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Thiomyristoyl Peptides as Cell-Permeable Sirt6 Inhibitors

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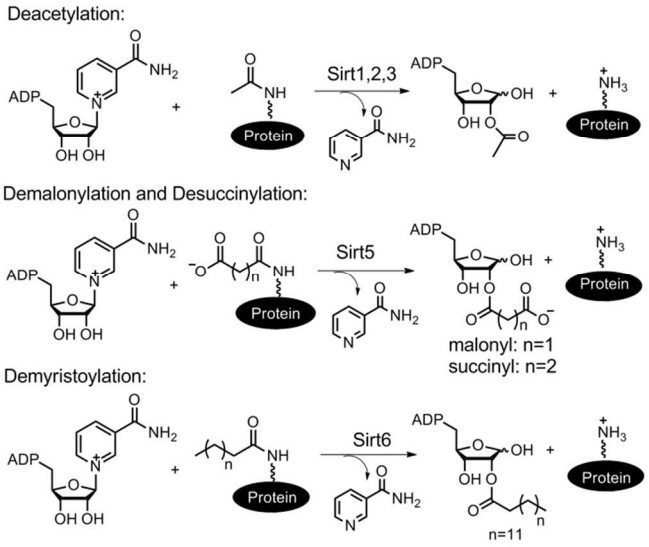
Sirtuins regulate a variety of biological pathways and inhibitors of sirtuins have been actively pursued as tool compounds to study sirtuin biology and as potential therapeutics. Here we demonstrate that thiomyristoyl peptides are potent and cell-permeable inhibitors of Sirt6, one of the seven human sirtuins, and will serve as starting point for the development of more specific Sirt6 inhibitors.

Sirtuins, a class of enzymes known as nicotinamide adenine dinucleotide (NAD)-dependent deacylases,[1] have been shown to regulate a variety of biological processes, including aging, transcription, stress response, and metabolism.[2, 3] Human possess seven sirtuins, Sirt1-7, which differ in their subcellular localization and in the substrate proteins they deacylate (Scheme 1). Among the seven sirtuins, Sirt6 has been shown to have very interesting biological fuctions. Sirt6 knockout mice show a premature aging phenotype, develop several acute degenerative processes by three weeks of age and die prematurely at one month of age.[4] Recently, it has been reported that male, but not female, transgenic mice overexpressing Sirt6 have a longer life span than wild-type mice.[5] Sirt6 promotes DNA repair and genome stability partially through deacetylation of telomeric histone H3 at lysine 9 and lysine 56 (H3 K9 and K56) and CtIP.[6-8] By deacetylating histone H3, Sirt6 has also been shown to regulate the transcription of genes that are controlled by several important transcription factors, such as HIF-1α,[9] NF-κB,[10] and c-Myc[11]. The important biological functions of Sirt6 suggest that Sirt6 can be a potential therapeutic target for human diseases.[9, 12, 13] Thus, Sirt6 inhibitors are of great interest for exploring the therapeutic potential of targeting Sirt6 and for further understanding the biology of Sirt6. Most inhibitors for Sirt1-3 do not inhibit Sirt6 efficiently.[14] At present, only a few weak Sirt6 inhibitors are available.[15] Nicotinamide is a weak inhibitor for sirtuins, including Sirt6.[16] Five small molecules from fenugreek seed extract have shown 25 − 50% inhibition at 100 μM against Sirt6.[18] Thioacetyl peptides and pseudopeptides have been reported as Sirt6 inhibitors, with the most potent one having an IC_{50} value of 47 μM.[17] Thus, more potent Sirt6 inhibitors are still needed.

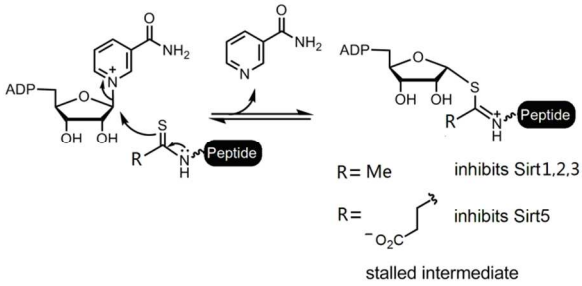


Scheme 1. Different sirtuins prefer to remove different acyl groups from protein lysine residues.

