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A fluorogenic assay for screening Sirt6 modulators

Jing Hu, Bin He, Shiva Bhargava, and Hening Lin*

Department of Chemistry and Chemical Biology, Baker Laboratory, Cornell University, Ithaca, NY 14853, USA

Abstract

A fluorogenic high-throughput assay suitable for screening Sirt6 modulators is developed based on the recently discovered efficient activity of Sirt6 to hydrolyze myristoyl lysine. Sirt6 modulators will be useful for investigating the function of Sirt6 and protein lysine fatty acylation.

Sirtuins are a class of evolutionarily conserved enzymes with nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylase activity (Fig. 1).^{1, 2} There are seven sirtuins in mammals, Sirt1-7,³ which are reported to regulate important biological processes, including life span, transcription, genome stability, and metabolism.^{4–6} Small molecules that can modulate sirtuin activity have been shown to have potential for treating several human diseases.^{7, 8} Sirtuin inhibitors can inhibit cancer cell growth and promote apoptosis.^{9–16} Sirt2 inhibitors were reported to provide beneficial effects in Parkinson's and Huntington's diseases.^{17, 18} Sirtuin activators can potentially be used to treat diabetes¹⁹ and promote longevity.²⁰ However, the effects of sirtuin activators are still controversial.^{21, 22}

To facilitate the development of small molecules that can regulate sirtuin activity, high-throughput assays for sirtuins have been developed. One is a fluorogenic assay that couples the deacetylation to the trypsin-catalyzed amide bond hydrolysis to release a fluorescent small molecule, 7-amino-4-methylcoumarin (AMC, Fig. 2).²³ The other method is a fluorescence resonance energy transfer (FRET)-based assay where a donor dye and an acceptor dye are connected to an acetyl peptide substrate. The deacetylation followed by trypsin digest disrupts the FRET signal.²⁴ The fluorogenic assay using AMC-acetyl peptide can be easily miniaturized and automated for high-throughput analysis and has been used for screening deacetylase inhibitors and activators.^{19, 20}

However, the fluorogenic assay has only been used for Sirt1, Sirt2 and Sirt3, the three sirtuins with high deacetylase activity. The other four human sirtuins, Sirt4-7, have very weak deacetylase activity.^{25–31} Because of this, there has been no reliable assay available for these sirtuins that can be used to analyze compounds that can modulate their activity. Correspondingly, not many inhibitors for these sirtuins have been reported. We recently discovered that Sirt5 is a demalonylase and desuccinylase³¹ while Sirt6 is a defatty-acylase

(removing long chain fatty acyl groups)²⁵ (Fig. 1). These novel activities are several hundred folds higher than the corresponding deacetylase activity. The more efficient desuccinylase/demalonylase activity of Sirt5 has enabled the development of a high-throughput assay for Sirt5 (Fig. 2).³² Here we report a high-throughput assay for Sirt6 using a fluorogenic AMC-myristoyl peptide substrate (Fig. 2). We believe this assay will be important for the development of Sirt6-specific inhibitors, which can be important tools to investigate the physiological function of Sirt6, the therapeutic potential of Sirt6 inhibitors, and the physiological function of protein lysine fatty acylation.

Initially, we tried a histone H3 lysine 9 (H3K9) AMC-myristoyl peptide. However, this peptide did not give increased fluorescence when incubated with Sirt6 and trypsin (data not shown). Thus, we decided to try different peptide sequences. Because the known defatty-acylation target of Sirt6 was TNF α ,²⁵ we made an AMC-myristoyl peptide based on the sequence of the TNF α peptide containing the fatty acyl lysine modification, AcEALPK(Myristoyl)-AMC (**4a**, Scheme 1), where Myristoyl stands for myristoyl lysine. For controls, we also synthesized the corresponding peptides with acetyl lysine or free lysine, AcEALPK(AcK)-AMC (**4b**, Scheme 1) and AcEALPKK-AMC (**4c**, Scheme 1). The syntheses of these peptides are shown in Scheme 1. The AcEALPKK-AMC peptide was first used to check whether it can be efficiently digested by trypsin to release the fluorescent AMC molecule. The results showed that 2.5 mg/ml of trypsin could hydrolytically release most of the AMC molecules in two hours in a reaction that contained 10 μ M of AcEALPKK-AMC (Fig. S1).

