Inhibitors

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Discovery of Macrocyclic Peptides Armed with a Mechanism-Based Warhead: Isoform-Selective Inhibition of Human Deacetylase SIRT2**

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Mass screening of available compound libraries by means of in vitro or cell-based assays is the most prevalent approach for the discovery of enzyme inhibitors. The major drawback of this approach is generally a poor cost–performance ratio, often giving many false-positive hits and therefore requiring extensive reevaluations of the hits to identify true inhibitors. Enzyme-mechanism-based drug design is an alternative approach,[1–3] in which an inhibitory functional group is ingeniously designed on the basis of knowledge of mechanism and embedded into an appropriate scaffold, such as a native enzyme substrate. Even though this approach represents a strategy with a high cost–performance ratio to obtain an initial hit(s), the primary design of such an inhibitor is rarely potent enough, particularly against an enzyme family that catalyzes various substrates or consists of multiple isoforms. In any case, in order to improve the potency of the initial hit(s) further elaboration of its structure is generally conducted by classical medicinal chemistry.

One example of mechanism-based designed inhibitors against yeast sirtuins is derived from a substrate peptide, H_2N-KSTGG-K^{Ac}-APRKQ-OH, in which the ε-*N*-acetyl group on the lysine residue (K^{Ac}) was replaced with ε-*N*-trifluoroacetyl group (K^{Tfa}).[4] Sirtuin belongs to a family of deacetylases, homologs of which are widespread in prokaryotes and eukaryotes, and deacetylates the ε-*N*-acetyl group on K^{Ac} in various cellular substrates in the presence of nicotinamide adenine dinucleotide (NAD^+) as a cosubstrate. Mechanistically, the carbonyl oxygen of the ε-*N*-acetyl group first attacks the C1′ of the *N*-ribose in NAD^+ to form an *O*-alkylamidate intermediate upon the release of a nicotinamide group, and following several reaction steps give the free ε-amino-K residue and 2′-*O*-acetyl-ADP-ribose (Scheme 1a).[5,6] The replacement of K^{Ac} with K^{Tfa} in the peptide marginally increases its affinity to yeast sirtuins, but reduces the rate of the formation of the *O*-alkylamidate intermediate by nearly six orders of magnitude, thus resulting in its inhibitory behavior (Scheme 1b).[4,7,8] Various other mechanism-based inhibitors against sirtuins, such as ε-*N*-thioacetyl-K (K^{Tac}) containing peptides,[4,9] have been reported, however, none of them showed spectacular inhibitory activities; for example, IC_{50} values of K^{Tfa} and K^{Tac} peptides were 61 μM and 1 μM, respectively.

Because of a biological importance of mammalian sirtuins (SIRTs) that are involved in the regulation of diverse cellular functions, it is of great interest to devise more potent inhibitors against SIRTs than the present examples. In this study, we chose human SIRT2, which is known to deacetylate K^{Ac} residues in α-tubulin[10] and histone H4K16,[11] thereby regulate cell-cycle progression. Since there are three isoforms (SIRT1–3) of class I SIRTs[12] and each SIRT plays distinct biological roles, we were interested in devising not only a SIRT-selective but also an isoform-selective inhibitor as an epigenetic probe and a potential therapeutic agent. We herein report a new methodology to construct a library of nonstandard macrocyclic peptides that contain a K^{Tfa} residue in the middle of the sequence by using a custom-made translation apparatus that enables genetic code reprogramming, and rapidly select potent inhibitors by using an in vitro display format. The nonstandard macrocyclic peptides that were identified exhibit a high inhibitory potency against SIRT2 with K_d (and IC_{50}) values in the low (single digit) nanomolar region as well as a remarkable isoform selectivity.

