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Thiosuccinyl Peptides as Sirt5-Specific Inhibitors

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Supporting Information

ABSTRACT: Sirtuins, a class of enzymes known as nicotinamide adenine dinucleotide-dependent deacetylases, have been shown to regulate a variety of biological processes, including aging, transcription, and metabolism. Sirtuins are considered promising targets for treating several human diseases. There are seven sirtuins in humans (Sirt1−7). Small molecules that can target a particular human sirtuin are important for drug development and fundamental studies of sirtuin biology. Here we demonstrate that thiosuccinyl peptides are potent and selective Sirt5 inhibitors. The design of these inhibitors is based on our recent discovery that Sirt5 prefers to catalyze the hydrolysis of malonyl and succinyl groups, rather than an acetyl group, from lysine residues. Furthermore, among the seven human sirtuins, Sirt5 is the only one that has this unique acyl group preference. This study demonstrates that the different acyl group preferences of different sirtuins can be conveniently utilized to develop small molecules that selectively target different sirtuins.

Sirtuins are a class of enzymes known as nicotinamide adenine dinucleotide (NAD)-dependent deacetylases.[1] Humans have seven sirtuins, Sirt1−7, which are known to play important roles in many biological processes, such as the regulation of life span, transcription, and metabolism.[2] Small molecules that can regulate sirtuin activity have been shown to have potential in treating several human diseases,[3] such as cancer,[4−7] diabetes,[8,9] and Parkinson's disease.[10] Although there are controversies regarding the link between sirtuins and longer life span[11] and the effectiveness of Sirt1 activators,[12] it is clear that sirtuins have important biological functions. Inhibitors that are specific for a particular sirtuin will be very useful for investigating the biological function and therapeutic potential of the specific sirtuin. The major obstacle in the development of small molecules that can regulate sirtuin activity is the fact that four of the seven human sirtuins (Sirt4−7) have either very weak or no deacetylase activity.[1,2] This poses two difficulties for the development of small molecules that can regulate sirtuin activity. First, it is hard to develop inhibitors or activators that target these sirtuins because no robust activity assay is available. Second, it is hard to tell whether the inhibitors/activators that target Sirt1, Sirt2, and Sirt3 can also target Sirt4−7.

Recently, our laboratory discovered that human Sirt5, a mitochondrial sirtuin with weak deacetylase activity, is an efficient demalonylase and desuccinylase (Figure 1).[13] The specificity for negatively charged malonyl and succinyl groups is determined by a conserved Arg and Tyr residue in most class-III sirtuins.[13] Several mitochondrial proteins are malonylated and succinylated on lysine residues.[13] Sirt5 serves to remove some of the malonyl and succinyl groups from the proteins, possibly as a mechanism for reversible regulation of protein activity in mitochondria.[13] Independently, Zhao and co-workers also identified lysine succinylation in *Escherchia coli* as a new post-translational modification.[14]

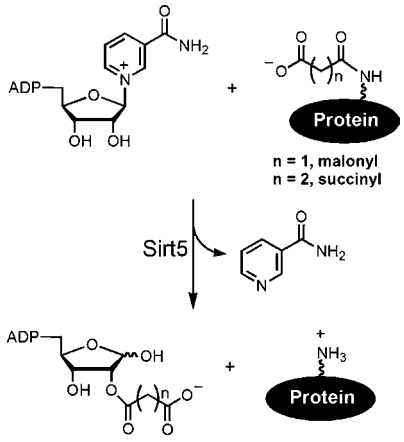

Figure 1. NAD-dependent demalonylation and desuccinylation reactions catalyzed by Sirt5.

The discovery of the robust enzymatic activity now provides a reliable assay for the development of Sirt5 inhibitors.[13] In addition, since Sirt5's acyl group preference is unique among all the human sirtuins,[13] we reasoned that we could take advantage of this to develop Sirt5-specific inhibitors. Such inhibitors would be valuable tools to study the biological function of Sirt5 in cells and to evaluate whether Sirt5 would be a good target for treating human diseases. Herein we report that thiosuccinyl peptides can be used as Sirt5-specific inhibitors.

