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A FRET-based assay for screening SIRT5 specific modulators

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ABSTRACT

A fluorogenic assay for SIRT5 has been developed to screen their small molecule modulators based on the recent discovery that SIRT5 is a demalonylase and desuccinylase. However, this assay uses a fluorogenic peptide containing 7-amino-4-methylcoumarin (AMC), which becomes the cause of false positive hits from the screening. To overcome this, we have developed an alternative method called a FRET-based assay, which will be reliable and useful for screening SIRT5 modulators in a high-throughput format since no AMC group present in this assay.

As Class III of histone deacetylases, sirtuins are originally known as a class of enzymes to remove posttranslational modification of acetyl group from lysine residue.[1,2] Different from Class I/II/IV of histone deacetylases, sirtuins carry out the deacetylation by using nicotinamide adenine dinucleotide (NAD) as a cofactor instead of Zinc (Fig. 1).[2] There are seven human sirtuins (SIRT1–7) that present diversity in cellular localization and function (Fig. 1).[3] Sirtuins involve many important biological functions, including life span, transcription, genome stability, metabolism and protein secretion.[4–6] Therefore, sirtuins as important drug targets have been extensively investigated. Small molecules that can modulate sirtuin activity have been shown to have potential for treating many human diseases, such as obesity, diabetes, inflammation, cancer, cardiovascular, neurodegenerative diseases and other ageing-related diseases.[7,8] For examples, SIRT1 inhibitors can induce cancer cell growth arrest and thus promote apoptosis.[9,10] SIRT2 inhibitors show beneficial effects in Parkinson's disease.[11] On the other hand, sirtuin activators have the potentials for treating diabetes[12] and promoting longevity.[13] However, there are still some controversies on the effects of sirtuin activators.[14,15]

To discover small molecules that can regulate sirtuin activity, high-throughput assays have been developed by the detection of either deacetylated peptide formation or nicotinamide formation using a coupled enzymatic assay. For the detection of nicotinamide formation, nicotinamidase and glutamate dehydrogenase as coupling enzymes have been used not only in sirtuin activity assay but also in other assays for nicotinamide-forming enzymes.[16] On the other hand, the detection of deacetylated peptide formation become the most common strategy for sirtuins' high-throughput assays. Among these, one is called a fluorogenic assay that couples the deacetylation of sirtuins to the trypsin-catalyzed amide bond hydrolysis to release a fluorescent small molecule, 7-amino-4-methylcoumarin (AMC, Fig. 2).[17] However, the use of this substrate containing AMC molecule has previously become the cause of false positive hits and thus bring the controversial on the effects of sirtuin activators (resveratrol and its analogues).[14,15] Not only AMC itself, but also other large aromatic groups would probably cause similar issues due to a hydrophobic binding pocket for aromatic groups.[18] The other method is a fluorescence resonance energy transfer (FRET)-based assay where a donor dye and a quencher dye are connected to an acetyl peptide substrate.[19] A FRET effect will occur in this acetylated peptide, which will cause the fluorescence of a donor dye quenched by a quencher dye. The deacetylation of sirtuins followed by trypsin digestion disrupts the FRET signal and thus releases the fluorescence from a donor dye (Fig. 3).[19]

All fluorogenic or FRET-based assays have been developed previously only for SIRT1, SIRT2 and SIRT3 so far, three sirtuins with high deacetylase activity. Until recent discoveries that SIRT5 is a demalonylase and desuccinylase (Fig. 1)[20] while SIRT6 is a defatty-acylase (removing long chain fatty acyl groups) (Fig. 1)[4], a fluorogenic assay for SIRT5 (Fig. 2) as well as for SIRT6 (Fig. 2) have been developed using a fluorogenic AMC-succinyl peptide

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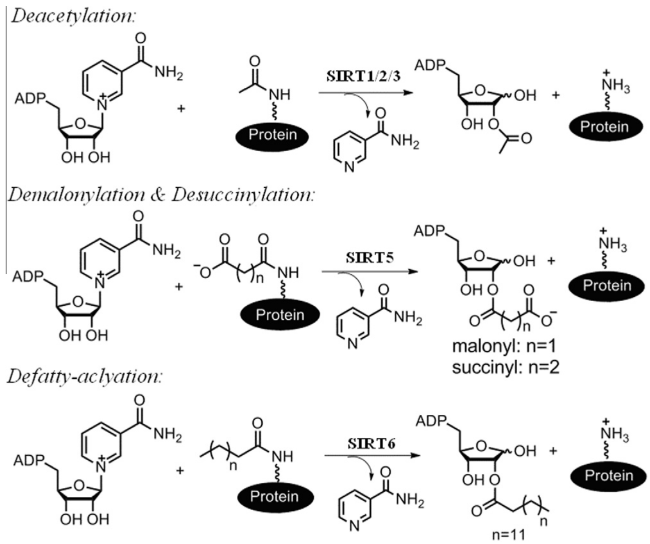

