸ Indeed, the cells treated with 40 nM of FK866 for 6 h showed 60% NAD⁺ depletion (determined with NAD⁺ cycling assay,⁵⁸ Figure 7B) with negligible loss of viability as compared to their control counterparts (Figure 7C). Subsequently, the cells were subjected to the *in situ* labeling protocol as described in Methods and Materials. NAD⁺ depletion caused reduced labeling of SIRT2 (Figure 7D, fluorescence) without altering SIRT2 protein level (Figure 7D, WB), in agreement with the notion that NAD⁺ concentration change influences sirtuin activity, not abundance. Increasing cellular NAD⁺ level has been suggested to create favorable metabolic profiles through the stimulation of sirtuin activity.^{59,60} Our activity-based chemical probe is capable of reporting the active sirtuin component in response to NAD⁺ availability in a highly complex surrounding. It should enable the accurate inventory of endogenous active sirtuin contents, leading to the delineation of the underlying role of NAD⁺–sirtuin pathway in disease onset and development. Similar strategy can be applied to other cell lines or primary tissue specimens. This innovation will facilitate the comprehensive analysis of sirtuin's functions in disease pathogenesis.

CONCLUSIONS

The “magnificent seven” human sirtuins play critical roles in various cellular processes, making them promising drug targets. However, the past few years have also witnessed controversies in sirtuin biology. The major challenge is to precisely attribute a certain phenotype to specific sirtuin activity. This is why we turned to the powerful “activity-based protein profiling” (ABPP) method.^{61,62} ABPP uses small molecule probes to directly interrogate the functional state of enzymes in their native matrix. ABPP probes, pioneered by Cravatt's group, have significantly expanded our knowledge of various enzymes in different physiological and pathophysiological settings and have suggested novel therapeutic targets for the treatment of disease such as cancer.^{63,64} In the current study, we took a highly

integrative chemical biology approach to inventory the functional state of sirtuins in their natural surroundings. We obtained a set of activity-based chemical probes that can assess the active sirtuin content in model systems. Thioacetyllysine peptides target sirtuin active sites, mimicking the chemistry that occurs for standard acetyllysine groups on the active sites of the enzymes. But more importantly, thioacetyllysine-generated thioimide complex is formed irreversibly and cannot react forward with efficiency, leading to mechanism-based trapping of the intermediate–enzyme complex.^{31,32} We took advantage of the unique property of thioacetyllysine to construct our chemical probes. All of the probes have three major components: (1) a thioacetyllysine warhead; (2) a photo-activatable functional group for covalent modification of the target enzyme; and (3) an alkyne moiety for click chemistry mediated conjugation to reporters.

These chemical probes have been subjected to photoaffinity labeling experiments with available recombinant human sirtuins in which some of them demonstrated good labeling efficiency and isoform selectivity. These data provide valuable information to enable the successful application of profiling native biological samples and also allow for the refinement of the probes to achieve improved selectivity and sensitivity. The “lead” probes were also applied to labeling of sirtuins in mammalian cell lysate and to *in situ* labeling. The identities of the labeled proteins can be further verified by either Western blot or MS. This strategy has the advantage of enriching active enzymes independent of protein abundance, allowing the capture of dynamic enzyme activity changes in response to environmental and cellular stimuli.

Ultimately we would like to employ some of these probes to monitor the subcellular localization and activity of sirtuin in live cells. Cell permeable and compartment-selective probes will be developed. For example, SIRT5 locates in the mitochondria. To make a mitochondria-targeting probe, a mitochondriotropic motif, triphenylphosphonium (TPP), can be tethered to probe 5. TPP is lipophilic and has a strongly delocalized cationic nature. It has been widely utilized to target various chemical probes to mitochondria.⁶⁵ The C-terminus of our tripeptide can be readily coupled with TPP derivatives through peptide bond formation. In the meantime, the labeling methods need to be fine-tuned to avoid significant damage to native cellular proteins and events. The click chemistry between terminal alkyne and azide requires Cu(I) catalyst, and Cu is known to be cytotoxic.⁶⁶ Cu-mediated click chemistry will not be applicable for live cell imaging. The Cu-free click reaction using cyclooctyne becomes an attractive alternative.⁶⁷ Currently we are focusing on the construction and application of cyclo-octyne-containing probes.

The successful development of activity-based chemical probes will provide platforms for sirtuin profiling in normal and malignant tissues, for sirtuin inhibitor discovery, for endogenous binding partner identification, and for physiological substrate search. All of these can be expected from our lab in the near future. Ultimately, comprehensive profiling of sirtuins using the chemical probes reported here and their variants in combination with quantitative proteomics methods may help unravel unknown cellular mechanisms controlled by these enzymes.