The major obstacle for developing more potent Sirt6 inhibitors is the very weak deacetylase activity of Sirt6.[19, 20] Recently, our laboratory discovered that human Sirt6 is an efficient defatty-acylase (removing long chain fatty acyl groups, Scheme 1). [21] We further demonstrated that Sirt6 promotes the secretion of

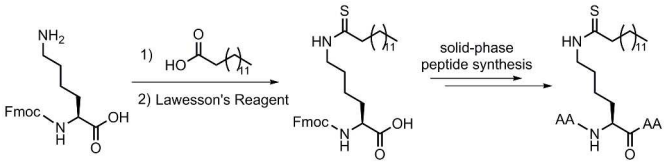
tumor necrosis factor α (TNFα) by removing the fatty acyl modification on Lys19 and Lys20 of TNFα.[21] The discovery of an efficient activity for Sirt6 has facilitated the development of a high-throughput assay that can be used to screen for Sirt6 modulators.[14] In the current study, we utilize this efficient defatty-acylase activity of Sirt6 to develop mechanism-based inhibitors for Sirt6. Herein, we report that thiomyristoyl peptides are potent and cell-permeable Sirt6 inhibitors.

It is reported that thioacetyl peptides can form a stalled covalent intermediate with NAD in the sirtuin active sites and inhibit Sirt1-3 (Scheme 2).[22-25] Our recent discovery that different sirtuins prefer different acyl groups as substrates (i.e. Sirt5 prefers malonyl and succinyl[25] while Sirt6 prefers long chain fatty acyl groups, Scheme 1)[21] suggests that we can target different sirtuins using different thioacyl lysine peptides. Indeed, we previously demonstrated that thiosuccinyl peptides could inhibit Sirt5 specifically (Scheme 2).[26, 27] Encouraged by this, we reasoned that thiomyristoyl peptides can be mechanism-based inhibitors for Sirt6.



Scheme 2 Mechanism-based inhibition of sirtuins by thioacyl lysine-containing peptides.

To make the thiomyristoyl lysine-containing peptides, we first synthesize the Fmoc-protected thiomyristoyl lysine as a building block (Scheme 3). Then we performed standard Fmoc solid-phase peptide synthesis to synthesize peptides with different sequences, including a tumor necrosis factor alpha (TNFα) peptide sequence and a histone H3 lysine 9 (H3K9) peptide sequence. Totally, we made five thiomyristoyl peptides, named BHJH-TM1, BHJH-TM2, BHJH-TM3, BH-TM4 and JH-TM5 (Table 1).



Scheme 3 Synthetic route for thiomyristoyl peptides.

We first assayed the inhibition of Sirt6 with these thiomyristoyl peptides using a pre-incubation method, which involved incubating Sirt6 with the thiomyristoyl peptides prior to the addition of the substrate peptide to initiate the reaction. The pre-incubation method allowed the stalled covalent intermediate to form without competition from the substrate peptide and thus normally gave better inhibition. The assays were carried out using 1 μM of Sirt6, 50 μM myristoyl peptide, KQTAR(MyK)STGGWW, and 0.5 mM NAD. The inhibition efficiencies of all tested thiomyristoyl peptides were excellent, with almost complete inhibition of Sirt6 at 1 μM concentration (data not shown). In order to differentiate the inhibitory potencies of these thiomyristoyl peptides, we then performed the assay without pre-incubation. All the assay conditions were the same as those used in the pre-incubation assay except that Sirt6 was added last to initiate the reaction and thus there was no pre-incubation of Sirt6 with the inhibitors before initiation of the enzymatic reaction. As shown in Table 2, all thiomyristoyl peptides except JH-TM5 could inhibit Sirt6 with low μM IC_{50} values (Table 2, Figure S1). Among these thiomyristoyl peptides, BH-TM4 from the H3 K9 sequence showed the best inhibition toward Sirt6 with an IC_{50} value of 1.7 μM. BHJH-TM1 and BHJH-TM3 from the TNFα sequence gave IC_{50} values of 2.8 μM and 8.1 μM, respectively. BHJH-TM2, which was also derived from the TNFα sequence, gave a higher IC_{50} value of 42.2 μM. This might be because the sequence before the thiomyristoyl lysine was one amino acid shorter in BHJH-TM2 than in BHJH-TM1 and BHJH-TM3, which could lead to fewer hydrogen bonding interactions involving main chain C=O and N-H in BHJH-TM2.[25] The same explanation could be applied to JH-TM5, which had a shorter sequence after the thiomyristoyl lysine and showed 18% inhibition of Sirt6 at 50 μM.