We then monitored whether the AcEALPK(Myristoyl)-AMC could be used to read out the activity of Sirt6. We first optimized the concentrations of the myristoyl peptide and Sirt6 (Fig. S2 and S3) and found that 1 μ M of Sirt6 and 10 μ M of peptide were optimal. After 1 μ M of Sirt6 was incubated with 10 μ M of AcEALPK(Myristoyl)-AMC for two hours at 37°C, an equal volume of 5 mg/mL trypsin solution (containing 8 mM nicotinamide to inhibit Sirt6) was added to the reaction mixture and the reaction mixture was incubated for two more hours. The fluorescence of the reaction mixture was then measured. Compared to the control without Sirt6, a 17.8-fold fluorescent increase was observed with 1 μ M of Sirt6 (Fig. 3). In contrast, when the acetyl peptide, AcEALPK(AcK)-AMC, was used as a substrate, Sirt6 increased the fluorescence by only 1.3-fold under identical conditions (Fig. 3). This was consistent with the early observation that the defatty-acylase activity of Sirt6 was hundreds fold more efficient than its deacetylase activity.²⁵

We then determined the kinetic constants of Sirt6 on the AcEALPK(Myristoyl)-AMC peptide. Using an HPLC assay, we measured the initial rate velocities as a function of substrate concentration and fit the data to the Michaelis-Menten equation (Fig. S4) to give the K_m and k_{cat} values as shown in Table 1. The catalytic efficiency (k_{cat}/K_m) for this substrate was a little lower (~4-fold) than that reported for longer peptide that did not contain AMC²⁵ (TNF- α K20 myristoyl, Table 1).

Next, we tested whether this fluorogenic assay is able to pick up compounds that can inhibit Sirt6. We chose several compounds that were reported to be sirtuin inhibitors, including nicotinamide,³³ Sirtinol,³⁴ AGK-2,¹⁷ Cambinol¹⁰ and Tenovin-1³⁵ (Fig. 4A). Because of the

lack of an efficient Sirt6 activity assay, these compounds were not tested in their ability to inhibit Sirt6. Using 200 μ M concentrations, we screened the ability of these compounds to inhibit Sirt6 in the fluorogenic assay. Among all the compounds tested, nicotinamide showed the best inhibition (57%) at 200 μ M. All other compounds showed less than 50% inhibition at 200 μ M (Fig. 4B). The dose-response curve (Fig. 5) for nicotinamide was obtained using this fluorogenic assay. From the dose-response curve, the IC₅₀ of nicotinamide for Sirt6 was determined to be 184 μ M, which was consistent with the value (153 μ M) obtained with the HPLC assay using a longer H3K9 myristoyl peptide that did not contain AMC. Thus, the fluorogenic assay could readily detect the known general sirtuin inhibitor, nicotinamide. This was the first time these known sirtuin inhibitors were tested on Sirt6 and the results pointed out that most known sirtuin inhibitors could not inhibit Sirt6 very well.

In summary, we developed a fluorogenic AMC-myristoyl peptide that could be used to screen for compounds that could modulate Sirt6 activity. Using this assay, we demonstrated that except nicotinamide, none of the known sirtuin inhibitors tested could inhibit Sirt6 efficiently, highlighting the need to develop better Sirt6 inhibitors. The fluorogenic assay we developed here should be easily miniaturized and automated for high-throughput screening to identify Sirt6-specific inhibitors or activators, which could be important tools to investigate the physiological function of Sirt6 and protein lysine fatty acylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
The deacylation reactions catalyzed by different sirtuins.

Fig. 2.

The fluorogenic assay for the deacetylases (Sirt1, 2 and 3) using AMC-acetyl peptides, the fluorogenic assay for Sirt5 using AMC-succinyl peptides and fluorogenic assay for Sirt6 using AMC-myristoyl peptides.

Fig. 3. Bar graph for deacylation of substrate AcEALPK(My rK)-AMC and AcEALPK(AcK)-AMC. The data were obtained as end point readings after incubation for 2 hrs with each substrate at 37°C and the experiments were performed in duplicate.

Fig. 4. Structures of known sirtuin inhibitors (A) and Sirt6 inhibition at 200 μ M (B) measured using the fluorogenic substrate AcEALPK(MyR)-AMC. Experiments were done in duplicate, and error bars represent deviation from the mean.

Fig. 5.
The dose-response curve measured using the fluorogenic substrate AcEALPK(MyK)-AMC.

Scheme 1. Synthesis of substrate **4a–c**

Reagents and conditions: (a) standard solid phase peptide synthesis; (b) ClCOO^iBu , NMM, 7-amino-4-methyl-coumarin; (c) 1% triisopropylsilane in TFA.

Table 1

Kinetics data for Sirt6 on the fluorogenic substrate, AcEALPK(Myristoyl)-AMC, and non-fluorophore containing peptide, TNF- α K20 myristoyl.

Substrate	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} M^{-1}$)
AcEALPK(Myristoyl)-AMC	9.7 ± 1.2	0.0028 ± 0.0001	2.7×10^2
TNF- α K20 myristoyl	4.5 ± 1.1	0.0050 ± 0.0004	1.1×10^3