METHODS AND MATERIALS

Reagents and Instruments

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. NMR spectra were acquired on a Bruker AVANCE III 500 MHz high-field NMR spectrometer, and the data were processed using Topspin software. Radiolabeled samples were counted in a Beckman LS6500 scintillation counter. HRMS spectra were acquired with either a Waters Micromass Q-tof Ultima or a Thermo Scientific Q-Exactive hybrid Quadrupole Orbitrap. Fluorescence scanning was performed on a Biorad Versa Doc 4000 MP Imaging System.

Synthetic Peptides

Synthetic peptides H3K9Ac, ARTKQTAR-(K-Ac)STGGKAPRKQLAS, p53K382Ac, KKGQSTSRHK(K-Ac)L-MFKTEG, and H3K9Suc, ARTKQTAR(K-Suc)STGGKAPRKQLA were synthesized and purified by Genscript. The peptides were purified by HPLC to a purity >95%.

Protein Expression and Purification

Plasmids of SIRT1 (full length), SIRT2 (38–356), SIRT3 (102–399), SIRT5 (34–302), and SIRT6 (1–314) were generous gifts from Dr. Hening Lin (Cornell University). The proteins were expressed and purified according to previously published protocols.⁶⁸ The identity of the protein was confirmed by tryptic digestion followed by LC-MS/MS analysis performed at the Vermont Genetic Network (VGN) Proteomics Facility. Protein concentrations were determined by Bradford assay.

Sirtuin Inhibition Assay

A typical reaction contained 500 μ M NAD⁺, 500 μ M peptide substrate (H3K9Ac for SIRT2, SIRT3, and SIRT6, p53K382Ac for SIRT1 and SIRT5), and varying concentrations of small molecule probe in 100 mM phosphate buffer, pH 7.5. The reactions were initiated by the addition of 10 μ M of sirtuin and were incubated at 37 °C before being quenched by 8 μ L of 10% TFA. The incubation time was controlled so that the conversion of substrate was less than 15%. The samples were then injected on an HPLC fitted to a Macherey–Nagel Nucleosil C18 column. NAD⁺, NAM, and AADPR peaks were resolved using a gradient of 0 to 20% methanol in 20 mM ammonium acetate. Chromatograms were analyzed at 260 nm. Reactions were quantified by integrating areas of peaks corresponding to NAD⁺ and AADPR. Rates were plotted as a function of small molecule probe concentration, and points were fitted to the following equation:

$$\nu(\%) = \nu_0(\%) - [\nu_0(\%)(10^x)/(10^x + IC_{50})]$$

where $v(\%)$ represents turnover rate expressed as percent enzymatic activity remaining, $v_0(\%)$ represents the uninhibited turnover rate expressed as an enzymatic activity of 100%. The variable x represents the $\log[\text{probe}]$ in nanomolar. IC_{50} values were derived from this equation.

Lineweaver–Burk Double-Reciprocal Plot Analysis

Peptide titration reactions containing 0, 10 μM , 25 μM , or 100 μM probe 5 were incubated with 800 μM NAD^+ and varying concentrations of p53K382Ac in 100 mM phosphate buffer, pH 7.5. The reactions were initiated by the addition of 10 μM SIRT5 and were incubated at 37 °C for 40 min 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^+ , NAM, and AADPR peaks were resolved using a gradient of 0 to 20% methanol in 20 mM ammonium acetate. Chromatograms were analyzed at 260 nm. Reactions were quantified by integrating areas of peaks corresponding to NAD^+ and AADPR. Double reciprocal plots were generated using Kaleidagraph and fit to a linear curve representative of the Lineweaver–Burk relationship.

^{14}C -Nicotinamide Base Exchange Assay

The reactions were carried out in 100 mM phosphate buffer, pH 7.5, containing 800 μM NAD^+ , 500 μM probe 4, 300 000 cpm of [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 to 20% methanol in 0.1% TFA. Fractions containing NAM and NAD^+ were collected, and the radioactivity was 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 with Kaleidagraph.

