**Email from Raj: Wednesday, November 30, 2016 1:42 PM**

RC: Have reviewed the data. Please answer all following on wiki as appropriate.

RC: 1) Seems IC50,NAM is about 4-5x higher than that for FdL2 peptide (IC50 there I assume was also measured at 3000uM NAD).

AU: As per XG, the IC50 NAM (36.8 uM) on FdL peptide was determined with 3000 uM NAD, 100 uM FdL peptide with 5U/rxn Enzo Sirt3, using Fluoroskan.

RC: Note we seem to observe partial inhibition of around 70% at saturating NAM (though this is an estimate). We don't want to get into the saturating range in our initial rate experiments, of course, but that shouldn't be a problem. Does Guan have the partial inhibition at saturating NAM for FdL2 peptide handy? If so it can posted side-by-side.

AU: XG’s data- with 3000 uM NAD, 100 uM FdL peptide with 5U/rxn Enzo Sirt3, using Fluoroskan.

|  |  |
| --- | --- |
| **[NAM],uM** | **% Activity** |
| 200 | 7.10 |
| 500 | 4.20 |
| 1000 | 2.90 |
| 2000 | 1.40 |

RC: 2) Did you work up the remaining concentrations of [NAD] from last week's reactions at 25uM NAM? Did they all show < 5% inhibition at 30 mins? You can post all the data from last 2 weeks to wiki in addition to answering this question.

AU: I have data for 50, 1000, 3000 uM NAD with 25 uM NAM for 30 min. I will post them on wiki.

RC: 3) Are the results with FdL2 peptide from today's report consistent with those from Guan using in-house enzyme? This would confirm that Enzo results can be extended to in-house in these studies.

AU:

XG data: In-house Sirt3, 3000 uM NAD, 250 uM FdL peptide, 30 min, NAM 25 uM, in 5% DMSO % activity remaining: 40.8% using Tecan.

AU data: 5U Enzo Sirt3, 3000 uM NAD, 250 uM FdL peptide, 30 min, NAM 50 uM, in 5% DMSO using HPLC

|  |  |  |
| --- | --- | --- |
| [NAM], uM | [P], uM, FdL | % activity |
| 0 | 2.376 | 100.0 |
| 50 | 1.216 | 51.2 |

RC: 4) These results also suggest that 100uM NAM used by Thomas did not significantly slow the reaction. How do the reaction conditions from today's report compare to those used by Thomas in our MST in presence of 100uM NAM? It seems you are only seeing very small % conversion (around 2%) of peptide substrate into product in 30 mins at saturating concentrations of both substrates?

AU: As per SM the MST buffer-

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| --- | --- | --- |
|   | MST Exp | AU-HPLC |
| Tris-HCl pH 8.0 | 47 mM | 50 mM |
| NaCl | 129 mM | 137 mM |
| KCl | 2.5 mM | 2.7 mM |
| MgCl2 | 0.94 mM | 1 mM |
| DMSO | 5% | 5% |
| Tween-20 | 0.05% | No Tween |

Amount of product produce in different concentrations of NAM in 30 min when both the substrates are saturating is shown below-

|  |
| --- |
| 5% DMSO, 3000 um NAD, 600 uM K122, 30 min, 5U/rxn Enzo Sirt3 |
| [NAM], uM | % Product produced |
| **0** | 2.88 |
| **25** | 2.75 |
| **50** | 2.44 |
| **100** | 2.02 |
| **200** | 1.64 |
| **500** | 1.03 |

RC: 5) You and Guan should choose a [NAM] that produces approximately the same % inhibition as 25uM in the case of FdL2 peptide and use that for the planned initial rate experiments with MnSOD and nonzero NAM. Once you have the results in absence of honokiol and both 0,nonzero NAM for MnSOD, provide me with that mixed fitting.

**AU:**

* As per XG’s data- In-house Sirt3, 3000 uM NAD, 250 uM FdL peptide (both substrate saturating); 30 min, NAM 25 uM, 5% DMSO %Activity remaining: **40.8%** using Tecan
* In case K122 peptide 600 uM, 3000 uM NAD (both substrate saturating, 30 min); 100 uM NAM will have comparable Sirt3 inhibition **(~30% inhibition, HPLC**).

RC: I'm not understanding this. 30% inhibition means 70% activity remaining. How is this comparable to 40.8% activity remaining?

AU: Sorry, my mistake. In XG’s case, ~40% activity remaining (60% inhibition).

In my case, then we should choose either 500 uM (% activity remain about 36%; 64% inhibition).

**Question:**

1.       Is it OK to fit mixed model with only one [NAM], e.g. 0 and 100 uM ?

RC: It's ok to identify trends if not quantitative estimates (see Guan's single NAM mixed fitting results for FdL2).

2.       While doing this experiment, I will not be including Honokiol? The combination of Honokiol and NAM will be done later?

RC: If you can do them together equally fast (0,200uM honokiol @ nonzero NAM) that's fine. As per our previous discussions, I believe you will only be doing spotchecks @ 0 NAM -- no full repeats.

