Discovery of Novel SIRT1 Modulators Using Differential Scanning Fluorimetry

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Overview

• Aim of this study was to use a label-free, biophysical binding technique (Thermal Shift Assay**)** for HTS discovery of novel small molecule modulators of SIRT1 enzyme. Workflow of secondary orthogonal/counter-screen assays was established to eliminate false positives and to progress the best hits towards detailed mechanistic studies and synthetic optimization.

Introduction

- Sirtuins (SIRTs), a subfamily of histone deacetylases (HDACs), are exciting therapeutic targets involved in metabolic diseases, neurodegeneration, cancer, inflammation and regulation of aging and lifespan.
- Both inhibitors and activators of SIRTs including blockers of protein-protein interactions (DBC1, AROS, etc.) are of interest. The discovery of novel modulators exemplified by SIRT 'activators' in particular, is hampered by the intrinsic limitations and issues associated with conventional assays.
- Here, we report the use of a simple and straightforward high-throughput screening assay (DSF, ThermoFluor™, Thermal Shift Assay**)** for HTS of a diverse, lead-like library of 9,000 small molecules against human SIRT1.
- Primary hits were processed through the orthogonal assay workflow consisting of :
  - DSF counter-screen on unrelated proteins
  - commercial biochemical SIRT-Glo™ assay (Promega)
  - LC-MS based substrate conversion assay
  - proliferation and cellular substrate(s) assays on a panel of cancer cell lines.

Methods

Thermal Shift Assay

Primary screening on recombinant hSIRT1 (produced in *E.coli*) was done in the presence of reporter dye Sypro Orange at 20 μM concentration of the tested compounds. Thermal scanning (40 to 85ºC at 0.05ºC/sec) with constant fluorescence read using 470/623nm filter set was performed in 384-well format using Applied Biosystems ViiA ™ 7 Real Time PCR System. All measurements were made in quadruplicates. Compounds producing both positive and negative Tm shifts of >0.5ºC upon derivative analysis of the melt curves were considered as hits. Selectivity counter-screens were done in a similar manner using a panel of diverse proteins: carbonic anhydrase, β-catenin, SH2 domains of Abl and Btk kinases.

Biochemical SIRT1 SIRT-Glo™ Assay

Commercial luminescent SIRT-Glo™ assay kit (Promega) was used to test for biochemical inhibition of the enzyme. The assay uses an acetylated, luminogenic peptide substrate that can be deacetylated by SIRT1 and it was performed according to the vendor protocols.

LC-MS substrate conversion assay

Readout of this label-free SIRT1 activity assay allowing measurements of up- or down-regulated substrate conversion in an *in vitro* reaction utilizing purified enzyme or a cellular extract was based on the quantitative differential LC-MS-based detection of acetylated and deacetylated forms of SIRT1 substrate KQTARK(Ac)STGG (histone H3K9).

Cell-based assays

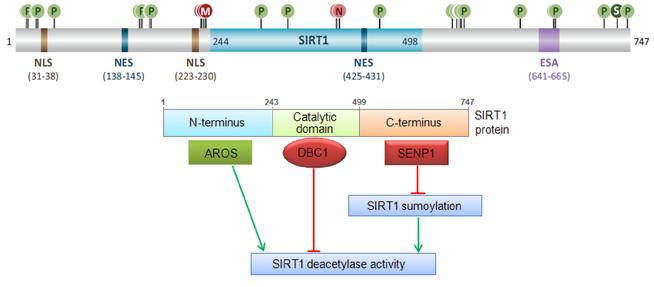
Several human hepatic (Huh7,HepG2) and prostate (DU145, PC3) cancer lines were used to test for antiproliferative effects of the compounds, as well as their modulatory effect on cellular markers associated with SIRT signaling.

Screening set selection

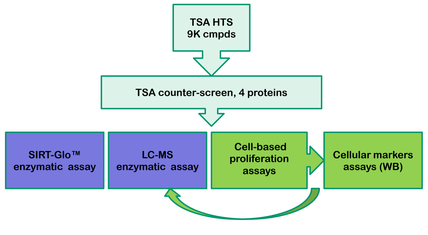
Library of ~9000 compounds consisting of an unbiased, diverse lead-like subset, as well as several targeted and hit expansion subsets, was selected from Enamine's 2,000,000+ small molecule collection through a combination of chemoinformatics, medicinal chemistry filtering, ligand-based searches and *in silico* docking strategies.