Labeling of Recombinant Sirtuin

A typical labeling experiment was performed as follows: In a 0.7 mL Eppendorf tube, purified recombinant human sirtuin (10 μM) was incubated with NAD^+ (500 μM) and activity-based probe at 37 °C for 10 min, allowing the formation of stable thioimide complex. The sample was transferred to a clear-bottom 96-well plate, placed on ice, and irradiated at 365 nm with a UV-lamp in a cold room for 1 h. Subsequently, ingredients of the click-chemistry reaction, including azide-fluor 545, CuSO_4 , reducing agent (tris(2-carboxyethyl)phosphine, TCEP), and stabilizing agent (tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine, TBTA), were added, and the sample was gently agitated at 250 rpm on a microshaker at RT for 30 min. The sample was then resolved on SDS-PAGE gel. To reduce the signal-to-noise ratio, the gel was destained to eliminate nonspecific binding of free dyes. This was done in a mixture of methanol/distilled water/acetic acid (v/v/v = 4/5/1) at ambient temperature for 4 h. Destained gel was then analyzed with in-gel fluorescence scanning using Biorad Versa Doc 4000 MP (excitation 532 nm, 580 nm cutoff filter, and 30 nm band-

pass). Finally, silver staining or Coomassie blue staining was applied to provide loading control.

Cell Culture

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg mL⁻¹ streptomycin. Cells were maintained in a humidified 37 °C incubator with 5% CO₂.

Overexpression of SIRT2 in HEK293 Cells

SIRT2 Flag was a gift from Eric Verdin (Addgene plasmid no. 13813).⁶⁹ The vector was transfected into HEK293 cells with Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's protocol. Over-expression efficiency was determined with Western blot.

Cell Lysate Labeling

Cells were harvested and lysed with RIPA buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific). Protein concentration was determined by Bradford assay. A typical labeling experiment contained 15 µg of protein, 500 µM NAD⁺, and 10 µM probe in 100 mM phosphate buffer, pH 7.5. The photoaffinity labeling, click conjugation to fluorescent dye, and visualization were similar to the protocols for labeling recombinant sirtuins as described above.

FK866 Treatment

Cells were treated with 0 or 40 nM FK866 for 6 h. Cells were harvested, rinsed, and resuspended in 1 mL of fresh medium. Fifty microliters of the cell suspension was removed for viability testing using trypan blue. The rest of the cell suspension was pelleted for NAD⁺ concentration measurement.

NAD⁺ Measurement

To the cell pellet was added 30 µL of ice-cold 7% perchloric acid, then the sample was vortexed for 30 s and then sonicated on ice for 5 min. The vortex-sonication cycle was repeated three times. The sample was then centrifuged for 3 min at RT. Clear supernatant was taken out and neutralized to pH 7 with 3 M NaOH and 1 M phosphate buffer (pH 9). NAD⁺ level was then measured using NAD⁺ cycling assay as described previously.⁷⁰

In Situ Labeling of SIRT2 with Activity-Based Probe

Cells overexpressing SIRT2 were treated with or without FK866 for 6 h. Medium was removed. Cells were rinsed with fresh medium three times. The cultures were replenished with fresh medium supplemented with 10 µM probe 1. The cells were irradiated at 365 nm on ice for 1 h. Subsequently, cells were harvested, rinsed, and pelleted by centrifugation. Whole cell lysate was isolated, subjected to click conjugation to azide fluor-545 and visualized as described above.

Western Blot

The cell lysate was resolved on a 12% SDS-PAGE gel and transferred to Immobilon PVDF transfer membrane (Millipore). The blot was blocked with 5% nonfat milk, probed with primary antibody targeting p53K382Ac (Cell Signaling), p53 (Abcam), or SIRT2 (Santa Cruz Biotechnology), washed with PBST, followed by incubation with anti-rabbit or anti-mouse HRP-conjugated secondary antibody. The signal was then detected by SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by 1R15GM123393 from NIH/ NIGMS (to Y.C.) and Start-up funds from Albany College of Pharmacy and Health Sciences (to Y.C.). MS analysis reported in this manuscript was performed at the VGN Proteomics Facility supported by P20GM103449 (NIGMS/NIH).

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Figure 1.

Photoaffinity probe 1 is a cell-permeable sirtuin inhibitor. (A) Structures of probes 1 and 2; (B) Western blot analysis showing increased acetylation level of p53 lysine 382 in HEK293 cells treated with probe 1.

Figure 2.

Photoaffinity labeling using first generation probe 1. (A) Concentration-dependent labeling of recombinant human SIRT2. SIRT2 (10 μM) was incubated with 500 μM NAD^+ and various concentrations of probe 1 in 100 mM phosphate buffer, pH 7.5. The samples were incubated at 37 $^{\circ}\text{C}$ for 10 min and then subjected to the UV irradiation–click conjugation protocol as described in Methods and Materials. (B) Effect of the competitor probe 2 on labeling of SIRT2. Silver staining is for the loading control.

Figure 3.