Thioacetyl peptides can inhibit sirtuins with deacetylase activities by forming a stalled covalent intermediate (Figure 2).[19−21] Because Sirt5 uses the same mechanism as the deacetylases to remove malonyl and succinyl groups,[13] we reasoned that thiosuccinyl or thiomalonyl peptides would be mechanism-based inhibitors for Sirt5. Because other sirtuins do not recognize malonyl and succinyl lysine peptides,[13] we predicted that thiomalonyl and thiosuccinyl peptides should be Sirt5-specific inhibitors. To test this hypothesis, we synthesized a histone H3 lysine 9 (H3K9) thiosuccinyl peptide (H3K9TSu, Figure 3). We chose to use the thiosuccinyl group because

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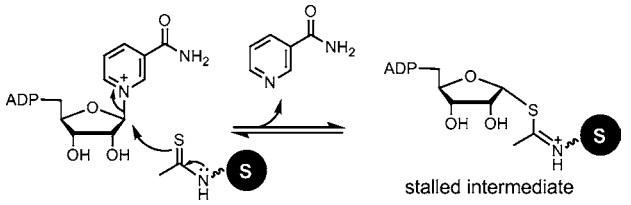

Figure 2. Mechanism-based inhibition of sirtuins with deacetylase activity by thioacetyl peptides. The thioacetyl peptides can undergo the first step of the sirtuin-catalyzed deacetylation reaction, forming the covalent 1′-*S*-alkylimidate intermediate. This intermediate is relatively stable and cannot proceed further in the deacetylation reaction pathway, thus inhibiting sirtuins.

succinyllysine is more stable (malonyllysine is prone to decarboxylation).[13]

The synthesis of H3K9TSu peptide is shown in Figure 3. L-Fmoc-Lys-OH was first coupled to the mono-*tert*-butyl succinate. The free carboxylate of Lys was then protected as the phenacyl ester. The fully protected Lys was then treated with Lawesson's reagent to introduce the thiosuccinyl functional group site-specifically to the side chain of Lys. The phenacyl ester was then removed with zinc in acetic acid, and the resulting compound was then used in standard Fmoc solid-phase peptide synthesis along with several L-Fmoc-amino acids to give the desired H3K9TSu peptide, KQTAR(TSuK)STGGKA. As a control, we also synthesized a H3K9 thioacetyl (H3K9TAc) peptide, KQTAR(TAcK)STGGKA, using published procedures.[19]

We then assayed the inhibition of Sirt1−3 and Sirt5 with the H3K9TSu and H3K9TAc peptides. The IC_{50} values are shown in Table 1. All of the assays were carried out using identical enzyme (1 μM) and substrate (0.3 mM acyl peptide, 0.5 mM NAD) concentrations. For Sirt5, we used an H3K9 succinyl peptide, KQTAR(SuK)STGGKA, as the substrate. For Sirt1−3, we used an H3K9 acetyl peptide, KQTAR(AcK)STGGKA, as the substrate. The Sirt5 or Sirt1−3 enzyme was added last to initiate the reaction, and thus, there was no preincubation of the sirtuin with the inhibitor before initiation of the enzymatic reaction. H3K9TSu did not inhibit Sirt1−3 even at 100 μM concentration, but it did inhibit Sirt5 with an IC_{50} value of 5 μM (the dose−response curves can be found in the Supporting Information). In contrast, the H3K9TAc peptide inhibited Sirt1−3 with IC_{50} values of 1−2 μM, but it did not inhibit Sirt5 at 100 μM. These results demonstrate that the H3K9TSu peptide is a Sirt5-specific inhibitor.

Many inhibitors have been developed for Sirt1, Sirt2, and Sirt3. We obtained several of them from commercial sources and tested whether they can also inhibit Sirt5 (Table 1). Suramin, which has been reported to be a Sirt5 inhibitor,[22] inhibits Sirt5 with an IC_{50} value of 25 μM but also inhibits Sirt1−3 (IC_{50} values from 5 to 75 μM). In addition, nicotinamide and AGK2 (a reported Sirt2-selective inhibitor)[10] also showed some inhibition of Sirt5 (Table 1). However, the IC_{50} values were much higher and they are not selective for Sirt5. Thus, the H3K9TSu peptide is not only the first but probably also the most potent Sirt5-specific inhibitor reported to date.