Genetic code reprogramming is a new means to ribosomally express various nonstandard peptides with desired structural diversities, that is executed by the reassignment of codons from proteinogenic amino acids to nonproteinogenic or artificial amino acids.[13–15] To facilitate genetic code reprogramming, we have devised a custom-made cell-free translation system integrated with the flexizyme (flexible tRNA acylation ribozyme) technology, referred to as FIT (flexible in vitro translation) system.[16] In the FIT system, arbitrary proteinogenic amino acids are omitted from the translation components to create vacant codons, and tRNAs that read the vacant codons are charged with nonproteinogenic amino acids by flexizyme; thus nonproteinogenic amino

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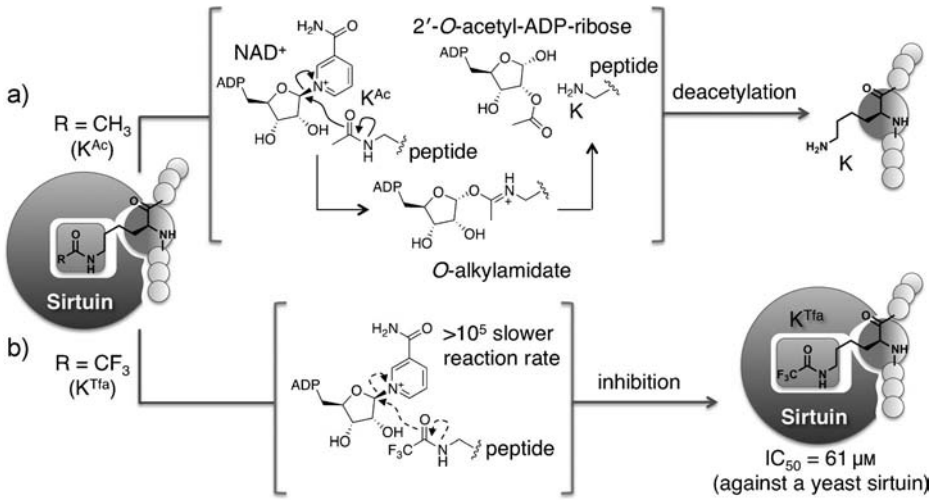


Scheme 1. Schematic illustration of a) deacetylation of acetylated lysine (K^{Ac}) on peptide catalyzed by sirtuin, and b) mechanism-based inhibition of deacetylation by a K^{Tfa}-containing peptide. a) The carbonyl oxygen atom of K^{Ac} attacks NAD^+ to form an *O*-alkylamidate intermediate, followed by the breakdown of the intermediate to give the corresponding deacetylated peptide. b) Substitution of K^{Ac} with K^{Tfa} leads to an unproductive intermediate, which significantly slows the initial attack of the oxygen atom of Tfa, thus resulting in inhibition of the activity of sirtuin.

acids are introduced to the translation system to express peptides that contain these nonproteinogenic amino acids. More recently, the FIT system has been coupled with an mRNA display method,[17,18] devising a new platform technology referred to as RaPID (random nonstandard peptide integrated discovery) system.[19] In comparison with other in vitro or in vivo display methodologies,[20–24] the RaPID system allows us to select active nonstandard peptides against desired targets from their highly diverse libraries that consist of multiple unique nonproteinogenic amino acids in the peptide chain. Herein we have utilized this RaPID system to construct such a library of peptides that contain K^{Tfa} residues as a mechanism-based warhead, and aimed at discovering anti-SIRT2 inhibitors.

In this study, we reprogrammed the AUG codon to two distinct artificial amino acids, one of which was α-*N*-(2-chloroacetyl)-L-tyrosine or α-*N*-(2-chloroacetyl)-D-tyrosine ($ClAc^LY$ or $ClAc^DY$) assigned to the initiator AUG codon, and the other of which was K^{Tfa} assigned to the elongator AUG codon. These double assignments of amino acids to a single AUG codon could be achieved by using an FIT system which lacks methionine and release factor 1 (RF1), but is supplemented with two aminoacyl-tRNAs, in which $ClAc^LY$ or $ClAc^DY$ and K^{Tfa} were charged onto initiator $tRNA^{fMet}_{CAU}$ and orthogonal elongator $tRNA^{Asn\text{-}E2}_{CAU}$,[25] respectively, by means of enhanced flexizyme (eFx).[15]