Table 1 Thiomyristoyl peptides synthesized.

Name	Sequence[a]	Peptide origin
BHJH-TM1	PKK(TMy)TG	TNFα K20
BHJH-TM2	PK(TMy)KTG	TNFα K19
BHJH-TM3	LPK(TMy)KT	TNFα K19
BH-TM4	ARK(TMy)ST	H3 K9
JH-TM5	GGK(TMy)G	

[a] TMy: thiomyristoyl.

For comparison, we also tested several commercial sirtuin inhibitors in parallel, such as AGK2, [28] sirtinol,[29, 30] cambinol[31] and tenovin-1.[32] None of these commercial sirtuin inhibitors can inhibit Sirt6 very well, although AGK2 and Sirtinol did show some inhibition at 300 μM (Table 2, Figure S2). Thus, the thiomyristoyl peptides are the most potent Sirt6 inhibitors reported so far.

Table 2. IC50 values (μM) of different inhibitors for different sirtuins. For Sirt6, an H3 K9 myristoyl peptide was used as the substrate in the inhibition assay. For Sirt1-3, an H3 K9 acetyl peptide was used as the substrate in the inhibition assay.

Inhibitor	Sirt6	Sirt1	Sirt2	Sirt3
BHJH-TM1	2.8	5.3	2.3	4.5
BHJH-TM2	42.2	10.7	1.9	17.5
BHJH-TM3	8.1	4.7	2.6	8.0
BH-TM4	1.7	4.4	2.6	5.6
JH-TM5	>50 (18%)[a]	58.9	0.7	>50 (43%)[a]
AGK2	>300 (28%)[a]	N.I[b]	66	>300 (22%)[a]
Sirtinol	>300 (41%)[a]	>300 (21%)[a]	64.6	150.1
Cambinol	N.I[b]	>300 (34%)[a]	134	N.I[b]
Tenovin-1	N.I[b]	>300 (17%)[a]	38.3	>300 (17%)[a]

[a] Values in parentheses show the the percentage of inhibition at the concentration listed before the parentheses. [b] N.I: no inhibition at 300 μM.

We also used TNFα peptide, EALPK(MyK)TGGPQWW, as substrate to test inhibition of BHJH-TM1, BHJH-TM2, BHJH-TM3 and BH-TM4 on Sirt6. All four compounds showed potent Sirt6 inhibition effects (Figure S14). To determine whether the thiomyristoyl peptides were specific for Sirt6, we tested whether they can inhibit Sirt1-3. We used a H3 K9 acetyl peptide, KQTAR(AcK)STGGWW, as the substrate for Sirt1-3. Surprisingly, all the thiomyristoyl peptides showed very potent inhibition activity for Sirt1-3 with IC_{50} values lower than the commercial inhibitors (Table 1, Figure S3-S11). These results indicated that Sirt1, Sirt2, and Sirt3 may have demyristoylation activity, too. To test this possibility, we used an HPLC assay to find out whether Sirt1-3 can hydrolyze a myristoyl peptide. Assays for both deacetylation and demyristoylation of Sirt1-3 were carried out under identical enzyme (0.5 μM) and substrate (50 μM acyl peptide, 0.5 mM NAD) concentrations. We used an H3 K9 acetyl peptide, KQTAR(AcK)STGGWW as the deacetylation substrate while an H3 K9 myristoyl peptide, KQTAR(MyK)STGGWW as demyristoylation substrate. The enzymatic reactions were quenched and then monitored by HPLC after 5 minutes of incubation. Indeed, Sirt1-3 can hydrolyze myristoyl peptide fairly efficiently (Figure 1). This result is consistent with a recent report by Denu and coworkers.[33] The defatty-acylase activity of Sirt1-3 can explain why thiomyristoyl peptides are not specific inhibitors for Sirt6, and further imply Sirt1-3 may have other physiological functions beyond deacetylation.

