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Studying SIRT6 regulation using H3K56 based substrate and small molecules

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ABSTRACT

SIRT6 is a modulator of chromatin structure having an important role in healthy ageing, and there is a crucial need to find specific modulators for it. Therefore, the activity of SIRT6 should be studied using a variety of methods. We examined the capability of SIRT6 to deacetylate a set of five fluorogenic substrates based on p53 and histone H3 sequences. The substrate designed around H3K56 deacetylation site exhibited the best signal-to-background ratio and was chosen for further studies. Nicotinamide is a known inhibitor for sirtuins, and it was found to be less potent inhibitor for SIRT6 than it is for SIRT1. In addition, we studied 15 other small molecule sirtuin modulators using the H3K56 based substrate. EX-527, quercetin and three pseudopeptidic compounds were found to be the most potent SIRT6 inhibitors, exhibiting over 50% deacetylation inhibition. These findings describe the first modulators of SIRT6 activity at the physiologically important H3K56 deacetylation site.

1. Introduction

A DNA binding nucleosome is formed with eight histone proteins; two copies of each histone subtype H2A, H2B, H3, and H4. The post-translational modifications on the lysine residues of the histone tails affect the chromatin structure. One of these modifications is the N^{ε}-acetylation of lysine residues that serves a unique purpose in neutralizing the positive charge of the lysine residues decreasing their attraction to the negatively charged DNA. This loosens the chromatin structure and enables the transcription of genes. The delicate acetylation status of these lysine residues is controlled by histone acetyltransferases and histone deacetylases (HDACs). Class III HDACs, also called sirtuins, have a broad range of functions in genomic stability, metabolism, ageing, and tumorigenesis. Sirtuins catalyse the N^{ε}-deacetylation reaction at lysine residues with a NAD-dependent mechanism, and thus their activity is regulated by the metabolic state of the cell.

Among the seven membered mammalian sirtuin family, SIRT6 deacetylates clinically relevant H3K9 and H3K56, maintaining the chromatin structure. H3K9 is deacetylated at telomeric chromatin when DNA is damaged, providing access for the repair machinery (Kawahara et al., 2009). H3K56 acetylation contributes directly to ribosomal RNA biogenesis and is connected to tumour proliferation and migration (Chen et al., 2012; Liu et al., 2012). In addition to H3, a few other deacetylation targets of SIRT6 have been identified (Kugel and Mostoslavsky, 2014). It has been recently shown that besides the deacetylation activity, SIRT6 also deacylates longer chain fatty acids from the N^{ε}-group of lysine residues and it has weak mono-ADP ribosylation activity (Jiang et al., 2013; Mao et al., 2011). However, the precise role of these two activities and their cellular function is not yet understood.

SIRT6 controls healthy ageing by affecting various cellular functions, such as telomere stability and glucose metabolism (Gertler and Cohen, 2013). SIRT6 also has a crucial role in several cancer subtypes, even though it is not known if SIRT6 functions as tumour suppressor or promoter, or both (Liu et al., 2013; Zhang and Qin, 2014). The overexpression of SIRT6 prolongs the lifespan of male mice and prevents diet induced obesity (Jiang et al., 2013; Kanfi et al., 2012). The lack of SIRT6, on the other hand, causes severe metabolic defects and premature ageing induced by abnormal telomeric structure (Mostoslavsky et al., 2006). Taken together, the activation or inhibition of SIRT6 offers possibilities to pharmacologically control metabolic disorders, cancer and possibly even ageing. Therefore it is highly desirable to efficiently and accurately screen for SIRT6 deacetylation modulators.

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The deacetylation activity of SIRT6 has been reported to be weak *in vitro* (Pan et al., 2011; Hu et al., 2013). In order to increase the signal obtained from the deacetylation reaction, we studied different substrates including sequences from the natural substrates and investigated them as part of a fluorometric assay. One of the natural substrate sequences increased the deacetylation signal, proving that the efficiency of the deacetylation reaction *in vitro* depends on the substrate sequence. A selection of compounds (including EX-527, quercetin, resveratrol, suramin, and sirtinol) reported to modulate the activity of other sirtuins was screened against SIRT6. Furthermore, the IC_{50} value for the physiological sirtuin inhibitor nicotinamide (NA) was determined.

2. Materials and methods

2.1. Chemicals

All chemicals were of commercial high purity quality. 7-Amino-4-methylcoumarin (AMC) labelled substrates Ac-HKK(Ac)-AMC (**S2**), Ac-AKK(Ac)-AMC (**S3**), Ac-TARK(Ac)-AMC (**S4**), and Ac-RYQK(Ac)-AMC (**S5**) were purchased from CASLO ApS (Denmark). Substrate Ac-RHKK(Ac)-AMC (**S1**), NAD, nicotinamide, recombinant SIRT6 enzyme, developer, fluorophore (5 mM 7-amino-4-methylcoumarin (AMC) in DMSO), and assay buffer (50 mM Tris–HCl, pH 8, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$) were from Cayman Chemical Company (USA). BSA (bovine serum albumin), DNA (deoxyribonucleic acid sodium salt from salmon testes), and DMSO (dimethyl sulphoxide) were from Sigma Aldrich (USA). Single-stranded 20-mer DNA oligonucleotides with a non-specific sequence were purchased from Oligomer (Finland). In the initial substrate profiling assays, SIRT6 was obtained from Cayman Chemical Company (USA).