The mRNA sequence library was designed to have a mixture of lengths consisting of AUG-$(NNC)_m$-AUG-$(NNC)_n$-UGC, in which the combination of $[m,n]$ is [3,4], [4,4], [4,5], [5,5], and [5,6], and UGC assigned cysteine (C) residue (see Figure 1a, and Table S1 in the Supporting Information). In this library design, five amino acids (M, Q, E, K, and W) would not appear in the random NNC region, and, more importantly, the AUG codon would also not appear in it; thus, $ClAc^LY$/$ClAc^DY$ and K^{Tfa} are ensured to appear only in the designated positions. RNA sequences for the ribosome binding site and $(GS)_3$ linker peptide were

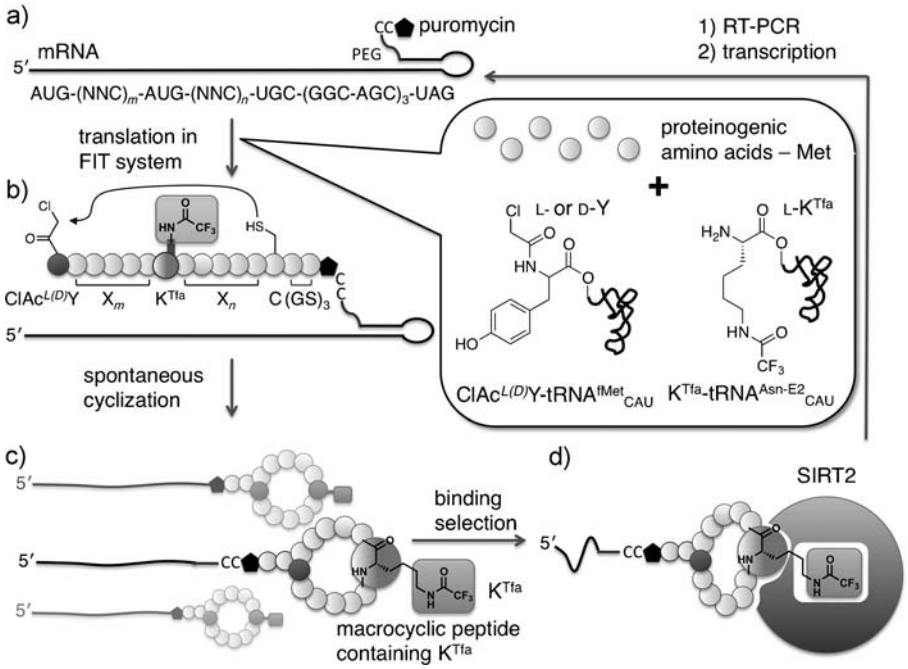


Figure 1. Schematic illustration of the construction of a library of macrocyclic peptides that contain a K^{Tfa} residue by the FIT system and selection of the nonstandard peptides. a) An mRNA sequence library containing two AUG codons. The initiation AUG codon is suppressed by $ClAc^{L(D)}Y\text{-}tRNA^{fMet}_{CAU}$ and the elongation AUG codon is suppressed by $K^{Tfa}\text{-}tRNA^{Asn\text{-}E2}_{CAU}$. b) The translated peptide library contains $ClAc^{L(D)}Y$ at the N terminus and K^{Tfa} at the middle of the random sequences. The peptide and its encoding mRNA are connected by puromycin, thus resulting in the peptide-displayed mRNAs. c) The ClAc group and cysteine residue spontaneously react with each other after translation to give a macrocyclic peptide that contains a single K^{Tfa} residue. d) SIRT2-binding peptides are selected from the library on the basis of SIRT2-binding activity. Selected mRNA sequences are amplified by reverse transcription and PCR (RT-PCR), and the amplified DNAs are transcribed to give an enriched mRNA library with the desired SIRT2-binding property.

embedded upstream and downstream of the mRNA library sequences, respectively, and this mRNA library was terminated with a UAG codon followed by a linker RNA region for the installation of a puromycin-CC-(poly(ethylene glycol) linker)-DNA fragment. Because RF1 was absent in the FIT system, the desired peptide–mRNA fusion with puromycin was effectively formed at the UAG ribosome-stalling position in generally greater than 10% yield, thus giving a complexity of the peptide library of 10^{12} or higher after all processes of the nonstandard-peptide library synthesis (Figure 1b and Table S1).