**Raj’s email: Thu 12/1/2016 10:54 AM**

RC: I've reviewed the report so far; please address the points below in addn to the ones you are currently addressing, and also add the following comments to wiki for future reference:

RC: quoting the product formation in 30 mins in terms of % substrate converted is arbitrary since it depends on the substrate concentration. Hence please provide the product formation in terms of moles and also provide next to it the number of moles of enzyme used in the respective experiments.

AU: I reported uM product produced in the table. Now pmole formed will be included. Sirt3 concentrations in terms of uM will be included in the table.

RC: given the results with NAM inhibition of MnSOD and FdL2, we see that stopping the reaction with NAM for the purpose of MST experiments on substrate binding is more effective for FdL2. Thomas is currently doing some MST experiments with unlabeled FdL2.

RC: If we want to use NAM to stop reaction in MST experiments (as opposed to or in addition to carbaNAD approach to getting Kd of NAD), we may want to use unlabeled FdL2 instead of MnSOD.

AU: Question: does these two method of stopping the reaction has different mode of action? Or does it matter what mode of inhibition used to stop the reaction and how does it affect MST data?

RC: In this regard, I may ask you to check rate of reaction for unlabeled FdL2 peptide in presence of fully inhibitory [NAM] using HPLC in near future.

AU: XG please comment.

XG: The related experiment can be designed.

1. Titration experiments for saturating non-AMC labeled FdL2 peptide concentration
2. Under saturating substrates concentration, IC50 NAM for in-house SIRT3 can be measured.

RC: We will do the MST expt after that. (Note: Alternatively, one could measure the rate of NAD conversion during such MST experiments, it would be more accurate to monitor reaction with Sirtainty, since it measures the rate of NAD conversion. However, problem is that this cannot be done in presence of NAM inhibitor.)

XG: Ture.

RC: BTW, in the future, one of you should do initial rate fitting with/without 120 min or last two time pts (misc task) to see whether they affect. Not relevant for current expts, but could save enzyme in future.

AU: OK, thanks

XG: I have put it into wiki/[miscellaneous tasks](http://pmc-at.wikispaces.com/miscellaneous%2Btasks) page. Will do it when get time.

**Dr Raj replied to Alo’s post on wiki-**

RC: This is not plotted on a linear scale. Also you should make a line plot.

AU: The plot in linear scale is provided below on left, and in Dixon format on right.

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RC: I won't have time to review further just now, I have given you both extensive feedback and it is not feasible to send many more emails at this time. Please do all the requested tasks and get back to me in one consolidated response, together.

XG: OK

RC: Guan needs to review and we need consensus.

RC: I thought Guan found IC50 around 37uM but used 25uM. But it sounds like her IC50 was lower than 25uM with in-house.

XG: NAM IC50 for SIRT3 is 37uM, which was measured in sirtuin assay buffer with Enzo SIRT3. The current data is in 5% DMSO with in-house SIRT3.

RC: I believe it would also be useful if you plotted on a linear scale the NAM titration for MnSOD. I think 500uM is in the plateau range.  See my previous email on this.

XG: The NAM titration for MnSOD data has been plotted on a linear scale by AU. Yes, 500uM is in the plateau range.

RC: If we had more than one [NAM], it would be good to include one in the plateau range, because it actually can help fitting the full mixed model. However, if only have one NAM, it may cause problems -- preferable to stay away from it.

XG: Only one NAM here, will stay away from plateau.

RC: Given the data, unless Guan has some imp reasons why she chose a conc with 60% inhibition, we can go lower here. Also check the estimated combined effect of 200uM honokiol and NAM. Guan, please look at it closely.

RC: Also check the extent of inhibition by 200uM honokiol for FdL2 and MnSOD and consider whether the combination of your proposed [NAM] and 200uM honokiol will still leave measurable activity.

XG: Yes. A combined inhibition effect of 200uM and NAM will be checked. 50, 100uM, and 200uM will be used. Will discuss with Alok for experimental details.

RC: One final point about this for now:

Guan previously did fitting studies using subsets of the data from NAM and NAD series (this was some time ago). The goal of this was to see how the parameter estimates are affected based on the chosen datasets. Alok asked about this today -- are more NAD and just one NAM enough?

XG: For mixed inhibition model fitting, 2 non-zero NAM concentrations would be good since it may not fit well with only one non-zero NAM.

RC: In the analyses requested, Guan may revisit those fittings and check whether it may be better for us to do the same number of total combinations of [NAM] and [NAD] but with fewer NAD and more NAM.

This is by no means required -- just something that should be considered. If it doesn't help, one NAM is ok.

XG: I have spent some time to revisit the previous fitting



In summary, 2 non-Zero NAM concentrations with 4 NAD concentration in in good agreement with 3-nonZero NAM + 5 NAD. 1 non-Zero NAM data are little off.

