We need answers to all the questions from my last couple of emails asap.

Sherry will follow up to make sure we get everything from you along with the updated schedule, if you don't send it.

Raj

I am talking about approximate values; do you mean there is no report of these Kms in the literature?

For Sirt1: The Km in the literature varies even with same peptide e.g.

For p53 peptide

Km = 10.3±2.6 uM (Kaeberlein et al, 2005)

Km = 25 uM (Rye et al., 2011)

Km = 61 uM (Blander et al., 2005) [in this case the sequence of p53 is not clear but contains same acetylated Lysine]

These are obviously relevant to screening. You should both take it seriously for planning purposes. As I said please provide a clear reply regarding when you are giving the lit and lab approximate values of these Kms and how it would affect your screening.

We are not clear whether you are asking lab determined Km for Sirt1 or Sirt3 on p53 or other peptides we have in the lab. We have both Sirt1 and Sirt3 from Enzo (bulk order).

**XG is planning to do following-**

XG is working on the refuting experiments using TeCan and HPLC. It will be finished by the end of this week.

[In-house SIRT3]=10ul
[FdL2 peptide]=50, 250 uM
[NAD+]=500, 2000 uM
[DHP1c]=0, 25, 50, 75, 100, 200uM
% DMSO= 5%
Time point=60min
Temp=37oC

If the customized peptides would be shipped to lab, then the standard curve will be the next experiments

If not, then XG will move to initial rate experiments. Km (NAD+) and Km(FdL2 peptide substrate) for in-house SIRT3 will be measured. The data will be available ~ 6/30/2016 (using TeCan).

If Km(NAD+) and Km(p53 peptide substrate) for in-house SIRT3 is the first priority, XG can do that first.

However, since p53 is not SIRT3 native peptide, we propose to do the experiments using AceCS2 instead.

Raj

" Km-type mechanism is supported by isothermal titration calorimetry experiments that show a roughly twofold increase in the affinity for peptide substrate binding to SIRT1-C in the presence of SRT1460 (Kd(-SRT1460) 5 25.7 uM and Kd(+SRT1460) 5 14.1 uM; data not shown)."

This sentence from Nature paper suggest that the Km for the substrate they used was 25.7 uM whereas the [peptide] they used was 0.5uM? This means [peptide] was around 2% of Km? This may partly explain why they saw significant levels of activation.

The above experiment was done using mass scpec. As you suggested , if we take 2% as stated above, and the literature Km varies from 10-61 uM (for p53) for Sirt1, the substrate (peptide) will be 0.2-1.22 uM. However, Pacholec et al., (2009) [**SRT1720, SRT2183, SRT1460, and Resveratrol Are Not Direct**

**Activators of SIRT1]** reported that the Km(p53) is 4.5 uM and the peptide substrate used for activation assay was 1uM. This peptide (p53) concentration will correspond to 22.22% of the Km.

By comparison, what are our %s for both [NAD+] and [peptide] (as %s of the respective Kms)?

XG(6.17.16): The km(p53) or km (NAD) measurement for enzo/ in-house SIRT3 will be obtained from initial rate experiment (0% DMSO + 0 uM modulator). As soon as we obtain the value, the % of the respective Km will be provided.

Raj

I am a little confused on why we chose certain conditions in our assays.

AU & XG(6.17.16): The conditions chosen are for SIRT3. The km (p53) and km(NAD) values reported in previous email were for SIRT1.

You said p53 Km is 4.5uM. Why did we start at 50uM peptide for nonsaturating peptide screening? That is clearly not nonsaturating.

Can we detect lower concentrations?

AU & XG(6.17.16): **For SIRT3**,  minimum p53 concentration used was 10uM with 2mM NAD+ for 5 and 10 min (Exp AU35). We have not done exp. for SIRT1 with p53. So the numbers 4.5 uM (Km\_p53(SIRT1) is not comparable to 50 uM ---[p53]\_SIRT3. Yes. We can go lower peptide concentrations.

You said SIRT1 NAD Km is around 100uM. Is it true that in order to match JMC we only used saturating NAD? This may be ok so far since we were refuting JMC.

AU & XG(6.17.16): The SIRT1 *Km* for NAD+ was reported as 94 uM in JBC 2010 paper. Yes, in order to match JMC  paper, we used 250uM FdL peptide + 500uM NAD conditions for SIRT1 and SIRT3.

What is Km of fluorophore conjugated peptide? I need to know peptide concentration used on nature as percent of Km peptide.

AU & XG(6.17.16): For Enzo SIRT1, km\_FdL1peptide =64uM; For Enzo SIRT3, km\_FdL2 peptide =32uM. Km\_peptide in nature 2007 paper was not clearly mentioned.