Labeling of human SIRT2 in HEK293 whole cell lysate. (A) HEK293 cells were transfected with pcDNA3.1-SIRT2 to overexpress SIRT2. Whole cell lysate was incubated with NAD^+ , with or without probe 1. The samples were then subjected to UV irradiation followed by click conjugation to azide fluor-545. The samples were resolved on SDS-PAGE gel and visualized with fluorescence scanning. When probe 1 was present, SIRT2 was robustly labeled. (B) Labeling of endogenous sirtuin with probe 1. Whole cell lysate isolated from HEK293 cells was treated under three different conditions: NAD^+ alone, NAD^+ with 10 μM probe 1, and NAD^+ with 10 μM probe 1 and 200 μM probe 2. The samples were then subjected to UV irradiation and click conjugation to fluorescent dye. At 10 μM concentration, probe 1 was able to label several proteins in the sample. The most intensively labeled band was confirmed to be SIRT2 with Western blot. This labeling can be competed out with excess nonclickable thioacetyllysine tripeptide probe 2.

Figure 4. SIRT6 is a lysine deacylase. (A) Reaction scheme of SIRT6 catalyzed deoctanoylation; (B) synthetic probe 3 with octanoyl group on the lysine residue supports SIRT6 catalyzed deacylation as evidenced by the formation of OADPR ($m/z = 686.52$). The hydrolysis product ADP-ribose (ADPR) was also detected.

Figure 5.

Probe 4 supports SIRT6 catalyzed base-exchange. (A) Reaction scheme of sirtuin catalyzed base-exchange using long chain acyllysine peptides as the substrates; (B) structure of probe 4; (C) schematic representation of the base-exchange experiment using carbonyl- ^{14}C labeled NAM as described in Methods and Materials; (D) kinetics of SIRT6 catalyzed base-exchange of $^{14}\text{C-NAM}$ into unlabeled NAD^+ using probe 4. The K_m value of the base-exchange was determined to be 528 μM .

Figure 6.

Activity-based probes targeting SIRT5 and SIRT6. (A) Chemical structures of probes 5 and 6; (B, D) dose-dependent labeling of SIRT5 and SIRT6 by probes 5 and 6, respectively; (C, E) partially purified recombinant SIRT1, SIRT2, SIRT3, SIRT5, and SIRT6 were mixed together and incubated with 500 μM NAD^+ and various concentrations of probe 5 or 6 in 100 mM phosphate buffer pH 7.5. The final concentration of each sirtuin isoform in the reaction was 10 μM . The samples were incubated at 37 $^{\circ}\text{C}$ for 10 min and then subjected to the UV irradiation–click conjugation process as described in Methods and Materials. SIRT5 was selectively labeled by probe 5 in a dose-dependent manner, while SIRT6 can be selectively labeled by probe 6 in the mixture.

Figure 7.

In situ labeling of SIRT2 by activity-based probe. (A) Schematic representation of NAD⁺ salvage pathway; (B) cellular NAD⁺ concentrations. HEK293 cells overexpressing SIRT2 were treated with or without 40 nM FK866. Cellular NAD⁺ was then assessed with cycling assay. (C) Viability of cells treated with FK866. HEK293 cells were treated with 0 or 40 nM FK866 for 6 h. Cells were harvested, rinsed, and resuspended in 1 mL of fresh medium. Fifty microliters of the cell suspension was removed for viability testing using trypan blue. FK866 treatment did not cause any significant loss of viability. (D) *In situ* labeling of SIRT2. SIRT2 overexpressing HEK293 cells treated with FK866 showed decreased labeling intensity compared to control cells. Western blot (WB) indicates unchanged SIRT2 level in whole cell lysates.

Acetyllysine Chemistry

Thioacetyllysine Chemistry

Scheme 1.

Acetyllysine Forms Imidate that Undergoes Both Base-Exchange and Deacetylation (top), while Thioacetyllysine Peptide Forms Stalled Thioimide at Sirtuin Active Site (bottom)

Scheme 2.
General Scheme of the Labeling Strategy

Table 1IC₅₀ Values of Activity-Based Chemical Probes^a

sirtuin	substrate	IC ₅₀ (μM)		
		probe 1	probe 5	probe 6
SIRT1	p53	39 ± 2.8	>5 mM	>1 mM
SIRT2	H3	7 ± 0.5	>5 mM	21.8 ± 4.0
SIRT3	H3	166 ± 23	>5 mM	>1 mM
SIRT5	p53	<i>b</i>	3.2 ± 0.4	>5 mM
SIRT6	H3	<i>b</i>	>5 mM	7.8 ± 1.1

^aIC₅₀ values were determined using HPLC assay as described in Methods and Materials.^bNot inhibited.