RC: in the line plot requested, please also make a sep plot for FdL2 peptide at saturating NAD. Basically this gives an approximate Dixon plot (for FdL2 you have the actual initial rate data as well). we'd like to compare them.

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| --- | --- |
| XG: Using Alok’s MnSOD/25uM NAM data, a rough fitting has been done. The initial rate was calculated based on 2 time points (0, 30 min) and 3 NAD concentrations (50, 1000, and 3000uM). MM and Mixed inhibition fitting have been done.The Dixon plot was generated as well (right).  | As requested, an approximate Dixon plot for FdL2 peptide was prepared as well (right). |
|  |  |
| **Mixed inhibition model fitting** |
|  |  |

RC: FdL2 saturates at much higher NAM. The activity is almost extinguished there. Prior to the plateau the Dixon slope (at high [NAD]) depends primarily on the ratio (1+Kex)/Kd,NAM.

RC: MnSOD saturates at lower NAM. Estimated now at perhaps 30% activity. The Dixon plot at high [NAM] depends on the ratio of (1+[NAM]/Kd,NAM) and(1+(1+Kex)[NAM]/Kd,NAM). Because Kex is smaller than in the case of FdL2, we see a plateau earlier; we can get an estimate of Kex from the % activity at the plateau.

RC: In the latter case there may be advantages to fitting the model with more than one nonzero [NAM]. Since the Dixon plot will display a plateau, it's slope may change quite a bit over the range of [NAM] are looking at.

RC: Final decision will be made after the above requested plots are made an analyzed, you provide info on the past fittings with more [NAM] fewer [NAD], and Alok uploads all the HPLC data @ 25uM NAM as well.

XG: Alok will upload the 25uM NAM HPLC data on wiki.

When you send all this info please also remind me whether you are doing  1 or 2 nonzero NAM for FdL2 peptide and honokiol.

XG: I am currently culturing the cells since synthesized FdL2 peptide was not delivered by Peptide 2.0. Zi will check with them for the exact delivery data Monday. At meanwhile, one set of experiments (5% DMSO) using Enzo FdL2 peptide was performed for the purpose of comparison to synthesized peptide. I have spent all my time to check the previous fitting, reviewing the questions, and answer question since morning. I will start prepare the summary on Comparison Enzo FdL2 peptide and synthesized FdL2 peptide.

XG: **One more point** need to be pointed out here: the model fitting was done using different time point to check

\*how close the kinetic parameter calculated using all time points with those using two time points

\*which time point get closest number



In summary (1) there are some variations between data calculated from all time points and those using two time points. (2) in terms of two time points, 30 min time point (~ 15% variation) give the closest number to that using all time points. We need to be careful to report those numbers calculated from two time points.

|  |  |  |  |
| --- | --- | --- | --- |
| [NAD], uM | with DMSO |  with 25 uM NAM | % activity |
| [P], uM | [P], pmoles | [P], uM | [P], pmoles |
| 50 | 6.24 | 311.92 | 6.05 | 302.34 | 96.93 |
| 1000 | 20.77 | 1038.36 | 16.50 | 825.00 | 79.45 |
| 3000 | 26.09 | 1304.50 | 21.65 | 1082.50 | 82.98 |
|   |   |   |   |
| [NAD], uM | with DMSO | with 200 uM Honokiol | % activity |
| [P], uM | [P], pmoles | [P], uM | [P], pmoles |
| 50 | 6.24 | 311.92 | 3.23 | 161.43 | 51.75 |
| 1000 | 20.77 | 1038.36 | 9.96 | 497.76 | 47.94 |
| 3000 | 26.09 | 1304.50 | 12.42 | 621.00 | 47.60 |
|   |   |   |   |
| [NAD], uM | with DMSO | with 25 uM NAM+200 uM Honokiol | % activity |
| [P], uM | [P], pmoles | [P], uM | [P], pmoles |
| 50 | 6.24 | 311.92 | 2.94 | 146.76 | 47.05 |
| 1000 | 20.77 | 1038.36 | 8.37 | 418.51 | 40.31 |
| 3000 | 26.09 | 1304.50 | 10.82 | 541.00 | 41.47 |

**Table 1: In-house Sirt3 5U/reaction (1.85 uM), 600 uM K122-MnSOD, 5% DMSO, 30 min at 37OC**

**Table 2: Enzo Sirt3 5U/reaction (0.214 uM), 3000 uM NAD, 600 uM K122-MnSOD, 5% DMSO, 30 min at 37OC**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **[NAM], uM** | **[P], uM** | **[P], pmoles** | % activity | % inhibition |
| 0 | 17.29 | 864.70 | 100.0 | 0.00 |
| 25 | 16.514 | 825.70 | 95.5 | 4.49 |
| 50 | 14.64 | 732.00 | 84.7 | 15.33 |
| 100 | 12.11 | 605.50 | 70.0 | 29.96 |
| 200.000 | 9.815 | 490.75 | 56.8 | 43.23 |
| 500.000 | 6.190 | 309.50 | 35.8 | 64.20 |