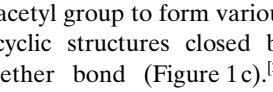
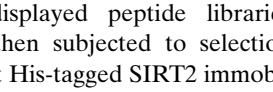
Translation of the mRNA library was initiated with ClAc^{L}Y and ClAc^{D}Y to give two independent libraries, referred to as ^{L}Y and ^{D}Y libraries. The C residue(s) installed at the UGC codon after the random region or UGC appear in the random region would spontaneously react with the N-terminal chloroacetyl group to form various macrocyclic structures closed by a thioether bond (Figure 1c).[26] The displayed peptide libraries were then subjected to selection against His-tagged SIRT2 immobilized on magnetic beads on the basis of strong affinity to SIRT2 (Figure 1d; see Figure S1 for more details of the RaPID selection procedures), resulting in saturated enrichment of active populations at the fifth and sixth rounds in ^{L}Y and ^{D}Y libraries, respectively (Figure S2).

The enriched cDNA pools from ^{L}Y and ^{D}Y libraries were cloned, and the peptide-encoding sequences of 21 and 16 clones, respectively, were analyzed (Table S2). Interestingly, the ^{L}Y library gave 14-mers consisting of Ac-^{L}Y-$(X)_5$-K^{Tfa}-$(X)_6$-C, while ^{D}Y library gave 13- and 14-mers consisting of Ac-^{D}Y-$(X)_5$-K^{Tfa}-$(X)_{5,6}$-C. Despite the fact that we did not observe convergent groups of sequences among these clones, the careful comparison of apparent peptide sequences showed nearly conserved residues (in a rate of over 70% in all clones) adjacent to the warhead K^{Tfa}, consisting of (I/V)-K^{Tfa}-RY in both libraries. Furthermore, among the clones that have the (I/V)-K^{Tfa}-RY sequence, the R(I/V)-K^{Tfa}-RY sequence appeared most frequently (10 clones out of 15), thus leading to the hypothesis that this sequence region might interact with the SIRT2 active site.

In order to see if selected peptides were able to inhibit SIRT2 enzymatic activity, every clone was expressed by the FIT system and the inhibitory activity of the individual peptide was assayed by a conventional fluorescence-based method by using a known SIRT2-substrate peptide (QPKK^{Ac}). MALDI–TOF analysis of the individual peptides clearly showed that all possessed the designated K^{Tfa} residue and macrocyclic structure (see Figure S3 for mass spectra of four representative clones and Table S3 for observed mass values of other clones). Although the expression level of all the peptides could be varied in 10–50 nM level under the assay conditions depending on sequence compositions, they all exhibited inhibitory activities to some extent (Figure S4), thus validating the effectiveness of our designed-library strategy.

In order to quantitatively determine how strongly selected macrocyclic peptides could bind to SIRT2, we chemically synthesized two representative peptides with a C-terminal carboxyamide modification, S2Li8 and S2iD7, both of which shared the R(I/V)-K^{Tfa}-RY motif, albeit originating from the different library sets, and analyzed their binding kinetics against SIRT2 by using a surface plasmon resonance (SPR) method. Interestingly, both peptides behaved nearly the same kinetically, and gave nearly identical K_d values of 3.8 and 3.7 nM, respectively (Table 1, S2iL8 and S2iD7; Figure 2a and b; Figure S5a and b).

