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Screening Compounds of Interest against USP5 Zf-UBD by SPR #2

Objective: To determine binding affinities of compounds of interest previously identified in a ^{19}F-NMR spectroscopy assay against USP5 zinc finger ubiquitin binding domain (Zf-UBD) with a surface plasmon resonance (SPR) assay.

Experiment & Results:

A) Chip Preparation

An SA chip was used in a Biacore T-200 system. The chip was equilibrated with 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 0.5% DMSO (v/v) and then primed with 3x60 s injections of 50 mM NaOH to all chip channels, 500 s, 300 s, 150 s injections of 0.05 mg/mL of biotinylated USP5$^{171-290}$ to channel 2, 3 and 4 respectively and 5x10 s injections of 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 0.5% DMSO (v/v) buffer to all chip channels. Approximately 7000, 6000, and 5000 RU of protein captured to channel 2, 3 and 4 respectively. Channel 1 was left blank as a reference channel.

B) Plate Preparation

Ubiquitin peptides, LRLRGG, FITC-LRLRGG and UBXML78 were used as positive controls. Controls and compounds were prepared in 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 0.5% DMSO (v/v) buffer. In experiment 1, 2 and 3, samples were diluted 1:4 in a 8-point concentration series starting at 500 µM for the peptides and 1 mM for the compounds in 96-well plates. In experiment 4, the compounds with the highest binding affinities were repeated with a 1:2 12-pt dilution series. The plates were sealed and centrifuged at 1000 RPM for 1 minute.

C) Assay

A multi cycle kinetics method was run for the sample plate with the following parameters:

- Contact time: 35 s
- Dissociation time: 120 s
- Flow Rate: 30 µL/min
- Running Buffer: 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 0.5% DMSO (v/v)

Sample injections were done sequentially by compound, from the lowest to highest concentration. Data was fitted with a steady state affinity model. Experimental results are summarized in Table 1. Please see attached Biacore result files (.bme) for fitted data.

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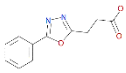
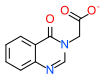
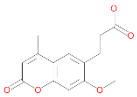
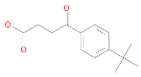
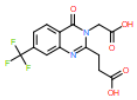
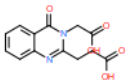
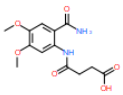
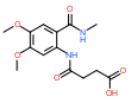
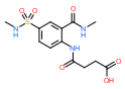
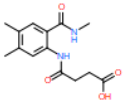
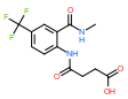
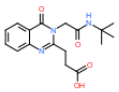
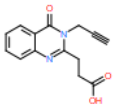
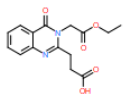
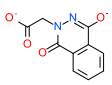
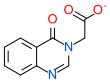
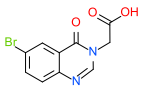
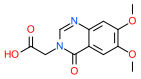
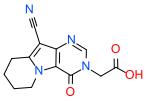
Table 1. Summary of SPR Assay Results

Compound	Compound Structure	Experiment 1 Average K_D (n=6)	Experiment 2 Average K_D (n=3)	Experiment 3 Average K_D (n=3)	Experiment 4 Average K_D (n=3)	Average K_D
LRLRGG		52 ± 2	161 ± 6	165 ± 6	145 ± 2	131 ± 53
FITC-LRLRGG					74 ± 2	
DAT180		365 ± 4				
DAT194		198 ± 2			231 ± 4	215 ± 23
DAT198		251 ± 6				
DAT201		138 ± 3			208 ± 2	173 ± 50
UBXML78		66 ± 6	62 ± 2	53 ± 0.5	56 ± 2	59 ± 6
UBXML70			147 ± 3		125 ± 5	136 ± 16
UBXML86			>1000			
UBXML87			>1000			
UBXML88			378 ± 32			
UBXML89			518 ± 22			
UBXML90			>1000			
UBXML93			238 ± 6			
UBXML94			347 ± 15			

