**Jin L, Galonek H, et al. Biochemical characterization, localization, and tissue distribution of the longer form of mouse SIRT3. (2009) Protein science, 18: 514-525.**

Enzyme assay

The partially purified full length and different fragments of mSIRT3L were tested for deacetylation activity with the mass spectrometry based assay. Saturating amounts of peptide substrate (20 M) and bNADþ (3 mM) were added to the reaction with enzyme concentration starting at 4 M and serial diluted in a 1:2 ratio. Reactions were incubated at 25oC and stopped at 0, 15, 30, 60, 90, 120, 150, 180 minute time points with 10% formic acid with 50 mM nicotinamide. The conversion of substrate to product was determined by mass spectrometry in conjunction with a Rapid Fire system (BioTrove).

Inhibition analyses

To identify the mechanism of inhibition of SRT1720, a small molecule SIRT3 inhibitor, and nicotinamide against mSIRT3L-54-334, we used a single acetylated peptide derived from the SIRT3 substrate AceCS2

(EILVVKRLPKTRSG-KAc-VMRRLLRKIITSEAQ, KAc is acetylated lysine). For compound inhibition against acetylated AceCS2 peptide, eight concentrations of nicatinamide (15, 3.75, 0.94, 0.23, 0.06, 0.015, 0.004, and 0.001 M) or nicotinamide (85, 28.3, 9.4, 3.2, 1.05, 0.35, 0.12, and 0.04 M) were used in the reaction. For each of the respective compound concentrations, the deacetylation rate was measured at five concentrations of acetylated peptide (5, 2.5, 1.25, 0.62, and 0.31 M) with mSIRT3L-54-334 (1.47 nM) and NAD+ (220 M) kept constant. Substrate inhibition was detected at high concentrations forcing the use of lower amounts of acetylated peptide. The reactions were carried out at 25oC. The time course was determined from the linear portions of the AceCS2 Km curve (ran at NAD+ Km) and the reaction was stopped with 10% formic acid with 50 mM nicotinamide and the conversion of substrate to product was determined by mass spectrometry.

For compound inhibition against NAD+, eight concentrations of SRT1720 (15, 3.75, 0.94, 0.23, 0.06, 0.015, 0.004, 0.001 M) or nicotinamide (80, 20, 5, 1.25, 0.31, 0.08, 0.02, and 0.005 M) were used. For each of the respective compound concentrations, the deacetylation rate was measured at eight fixed concentrations of NAD+ (2000, 1000, 500, 250, 125, 63, 31, and 16 M) with mSIRT3L-54-334 (1.47 nM) and acetylated AceCS2 peptide (2 M) kept constant at 25oC. The time course was determined from the linear portions of the NAD Km curve (ran at AceCS2 Km) and the reaction was stopped with 10% formic acid with 50 mM nicotinamide and the conversion of substrate to product was determined by mass spectrometry.

To determine the inhibition constants (Ki) and mechanism of action toward NAM and SRT1720, the initial velocities of several series of reactions were calculated by global non-linear regression. Briefly, varying concentrations of inhibitor were titrated against fixed concentrations of substrate (NAD+ or AceCS2). The observed rates of reaction with respect to substrates and inhibitors were then globally fit to the mixed noncompetitive inhibition equation using the software package GraFit 6.0.5 (Erithacus Software).



where, Ki and Ki are the competitive and uncompetitive inhibition constants, respectively. The mechanisms of inhibition are determined from the alpha constant value.  = 1, indicates noncompetitive inhibition,  >>1, competitive inhibition and  << 1 uncompetitive inhibition.

Results

As part of the characterization of the purified mSIRT3L-54-334 protein, we have tested two deacetylase inhibitors, nicotinamide (NAM) and SRT1720.

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| SRT1720 inhibits mSIRT3L-54-334 with Ki of 0.34 M against NAD+. The  value is significantly smaller than 1 indicating uncompetitive inhibition. However, SRT1720 inhibits mSIRT3L-54-334 in a competitive manner ( value >>1) against AceCS2 peptide with a Ki value of 0.56 M. This suggests that SRT1720 binding requires NAD+ to bind first to the enzyme and that SRT1720 competes for the same binding site of AceCS2 peptide or works as an allosteric competitive inhibitor to mSIRT3. |
| NAM has an a value much greater than one against NAD+ and AceCS2 peptide suggesting a competitive inhibition mechanism toward both substrates with Ki values of 2.84 M against NAD+ and 4.62 M against AceCS2 peptide. NAM has been reported as a non-competitive inhibitor toward both NAD+ and the peptide substrate and is part of the base-exchange reaction in SIRT1, SIRT2, yeast and bacterial sirtuins. However, it is likely that mSIRT3 has a different mechanism of inhibition for NAM. A more complete kinetic analysis of SIRT3 is ongoing to propose a reaction mechanism. |  |