Table 1: Binding and inhibitory properties of peptides studied.[a]

Peptide	Sequence	k_a [$\times10^6$ M s^{-1}]	k_d [$\times10^{-3}$ s^{-1}]	K_d [nM]	IC_{50} [nM] SIRT2	SIRT1	SIRT3
S2iL8		1.2	4.7	3.8	3.2	47	480
S2iD7		1.3	4.8	3.7	3.7	32	240
lin-S2iL8	Ac^{L}YSNFRIK^{Tfa}RYSNSSC^{R}–NH$_2$	0.98	13	13	6.1	n.d.	n.d.
lin-S2iD7	Ac^{D}YHDYRIK^{Tfa}RYHTYPC^{R}–NH$_2$	1.6	17	10	5.5	n.d.	n.d.
RIK^{Tfa}RY	AcRIK^{Tfa}RY–NH$_2$	0.82	67	82	31	280	1000
SDK^{Tfa}TI	AcSDK^{Tfa}TI–NH$_2$	n.d.	n.d.	n.d.	$>10^4$	n.d.	n.d.

[a] The values of k_a, k_d, and K_d were determined by SPR, and IC_{50} values were determined by fluorescence-based in vitro assays. n.d. = not determined.

Encouraged by the strong affinities observed in S2iL8 and S2iD7, we measured their inhibitory activities by titrating the SIRT2-catalyzing deacetylation rate of K^{Ac} in the aforementioned fluorescence-based assay. The IC_{50} values in both peptides are again very similar (3.2 and 3.7 nM, respectively) and consistent with the K_d values (Table 1, S2iL8 and S2iD7; Figure 2c and d). To evaluate their isoform selectivity against class I SIRTs, the same titration experiments were performed against SIRT1 and SIRT3 with their corresponding peptide substrates (RHKK^{Ac} and QPKK^{Ac}, respectively). Both macrocyclic peptides possess similar isoform selectivity with approximately 10-fold and 100-fold elevated IC_{50} values against SIRT1 and SIRT3, respectively. However, S2iL8 seems to be more isoform selective over S2iD7; other residues outside of the R(I/V)-K^{Tfa}-RY motif possibly contribute to the observed selectivity (Table 1, S2iL8 and S2iD7; Figure 2c and d).

The macrocyclic form of the peptides might preorganize the structures and might thus contribute to the increase in their target-binding affinities.[19,27,28] Therefore, to evaluate such an effect on the selected peptides, we synthesized linear counterparts of S2iL8 and S2iD7, lin-S2iL8 and lin-S2iD7, respectively (Table 1), in which the N-terminal chloroacetyl group is substituted with an acetyl group, and the C-terminal cysteine residue is alkylated with iodoacetamide (C^{R}). SPR