Results

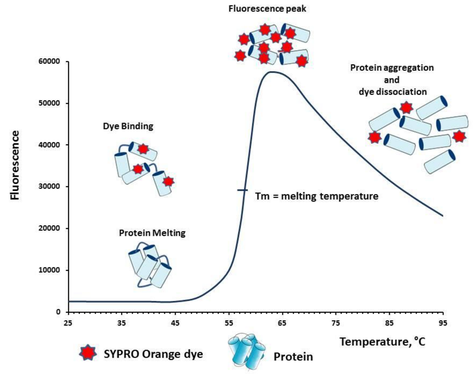
SIRT1 is regulated via multiple posttranslational modifications and protein-protein interactions



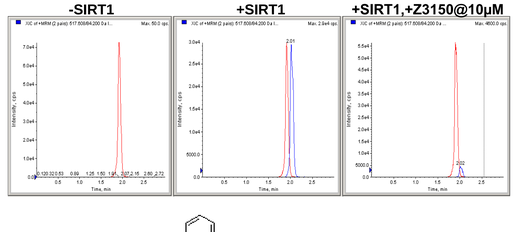
Discovery workflow

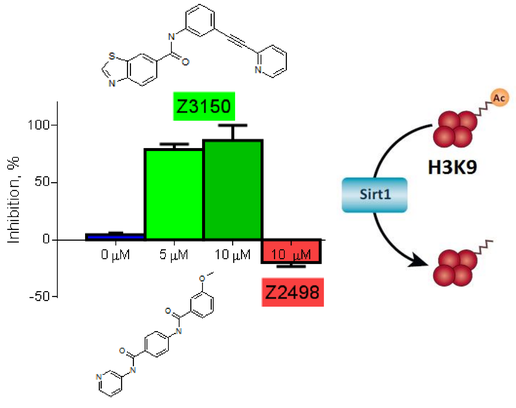


TSA principle

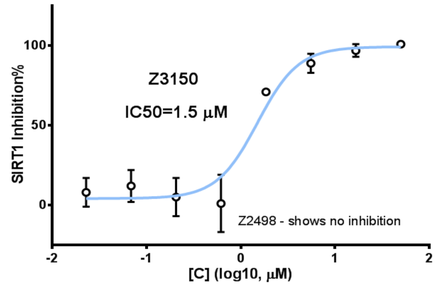


LC-MS assay detects down- and up- modulators

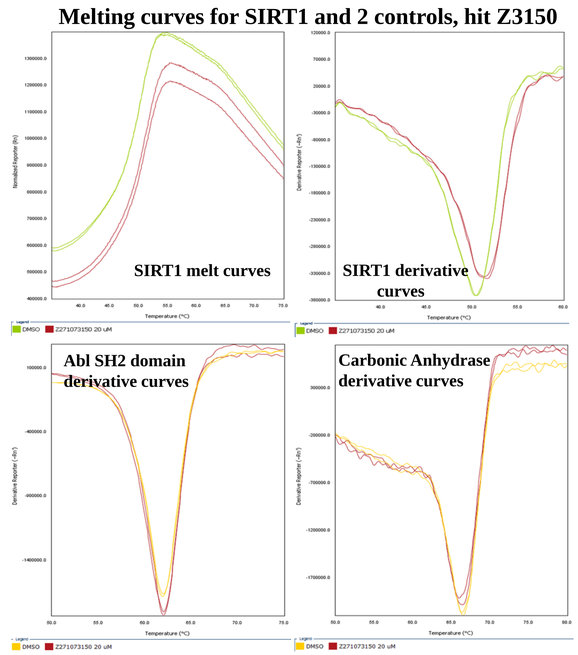




Alternative biochemical assay SIRT-Glo™



Melting curves for SIRT1 and 2 controls, hit Z3150



Cell-based characterization of the most interesting SIRT1 effectors is currently in progress and includes cell viability assays on a panel of cell lines as well as Western blot/IP analysis of the relevant markers (Foxo1/3, p53, SIRT1) and measurements of SIRT1 activity in compound-treated cell lysates.

Conclusions

- We have used Thermal Shift Assay (TSA, DSF) to identify potential SIRT1 binders via HTS of a rationally designed set of 9000 chemical compounds. As a result, ~50 chemically diverse TSA hits displaying positive or negative thermal melt shifts of 0.5-2°C were identified.
- These apparent SIRT1 protein binders were further characterized using the secondary screening workflow consisting of TSA selectivity counters-screen, a conventional enzymatic biochemical SIRT1 assay, LC-MS based peptide substrate conversion assay and cell-based assays.
- Two chemical series and several singletones of interest were identified. Spectrum of the observed activities includes both inhibitory and possible activatory effects. Further mechanistic studies are required to clarify possible orthosteric and allosteric mechanisms of SIRT1 modulation.