Figure 1. The deacylation reactions catalyzed by different sirtuins.

and AMC-myristoyl peptide, respectively.[21,22] As we discuss above, AMC molecule became an issue of accuracy during the screening.[14,15] Additionally, AMC molecule has to be installed at the C-terminal of a fluorogenic peptide, which will cause less binding affinity to sirtuin. Therefore, an alternative assay for these sirtuins still needs to be further developed.

Here we report that an alternative method called a FRET-based assay for SIRT5 has been developed, which can be used to screen for SIRT5 inhibitors. We also demonstrate that combined with a secondary screen of SIRT1/2/3, we can identify inhibitors that are selective or not for SIRT5.

The design of a FRET-based assay of SIRT5 is a succinyl peptide containing a pair of donor dye and quencher dye at the end of each terminal, respectively (Fig. 3). Compared to other FRET pairs, a FRET pair of 4-(dimethylaminoazo)benzene-4-carboxylic acid/5-((2-Aminoethyl)aminonaphthalene-1-sulfonic acid, DABCYL/EDANS exhibits better sensitivity at E_x/E_m (maximum excitation and emission) = 340 nm/490 nm.[23] Therefore, we chose DABCYL/EDANS as a pair of donor dye and quencher dye (structures shown in Fig. S1, in the Supporting information). The synthetic route to this FRET peptide is following the standard procedure of solid phase peptide synthesis shown in the Supporting information (SI). Because one of the known succinylated proteins is glutamate dehydrogenase,[20] we made a FRET peptide based on the sequence of the glutamate dehydrogenase containing the succinyl lysine residue, (DABCYL)ISGASE(SuK)DIVHSE(EDANS)G (Fig. 3), where SuK stands for succinyl lysine. For controls, we also synthesized the corresponding peptides with acetyl lysine and free lysine, (DABCYL)ISGASE(AcK)DIVHSE(EDANS)G and (DABCYL)ISGASEKDIVHSE(EDANS)G.

With these FRET peptides in hand, we first used (DABCYL)ISGASEKDIVHSE(EDANS)G to check whether it could be efficiently digested by trypsin to disrupt the FRET signal and then give the fluorescence. The results showed that 6.25 U trypsin could digest the peptide very efficiently and give about 15 times higher fluorescence compared to the control without trypsin in a one-hour reaction containing 10 μM (DABCYL)ISGASEKDIVHSE(EDANS)G (Fig. S2, SI).

We then determined the kinetic constants of SIRT5 on the (DABCYL)ISGASE(SuK)DIVHSE(EDANS)G peptide versus (DABCYL)-ISGASE(AcK)DIVHSE(EDANS)G 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 to give the K_m and k_{cat} values as shown in Table 1. The catalytic efficiency (k_{cat}/K_m) for the succinyl peptide was much higher than that for the acetyl peptide (Table 1), which is consistent with the results from the non-fluorophore containing peptide.[20] This result demonstrate that (DABCYL)ISGASE(SuK)DIVHSE(EDANS)G peptide is a substrate for SIRT5, a desuccinylase.

Encouraged by this, we next test the (DABCYL)ISGASE-(SuK)DIVHSE(EDANS)G peptide by coupled the SIRT5 enzymatic reaction with trypsin digestion. In brief, the assay was carried out in one-hour incubation with 1 μM SIRT5 and 10 μM peptide followed by one-hour incubation with 6.25U trypsin. As shown in Figure 4, the fluorescence was increased over 10-fold compared with the control without SIRT5. In contrast, no fluorescence increase was observed when SIRT1, 2 or 3 was used instead of SIRT5 (Fig. 4). The fluorescence also show a dose-dependent manner against the concentrations of SIRT5 (Fig. S3, SI). These results demonstrate that the (DABCYL)ISGASE(SuK)DIVHSE(EDANS)G peptide is a suitable FRET substrate for SIRT5 activity assay.