UBXML95		398 ± 23		
DAT19b		377 ± 15		
DAT22b		320 ± 10		
DAT53b		253 ± 6		
DAT76b		116 ± 2	102 ± 2	109 ± 10
DAT80b		95 ± 3	93 ± 1	94 ± 1

Conclusions & Future Directions:

In general, I've found that using a 96-well plate format instead of a 384-well format has been more useful for my purposes for the SPR assay, as the USP5$^{171\text{-}290}$ captured on the SPR chip is stable at room temperature for the time it takes the assay to run. Running a 96-well plate with the method I am using takes about 11-12 hours, whereas a 384-well plate which can hold more compound titrations takes approximately 36 hours to run. In the past, I've found the signal of binding to the protein decreases over time so running a longer experiment, although cost-effective is unreliable for binding affinity measurements due to the instability of the protein. For this reason, I use 1 SA chip per 96-well plate for experiments, which is why the affinity measurements of the compounds of interest from the ^{19}F NMR assay have been done over multiple experiments.

The control ubiquitin peptide, LRLRGG K_D increased by 3-fold from experiment 1 to experiment 4. To test another peptide sample, I included FITC-LRLRGG in experiment 4. This is the same peptide with a fluorescent tag so the binding affinity should be similar to that of LRLRGG peptide. The binding affinity of FITC-LRLRGG was 74 µM. It is possible I'm seeing the increase in binding affinity of LRLRGG peptide due to contamination of my LRLRGG peptide stock or due to multiple freeze thaw cycles. Based on previous FP/SPR experiments the measured K_D was approximately 30-40 µM. I'll have to re-order my LRLRGG peptide and aliquot the stocks so I won't face this issue in the future. Since, my positive control LRLRGG ubiquitin peptide was not consistent, how do I know my data for my compounds is still reliable? Another positive control used was UBXML78, which was included in all titration plates for the experiments. On average UBXML78 had the best binding affinity from the series of compounds tested with a K_D of approximately 59 µM, and measurements were consistent for all the experiments.

The next thing I did was to see if I could correlate the ^{19}F NMR assay results with the SPR binding affinity measurements. Please see the attached .icb file in which I clustered the data to see if there were

patterns with the ^{19}F NMR chemical shift, change in peak area and the SPR binding affinities. You can download the free software (ICM Browser) to view the icm file form Molsoft. Generally, a chemical shift greater than 1 ppm in the ^{19}F NMR assay results in binding affinities less than 400 µM (Figure 1). I can use this trend, to prioritize testing compounds with SPR based on the NMR screen. This will limit testing of very weak binding compounds.

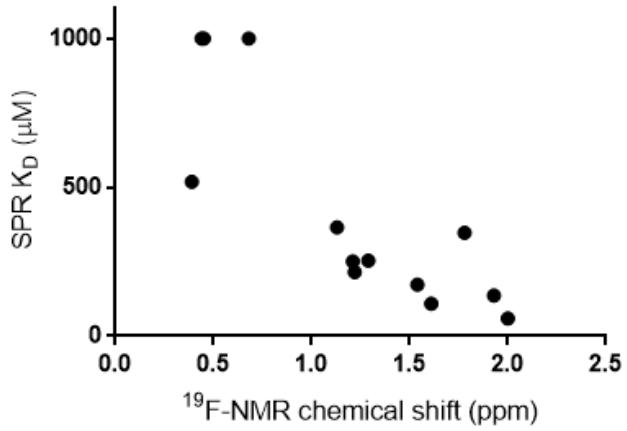


Figure 1. Relationship between ^{19}F NMR chemical shift and measured SPR binding affinities

Next, I'll be doing hit expansions of a few of the compounds of interest and using commercial substructure searches and docking to select compounds for the next set of screening. This will hopefully increase my understanding of the structure activity relationship (SAR) of the compounds and their potency against $USP5^{171-290}$.