We also performed kinetic studies to find out whether H3K9TSu is competitive with substrate. Initial velocities (V) at saturating NAD concentrations were determined at various concentrations of H3K9Su as the substrate and H3K9TSu as the inhibitor. At different inhibitor concentrations, V was plotted versus substrate concentration ([S]) at each inhibitor concentration, and the data were fitted to the Henri−Michaelis−Menten equation.[23] As shown in Figure 4A, the apparent K_m value for H3K9Su increased with increasing concentrations of H3K9TSu inhibitor. Furthermore, plots of $1/V$ versus $1/[S]$ revealed a series of lines that intersected at the $1/V$ axis at each inhibitor concentration (Figure 4B). The features of the double reciprocal plot are consistent with H3K9TSu being a competitive inhibitor.

Finally, to extend the application of thiosuccinyl peptides as Sirt5 inhibitors, we synthesized several shorter thiosuccinyl peptides. The IC_{50} values are shown in Table 2. The longer thiosuccinyl peptide is a more potent inhibitor for Sirt5 (Table 2; compare entry 1 to entries 2−6). However, for the five-residue thiosuccinyl peptides, the IC_{50} value can still be as low as 25 μM (Table 2, entries 4−6). Interestingly, peptides with the thiosuccinyllysine residue at the C-terminus or N-terminus (Table 2, entries 2 and 3) were less potent than peptides with the thiosuccinyllysine residue in the middle (Table 2, entries 4−6). Thus, it may be possible to obtain Sirt5 inhibitors with lower molecular weights.

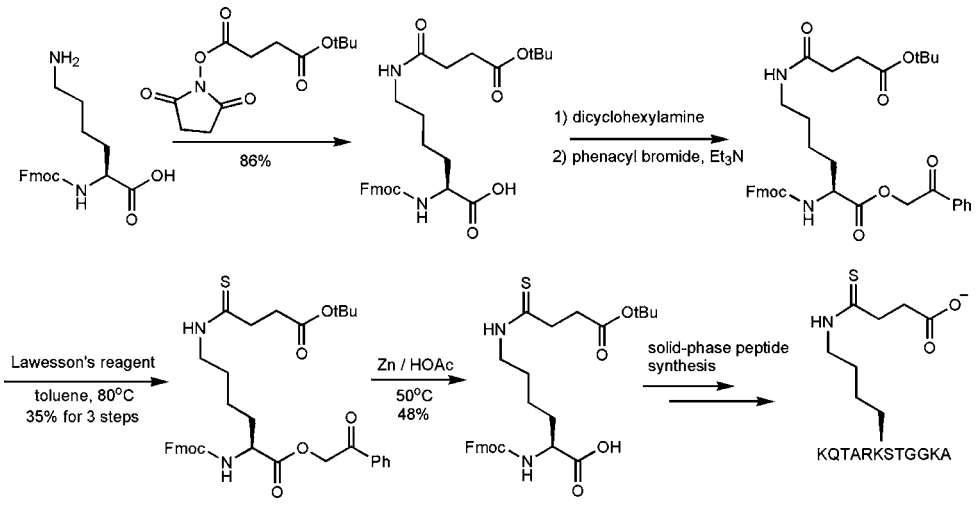

Figure 3. Synthesis of the H3K9TSu peptide.

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Table 1. IC_{50} Values of Different Inhibitors for Different Sirtuins (Reported Values Are Shown in Parentheses)

	IC_{50} (μM)					
enzyme	H3K9TSu	H3K9TAc	nicotinamide	AGK2	suramin	sirtinol
Sirt1	>100[a]	1	120 (<50[14])	200 (>40[10])	5 (0.3[15])	200 (131[16])
Sirt2	>100[a]	2	75 (32[17])	150 (3.5[10])	10 (1.2[15])	100 (38[18])
Sirt3	>100[a]	2	50	200 (>40[10])	75	150
Sirt5	5	>100[a]	150	>100[b]	25 (22[20])	>100[a]

[a]No inhibition at 100 μM. [b]40% inhibition at 100 μM.