We next tested whether this FRET-based assay could be utilized to pick up compounds that can modulate SIRT5 activity. We chose several compounds known to be sirtuin inhibitors, including nicotinamide,[24] suramin,[25] splitomicin,[26] AGK2[11] and sirtinol[27] (structures shown in Fig. S4, SI). Suramin was reported to inhibit SIRT5's deacetylase activity with IC50 value of 22 μM.[25] We measured its IC50 value for the desuccinylase activity of SIRT5 using an HPLC-based assay and obtained a similar value of 25 μM.[28] Other compounds are not efficient at inhibiting SIRT5, with IC50 values >100 μM.[28] Using the concentration of 30 μM, we screened

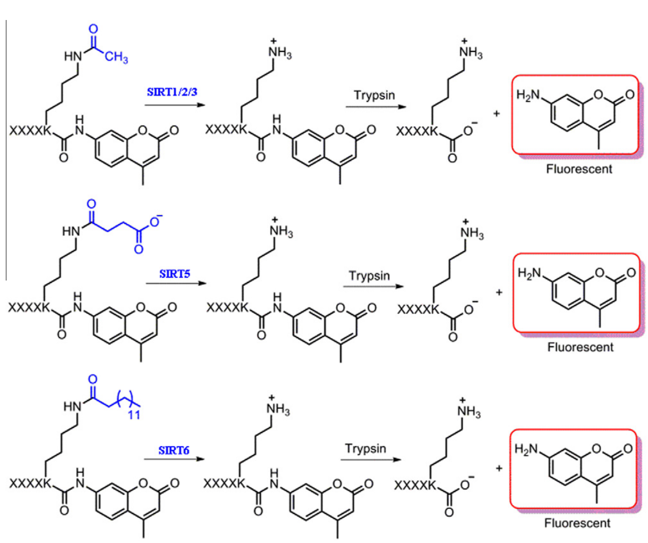

Figure 2. Fluorogenic assays for different sirtuins.

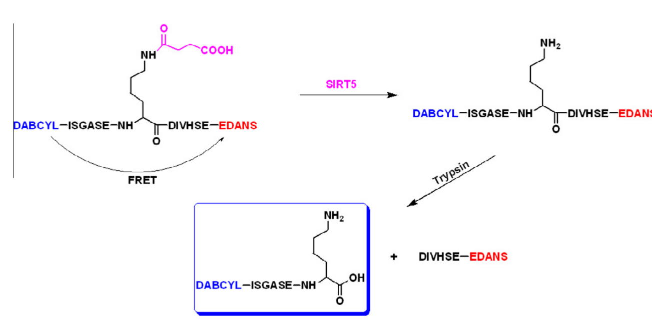

Figure 3. A FRET-based assay of SIRT5 using a FRET pair of DABCYL and EDANS.

Table 1
The kinetic parameters of SIRT5 on FRET peptides

Kinetics	FRET-Ac[a]	FRET-Su[b]
K_{cat} (S^{-1})	ND[*]	0.0050 ± 0.001
K_m (μM)	ND[*]	2.3 ± 1.0
k_{cat}/K_m (S^{-1} M^{-1})	11	2142

[a] (DABCYL)ISGASE(AcK)DIVHSE(EDANS)G peptide.
[b] (DABCYL)ISGASE(SuK)DIVHSE(EDANS)G peptide.
[*] The k_{cat} and K_m values for SIRT5 cannot be determined because the $V \sim [S]$ plot is linear (K_m is much greater than the highest substrate concentration tested). Thus only k_{cat}/K_m value can be obtained.

these compounds' capability to inhibit SIRT5 desuccinylase activity in this FRET-based assay. As shown in Figure 5, the fluorescence was close to background level in the presence of 30 μM suramin. In contrast, other compounds did not significantly decrease the fluorescence. These results are consistent with no inhibition of these compounds for the desuccinyltion of SIRT5 reported previously.[28]

We envisioned that this FRET assay containing succinyl lysine peptide for SIRT5 can be coupled with a secondary assay containing acetyl lysine peptide for SIRT1/2/3 to eliminate compounds that can also inhibit SIRT1/2/3. We used the (DABCYL)-ISGASE(AcK)DIVHSE(EDANS)G peptide to test whether suramine can also inhibit SIRT1/2/3. This FRET peptide containing acetyl lysine residue is a reasonable substrate for SIRT1, 2 or 3. Using SIRT1 and this FRET peptide, we tested whether suramine can also inhibit the enzymatic activity of SIRT1. As shown in Figure 6, suramine also can significantly decrease the fluorescence produced by SIRT1 to almost background level. The result is consistent with literatures that suramine is not a SIRT5-specific inhibitor[25] and further demonstrates that this FRET assay containing succinyl lysine peptide for SIRT5 can be coupled with a secondary assay containing acetyl lysine peptide for SIRT1/2/3 to screen for compounds that selectively modulate SIRT5 activity.