Also, Kaeberlein et al, 2005 reported that Km (FdL peptide) = 87.6±19.7

Is SIRT3 NAD Km around 600uM? Then our lowest concentration of NAD is around 1/6 of that? I want to compare this % to nature peptide concentration as % of Km.

AU & XG(6.17.16): Enzo SIRT1 km\_NAD+=558 uM (FdL1 peptide); Enzo SIRT3 km\_NAD+=2034 uM (FdL2 peptide). This is reported in Enzo user manual.

Also, Lei Jin et al 2009 reported that Km NAD for Sirt3 is 600 uM.

Please revert quickly and clearly and give these comments both of your attention since this is an example of how we can lose time with insufficient exptl planning.

Raj

RC (6.17.16): In order to run modulation assays under conditions similar to those reported for previously reported allosteric activators (resveratrol/SRT compounds), we may want to look into SI of the attached paper (which we have all seen before).

In particular, please look up the SI and determine:

a) what was [peptide] used in the EC1.5/max activation assays?

b) what was the time used in the EC1.5/max activation assays?

XG(6.17.16): **SIRT1** activity was monitored using a 20 amino acid peptide (Ac-Glu-Glu-Lys(biotin)-Gly-Gln-Ser-Thr-Ser-Ser-His-Ser-Lys(Ac)-Nle-Ser-Thr-Glu-Gly–Lys(MR121 or Tamra)-Glu-Glu-NH2) derived from the sequence of p53. The mass spectrometry assay was conducted as follows: **0.5 μM peptide substrate**, 120 μM βNAD+, 10 nM SIRT1, and reaction buffer (50 mM Tris-acetate pH 8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 5 mM DTT, 0.05% BSA). Reactions were incubated for **25 minutes at 25oC**. Test compounds were added to the reaction or vehicle control, DMSO. After the incubation with SIRT1, 10% formic acid with 50 mM nicotinamide (Sigma) was added to stop the reaction. ***Milne JC et al. Nature, 45(2007)712-716.***

RC(6.17.16): you also know the (p53?) peptide Km for SIRT1, please report it as well and hence the [peptide] as % of Km.

XG(6.17.16): *Km* value for the p53 peptide(Native peptide 3\_TableS1 below) to be 4.5 uM by the HPLC method. The SIRT1 *Km* for NAD+ was similarly determined by HPLC and was found to be 94 uM. Data were reported in ***Pacholec M et al. Journal of Biological Chemistry 285(2010) 8340- 8351.***

RC(6.17.16): We will use this data to compare to our assay conditions, including those for saturating peptide, nonsaturating NAD+.

XG(6.17.16): The substrate peptide used in Nature 2007 paper was fluorophore conjugated peptide. The substrate peptide used in JBC 2010 paper was native p53 peptide. The conditions used in JBC2010 paper may be used for comparison with our experimental conditions.

\*\*\*The Km of human SIRT1 enzyme for acetylated peptide substrate was examined using the SIRT1 mass spectrometry assay. To determine the Km, the linear deacetylation rate was determined at 12 concentrations of acetylated peptide substrate (**50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.098, 0.049, and 0.024 μM**) for each of the compound concentrations and for the vehicle control. SIRT1 enzyme, **2 mM NAD+, and 0-50 μM acetylated peptide substrate** were incubated with 0-100 μM compound at **25°C**. At **0, 3, 6, 9, 12, 15, 20, and 25 minutes**, the reaction was stopped with 10% formic acid with 50 mM nicotinamide and the conversion of substrates to products determined by mass spectrometry. . ***Milne JC et al. Nature, 45(2007)712-716.***

\*\*\* For the Km value determinations of the native peptide, the **peptide** concentrations were varied **from 0.78 to 100 uM** and **NAD+ was kept at 2 mM**. For the Km value for NAD+, NAD+ was varied from 0.5 to 1000 uM and the native peptide was kept at 75 uM. Time points are 0, 5, 15,30, 45 min. The HPLC column temp. was 25oC. ***Pacholec M et al. Journal of Biological Chemistry 285(2010) 8340- 8351.***

RC(6.17.16): Please also remind me of a) the [NAD+]'s we have used in the saturating peptide, nonsaturating NAD+ assays, along with the time (5 and 30 mins?)

XG(6.17.16): In saturating peptide, the NAD+ concentrations used were 100, 200uM for endpoint experiments at 5, 30 min time points.

b) the [peptides] we have used in nonsaturating peptide, saturating NAD+ assays.

XG(6.17.16): In saturating NAD+, the peptide concentrations used were 50uM for endpoint at 5, 30 min time points.

These can be posted on wiki.

Raj