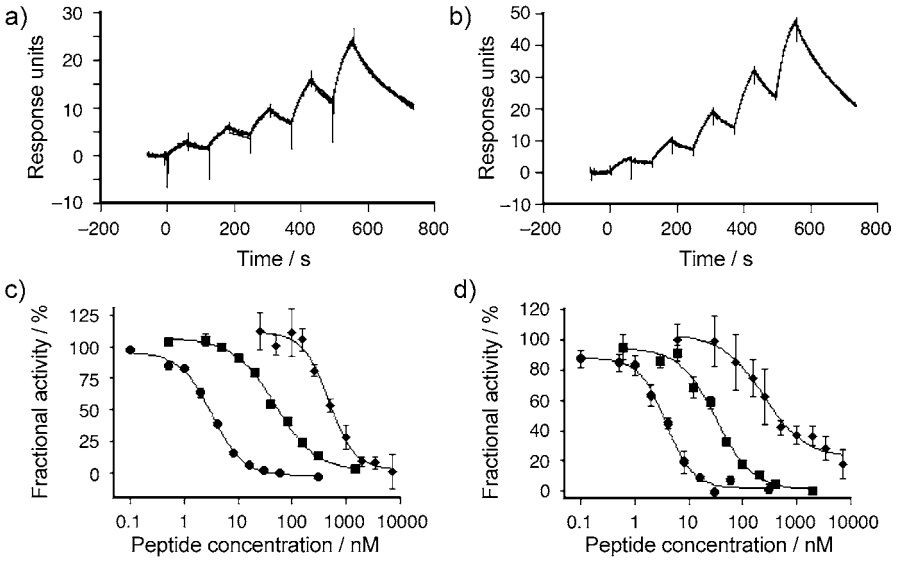


Figure 2. Binding and inhibitory properties of macrocyclic peptides. SPR sensorgrams of a) S2iL8 and b) S2iD7 against SIRT2. The peptide was used in five different concentrations (1, 2, 4, 8, and 16 nM) with a SIRT2-immobilized sensor chip. The binding curve was analyzed by the single-cycle kinetics method. The observed data were superimposed to theoretical fitting curves based on a 1:1 binding model (see Figure S5 to distinguish the observed data in black and the theoretical fitting curve in red). Inhibitory potencies of c) S2iL8 and d) S2iD7 against SIRT2 (●) and its isoforms SIRT1 (■) and SIRT3 (◆). The inhibitory activities were measured by means of in vitro fluorescence-based assays (see details in material methods section in the Supporting Information) and plotted as the fractional activities of SIRT2 or its isoforms at varying inhibitor concentrations. The standard deviation of each data point was generated by four independent experiments, and the data were fitted to a sigmoidal curve.

analysis showed that both linear peptides have slightly higher dissociation rates (k_d) from SIRT2 than cyclic peptides, thus reflecting the elevation of K_d values by approximately three times. Inhibitory activities against SIRT2 were also consistent with the observed K_d values, giving slightly higher IC_{50} values than those of macrocyclic ones (Table 1 and Figure S5c–f). The data suggested that in these particular peptides the macrocyclic structure indeed increased the affinity to SIRT2, but the degree of increased affinity turned out to be rather less significant. As aforementioned, the comparison of clone sequences has suggested that R(I/V)K^{Tfa}RY sequence might be the critical motif responsible for binding activity. To this end, a short peptide, RIK^{Tfa}RY bearing an N-terminal acetyl and a C-terminal amide group, was synthesized and its binding and inhibitory activities against SIRT2 were determined. Similar to the linear peptides, the dissociation rate of RIK^{Tfa}RY was further elevated, but the K_d value remained below 100 nM (Figure S6a). Most importantly, the IC_{50} values of RIK^{Tfa}RY against SIRT2, SIRT1, and SIRT3 were 31 nM, 280 nM, and 1000 nM, respectively (Table 1, RIK^{Tfa}RY and Figure S6b), thus exhibiting the selective inhibitory potency against SIRT2 over other isoforms, such as the full-length macrocyclic peptides. In sharp contrast, a 5-mer peptide derived from an α-tubulin substrate motif (Table 1, SDK^{Tfa}TI and Figure S6c) had an IC_{50} value that was nearly three orders of magnitude higher, thus confirming that the selected sequence motif is critical for the observed potency.

In conclusion, we have ribosomally expressed a highly diverse library of thioether-containing macrocyclic peptides based on the concept of a mechanism-based warhead, and utilized the RaPID system to enrich "binding-active" sequences that possess K^{Tfa} as a warhead against SIRT2. The selected peptides are not just binders but also potent inhibitors of SIRT2 and gave consistent K_d and IC_{50} values of approximately 3–4 nM. Remarkably, these peptides are SIRT2-selective inhibitors that also discriminate against other isoforms. Thus, this proof-of-concept experiment validates that introduction of a mechanism-based warhead into random sequences of peptides facilitates inhibitor discovery. The methodology developed herein is rationally applicable to the development of inhibitors against various other posttranslational-modification enzymes by recruiting appropriate mechanism-based warheads, for example, hydroxamic acid against deacetylases,[29–31] and propargylamine against demethylases.[32]

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