Finally, we tested whether this SIRT5 FRET-based assay can measure the IC50 value. Accordingly, the dose-response curve for suramine was obtained using this FRET-based assay (Fig. 7). From the dose-response curve, the IC50 value of suramine for SIRT5 was determined to be around 2 μM, which is close to the value (3 μM) obtained by an HPLC assay using the FRET peptide of (DABCYL)ISGASE(SuK)DIVHSE(EDANS)G peptide (Fig. S5, SI).

In summary, using a succinyl lysine peptide containing a FRET pair of DABCYL/EDANS, we have developed a FRET-based assay for SIRT5 that can be used to screen for compounds that can modulate SIRT5 activity. Coupled with a secondary FRET-based assay for SIRT1/2/3's deacetylase activity, inhibitors selective for SIRT5 could be identified. This assay is a 'mix-and-measure' type of assay and should be easily miniaturized and automated for a high-throughput screening to identify SIRT5-specific inhibitors or activators.

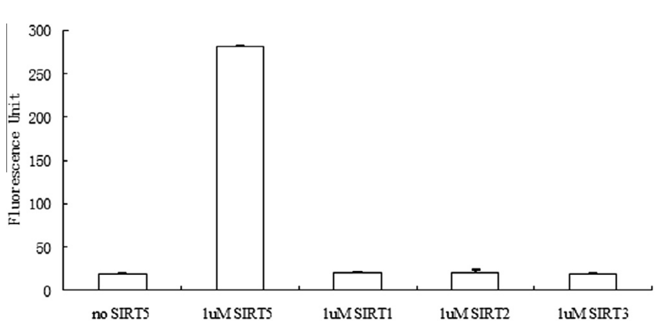

Figure 4. Fluorescence from (DABCYL)ISGASE(SuK)DIVHSE(EDANS)G without or with different sirtuins.

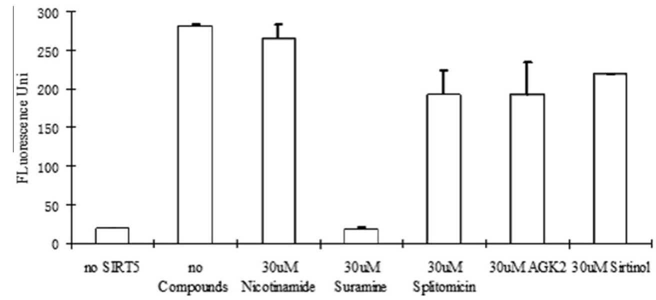

Figure 5. Using the FRET-based assay to screen SIRT5 inhibitors. Reactions without SIRT5 and with SIRT5 but without small molecules added were used as controls.

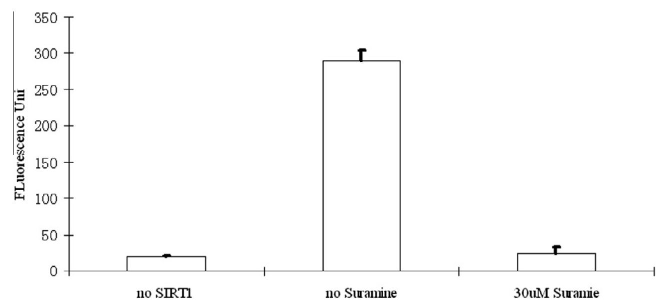

Figure 6. A second screen with (DABCYL)ISGASE(AcK)DIVHSE(EDANS)G and SIRT1.

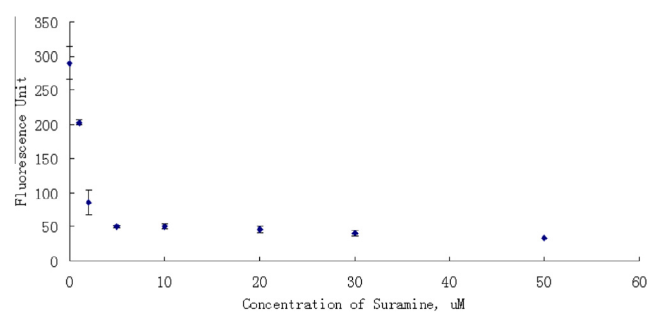

Figure 7. The dose-response curve measured using SIRT5 and (DABCYL)-ISGASE(SuK)DIVHSE(EDANS)G peptide.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.03.018.

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