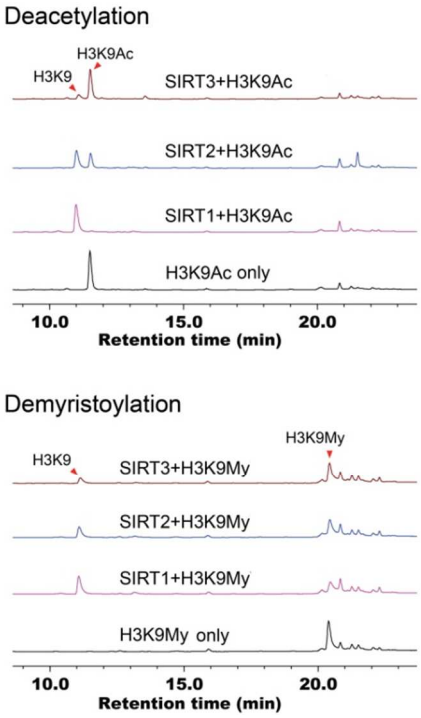


Figure. 1 Sirt1, Sirt2, and Sirt3 can catalyze both deacetylation and demyristoylation *in vitro*.

Because Sirt6 has also been shown to have deacetylase activity, we wanted to test whether the thiomyristoyl peptides could also inhibit the deacetylase activity of Sirt6 and whether thioacetyl peptides could inhibit Sirt6. We tested the effect of the thiomyristoyl peptide BH-TM4 on the deacetylase activity of Sirt6 with an H3 K9 acetyl peptide as the substrate. BH-TM4 could inhibit Sirt6 very well in both the demyristoylation and deacetylation activity assay with IC_{50} values of 1.7 μM and 8.2 μM, respectively (Table 3 and Figure S12-S13). The higher IC_{50} value for the deacetylation activity is likely because the much higher concentration of Sirt6 (10 μM) used. Sirt6's deacetylase activity is much weaker and we had to use much higher concentrations of Sirt6 in order to detect the deacetylation activity. To test whether thioacetyl peptides could inhibit Sirt6, we synthesized an H3 K9 thioacetyl peptide, ARK(TAc)ST, and tested its effects on both the demyristoylase and deacetylase activity of Sirt6. ARK(TAc)ST only showed 22% and 37% inhibition of the demyristoylase and deacetylase activity, respectively, at 100 μM. In addition, AGK2, a known Sirt2 inhibitor, showed 28% and 27% inhibition of the demyristoylase and deacetylase activity, respectively, at 100 μM. These data demonstrated that the thiomyristoyl lysine is important for inhibiting the two different activities of Sirt6.

Table 3. IC_{50} values (μM) of different inhibitors against the two different activities of Sirt6.

	Demyristoylation[a]	Deacetylation[b]
ARK(TMy)ST (BH-TM4)	1.7	8.2
ARK(TAc)ST	>100 (22%)[c]	>100 (37%)[c]
AGK2	>300(28%)[c]	>300 (27%)[c]

[a] An H3 K9 myristoyl peptide was used as the substrate. [b] An H3 K9 acetyl peptide was used as the substrate. [c] Values in parentheses show the percentage of inhibition at the concentration listed before the parentheses.