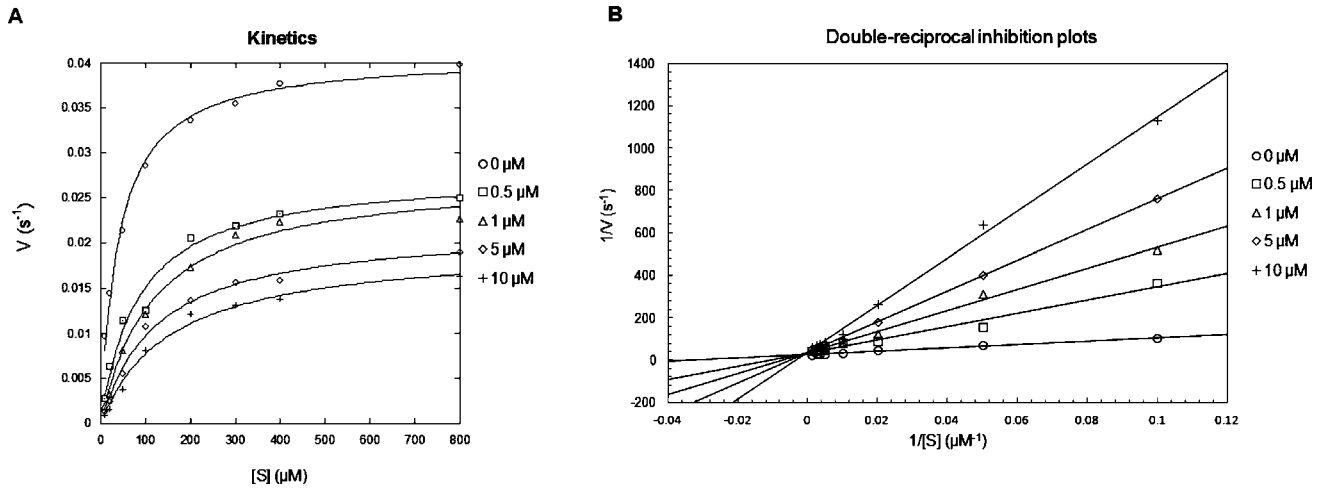

Figure 4. (A) Henri–Michaelis–Menten plots and (B) double-reciprocal plots showing the effects of H3K9TSu inhibitor on the velocity of Sirt5 desuccinylation.

Table 2. IC_{50} Values of Shorter Thiosuccinyl Peptides for Sirt5

	thiosuccinyllysine peptide	IC_{50} (μM)
1	KQTAR(TSuK)STGGKA (H3K9TSu)	5
2	KQTAR(TSuK)	100
3	(TSuK)STGGKA	100
4	AR(TSuK)ST	30
5	Ac-AR(TSuK)ST-NH_2	40
6	Ac-RR(TSuK)RR-NH_2	25

In summary, we have shown that the H3K9TSu peptide is a mechanism-based and competitive inhibitor specific of Sirt5 and that shorter peptides with thiosuccinyllysines can also inhibit Sirt5. This opens up possibilities for the development of more potent and more cell-permeable inhibitors specific for Sirt5 to study the biological function of Sirt5 and explore the therapeutic potential of Sirt5 inhibition. Since protein succinylation and malonylation (which are controlled by Sirt5) have not been studied to date, small-molecule inhibitors specific for Sirt5 will also be valuable tools for controlling the levels of protein succinylation and malonylation, which can facilitate the study of the biological function of these protein post-translational modifications.

■ ASSOCIATED CONTENT

Supporting Information

Experimental methods for the synthesis of peptides, HPLC assays, kinetics studies, NMR spectra, and complete refs 7, 8, and 11–13. This material is available free of charge via the Internet at http://pubs.acs.org.

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