2.2. Recombinant SIRT6 production

Expression vector hSIRT6-pGEX-6P3 containing the coding sequence of the human SIRT6 was kindly provided by Prof. Katrin Chua (Stanford, USA). GST-tagged SIRT6 was produced by fermentation in *Escherichia coli* BL21(DE3)-pRARE. After induction with 0.1 mM IPTG for 20 h at +16 °C, the soluble overexpressed protein was affinity purified on glutathione agarose (Sigma). The purity of the GST-SIRT6 was >80%, as detected by SDS–PAGE analysis.

2.3. SIRT6 inhibition assay

The known sirtuin modulators were tested for their inhibitory activity against SIRT6 in black half-volume 96-well plates. In each well, the reaction mixture contained 320 μM substrate **S5**, 3 mM NAD, in-house recombinant SIRT6 (4.5 μg/well), and SIRT assay buffer giving total volume of 50 μl. Under the defined assay conditions the used enzyme amount (4.5 μg/well) is equal to the enzyme activity of 290 pmol/mg protein/min. All compounds were tested at 200 μM concentration. The IC_{50} of NA was calculated from two independent determinations using 11 inhibitor concentrations. During compound screening the final DMSO concentration was 5% (see Supplementary Information for the effect of DMSO on SIRT6). To initiate the reaction, SIRT6 was added to the reaction wells. The reactions and the remaining SIRT6 were incubated at 37 °C for 90 min. After that, 50 μl of developer solution (6 μg/μl trypsin, 40 mM NA) was added to all wells and SIRT6 to the control wells. The plates were incubated for 30 min at room temperature and the fluorescence was measured with excitation and emission wavelengths of 380 and 440 nm, respectively, using EnVision 2104 Multilabel Reader (PerkinElmer, Finland). In the initial substrate profiling assays, the substrates were used at 400 μM concentration. The results were calculated after the subtraction of the background fluorescence in the control wells.

3. Results and discussion

3.1. Fluorogenic substrates

The collection of fluorogenic substrates for the profiling test is presented in Fig. 1. The substrate **S1** (Ac-RHKK(Ac)-AMC) is based on the amino acids 379-382 of the human tumour suppressor protein p53. SIRT1 and SIRT2 are known to deacetylate p53 at lysine 382 (Vaziri et al., 2001) and the sequence surrounding the K382 is often used as a substrate in fluorometric sirtuin assays (Marcotte et al., 2004). We have previously shown that peptides containing an N^{ε}-thioacetylated lysine can inhibit SIRT6 (Kokkonen et al., 2012). Two most potent sequences were HKK(thioAc)LM and AKK(thioAc)LM, which are also based on p53. Therefore substrates **S2** (Ac-HKK(Ac)-AMC) and **S3** (Ac-AKK(Ac)-AMC) were included in this study.

The major, well-established physiological deacetylation substrate of SIRT6 is histone 3 (H3). SIRT6 targets lysines 9 (H3K9) and 56 (H3K56) on the H3 sequence and has been shown to efficiently deacetylate these sites *in vivo* (Kawahara et al., 2009; Michishita et al., 2009). H3K9 is the specific regulation site of chromatin at telomeres (Michishita et al., 2008; Tennen and Chua, 2011) while the acetylation status of H3K56 controls DNA damage response and genomic stability (Michishita et al., 2009; Yuan et al., 2009). Substrate **S4** (Ac-TARK(Ac)-AMC) and substrate **S5**

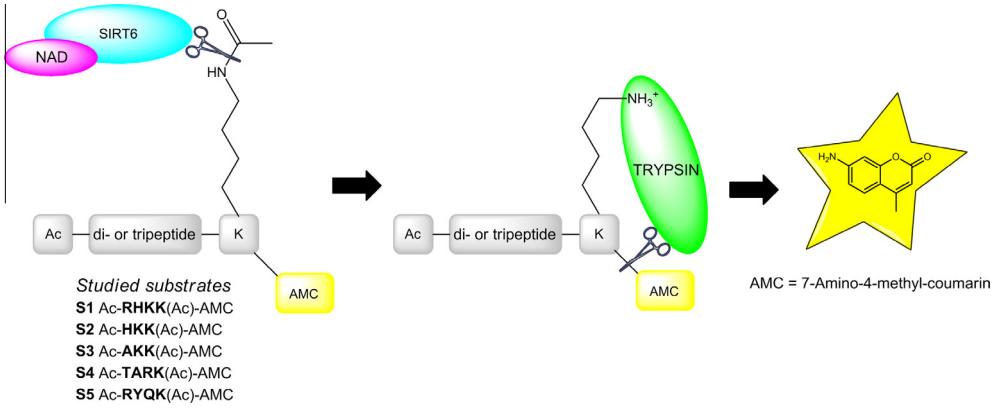

Fig. 1. The principle of the SIRT6 deacetylation assay and the studied fluorogenic substrates. In the first reaction step, SIRT6 deacetylates the N^{ε}-acetylated lysine of the substrate in abundance of NAD. In the second step, the added trypsin detaches the fluorophore (AMC) from the substrate, producing fluorescence directly proportional to the SIRT6 deacetylation activity.