

Abstract

SIRT1 is an NAD⁺-dependent protein deacetylase that modulates the activity of several proteins including PPAR- γ co-activator (PGC-1 α) and FOXO1. Activation of SIRT1 induces mitochondrial biogenesis, resulting in lower blood glucose and improved insulin sensitivity. Small molecule activators of SIRT1 may be therapeutically beneficial for the treatment of diseases of aging such as diabetes. In order to identify small molecule activators of SIRT1, we have developed a high throughput mass spectrometry (MS) assay. Deacetylation results in the removal of an acetyl group from a lysine residue in the target substrate protein or peptide resulting in a 44 AMU mass change. This assay uses native peptide sequences as substrates and both substrate and product are directly monitored. Percent conversion of substrate is calculated and used to determine compound activity. MS throughput is 4 seconds/well allowing screening of large compound libraries. This proprietary assay is being used to develop novel small molecule therapeutics.

Introduction

SIRT1 is an enzyme that catalyzes the NAD⁺-dependent deacetylation of a number of important cellular proteins including p53, PGC1 α , and the FOXO transcription factors according to the schematic shown in Figure 1. Deacetylation results in the removal of an acetyl group from the ϵ -amino group of a lysine residue in the target substrate protein. SIRT1 belongs to a family of enzymes that is comprised of seven members in humans (SIRT1-7). These enzymes share a conserved catalytic core domain, and possess NAD⁺-dependent deacetylation and/or ADP-ribosylation activity.

The Human Sirtuin Enzyme Family



Sirtuin	Deacetylation domain	Key Advantages:
hSIRT1	Green	<ul style="list-style-type: none"> • Unique NAD⁺-dependent deacetylases • Links to human disease • Crystal structures • Selectivity achievable
hSIRT2	Red	
hSIRT3	Blue	
hSIRT4	Yellow	
hSIRT5	Purple	
hSIRT6	Light Green	
hSIRT7	Light Blue	

Activation of SIRT1 *in vivo* induces mitochondrial biogenesis, resulting in lower blood glucose and improved insulin sensitivity. Activators of SIRT1 may be therapeutically beneficial for the treatment of a number of age-related diseases such as metabolic and neurodegenerative.

To identify small molecule activators of SIRT1 and other sirtuin deacetylases, a robust high throughput enzyme assay is required for screening large collections of compounds. The assays described to date in the scientific literature are either coupled enzyme assays not ideal for mechanistic studies or radioactive assays that are low sensitivity and throughput. Sirtris Pharmaceuticals, in collaboration with BioTrove Inc., have developed a high throughput mass spectrometry assay that enables the direct readout of deacetylation activity against peptide substrates.

SIRT1 HTMS Assay

The activity of SIRT1 enzyme in the presence or absence of test compounds can be measured by mass spectrometry. In this cell-free assay, SIRT1 enzyme, NAD⁺, and acetylated peptide substrate are incubated at 25 °C for 25 minutes. The reaction is stopped with 10% formic acid and the conversion of substrates to products is monitored by mass spectrometry. The removal of an acetyl group results in the loss of 44 AMU from the original peptide substrate. The ability of test compounds to inhibit or activate SIRT1 enzyme can be monitored by comparing the % conversion of substrate to product in the presence of compound to that in a control reaction.

Reagent	Source	[Reaction]
hSIRT1 Enzyme	Sirtris	1-100 nM
Tri-Acetylated Peptide	BioPeptide	1-30 μ M
NAD ⁺	Sigma	100-3000 μ M
Nicotinamide	Sigma	
SIRT1211 (inhibitor)	Sirtris	0.01-100 μ M
BSA	Sigma	0.05%
DTT	Sigma	5 mM
Tris-HCl	Sigma	50 mM
NaCl	Sigma	137 mM
KCl	Sigma	2.7 mM
MgCl ₂	Sigma	1 mM

MS Method Development

MS conditions were developed using BioTrove's RapidFire™ High Throughput Mass Spectrometry System interfaced to a Sciex API-4000 triple quadrupole mass spectrometer.

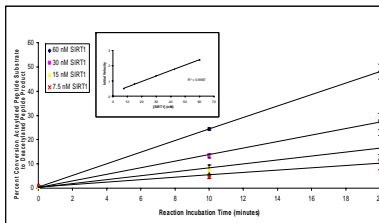


Results

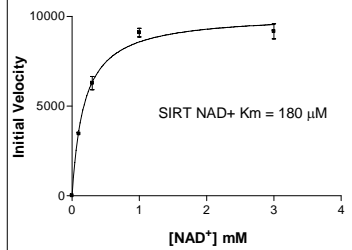
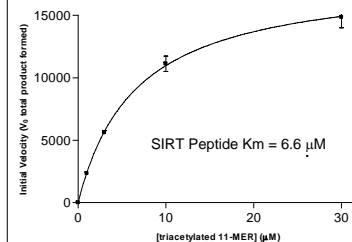
- Enzyme Titration and Time course
- Peptide Substrate Km
- NAD⁺ Km
- Substrate and Product Detection
- DMSO Tolerance
- Z' Determination
- SIRT1 Inhibition
- Final Assay

SIRT1 Enzyme Titration Time course

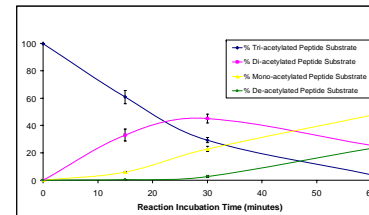
- 5.0 μ M Triacetylated peptide substrate
- 500 μ M NAD⁺
- 37 °C incubation; quench with 1% formic acid



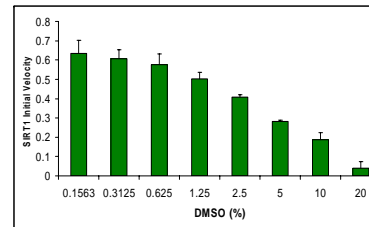
SIRT1 Triacetylated Peptide Substrate Titrations



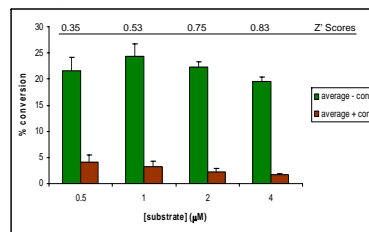
Simultaneous Detection of Substrate Depletion and Product Formation



DMSO Tolerance

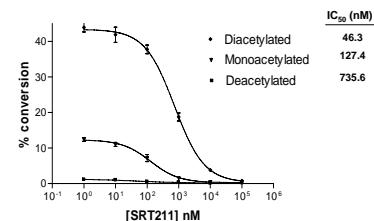


Z' Experiment



- Z' was calculated at varying [substrate] to determine optimal screening conditions

SIRT1 Inhibition



SIRT1 MS Final Assay Conditions

- Final assay volume = 100 μ L
- 10 nM SIRT1
- 5 μ M Triacetylated Peptide Substrate
- 120 μ M NAD
- MS Throughput of 7 seconds per well

Conclusion

Activators of SIRT1 may be therapeutically beneficial for the treatment of age-related diseases such as neurodegenerative and metabolic. To identify and optimize small molecule SIRT1 activators, a robust enzyme assay is required. Sirtris Pharmaceuticals, in collaboration with BioTrove Inc., have developed a high throughput MS assay that is suitable for HTS, SAR support, and mechanistic studies. The assay developed employs native, untagged peptides as substrates, and allows the simultaneous detection of both substrate and product peptides. This assay has enabled the identification and development of small molecule SIRT1 activators that are currently in pre-clinical development.

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*BioTrove Inc.