Finally, we tested whether the thiomyristoyl peptides were cell-permeable and could inhibit Sirt6 in live cells. Peptides are generally considered to have low cell permeability. However, it has been known that hydrophobic fatty acyl groups could increase the permeability.[34] Thus, the thiomyristoyl peptides may be cell-permeable. Previously, we demonstrated that Sirt6 can regulate the fatty acylation of TNFα [21] Thus, we examined whether the thiomyristoyl peptides could increase the lysine fatty acylation level of TNFα using a method previously described.[21] Flag-tagged TNFα was transfected into human embryonic kidney (HEK) 293T cells. The cells were cultured in the presence of the four different Sirt6 inhibitors and an alkyne-tagged fatty acid analog (Alk14). TNFα was immunoprecipitated and conjugated to rhodamine-azide (Rh-N_3) using click chemistry. A protein would be fluorescently labelled if it was fatty acylated by Alk14.[21] As shown in Figure 2, TNFα from the cells treated with the four Sirt6 inhibitors had increased fluorescent labelling (and thus increased fatty acylation levels) compared to TNFα from the control cells, suggesting that these thiomyristoyl peptides could inhibit SIRT6 in cells. Among them, BHJH-TM3 was the most potent at inhibiting Sirt6-catalyzed defatty-acylation of TNFα in cells. We further determined the dose response of BHJH-TM3. After transfection of TNFα, HEK293T cells were treated with different concentrations of BHJH-TM3 (0, 5, 10, 50, 100 and

200 μM), followed by the treatment of Alk14. The fluorescent labelling of TNFα increased almost 2 times at concentration of 5 μM and increased about 4 times at the concentration of 200 μM (Figure 2).

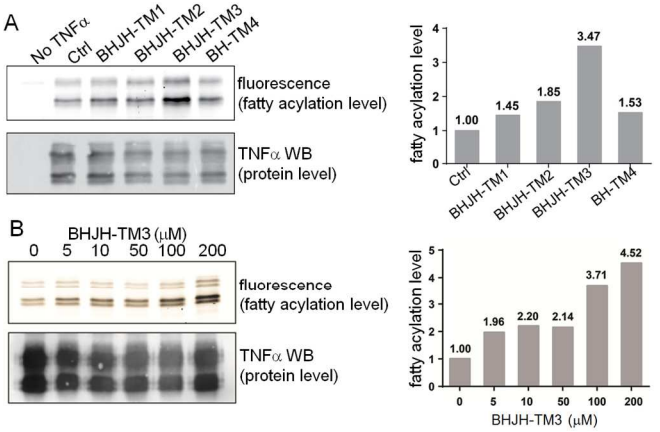


Figure. 2 Thiomyristoyl peptides inhibited Sirt6 and increased TNFα fatty acylation in mammalian cells. (A) HEK293T cells expressing TNFα were treated with 200 μM of different thiomyristoyl peptides. The TNFα fatty acylation level and protein level were detected (left) and quantified (right). (B) HEK293T cells expressing TNFα were treated with different concentrations of BHJH-TM3. The TNFα fatty acylation level and protein level were detected (left) and quantified (right).

Conclusions

In summary, we have demonstrated that thiomyristoyl peptides are potent and cell-permeable inhibitors for Sirt6. Although they are not specific for Sirt6 as they also inhibit Sirt1-3, this is the first time that potent and cell-permeable Sirt6 inhibitors are developed. These inhibitors should be useful starting points for the development of inhibitors that are more specific and more potent for Sirt6. Interestingly, we found that Sirt1-3 also have demyristolyation activity *in vitro*, which suggests that Sirt1-3 may have physiological functions beyond deacetylation.

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Notes and references

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‡ Electronic Supplementary Information (ESI) available: Experimental details and spectral/analytical data. See DOI: 10.1039/c000000x/

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Graphic Abstract

Potent mechanism-based Sirt6 inhibitors