

Screening compounds of interest against USP5 Zf-UBD with a surface plasmon resonance assay

Objective: To determine binding affinities of compounds of interest 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 primed with 50 mM NaOH, 0.05 mg/mL of biotinylated USP5¹⁷¹⁻²⁹⁰ and 20 mM Hepes pH 7.4, 150 mM NaCl, 0.005% Tween-20 (v/v), 1% DMSO (v/v) buffer. The chip was treated with 3x60 s injections of 50 mM NaOH. ~6500-7000 RU biotinylated USP5¹⁷¹⁻²⁹⁰ at 0.05 mg/mL was captured to channels 2, 3, and 4 of the chip. Channel 1 was left blank as a reference channel.

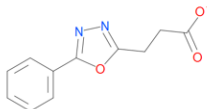
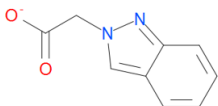
B) Plate Preparation

Ubiquitin peptides, RLRGG, LRLRGG and full length ubiquitin 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) \pm 1% DMSO (v/v). Samples were diluted 1:2 in 12-point concentration series starting at 500 μ M for peptides, 62.2 μ M for ubiquitin and 2 mM for compounds in a 96-well plate (n=1). The plate was sealed and centrifuged at 1000 RPM for 1 minute.

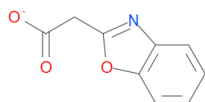
C) Assay

Sample injections were run in order of concentration, beginning with the lowest concentration. Data was fitted with steady state affinity model. 12 compound hits from a preliminary [19F NMR assay](#) were included in the first experiment. The compounds are summarized in Table 1. A second experiment was done with the best compounds (DAT180, DAT194, DAT198, DAT201) for confirmation of binding affinities. Negative controls (DAT185, DAT188) that showed no significant shift in the 19F NMR assay were used as negative controls for experiment 2. The average K_d values of 2 experiments are summarized in Table 2. Please see the attached document for the raw data of fitted binding curves and sensograms for each sample/experiment.

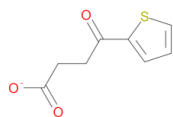
Table 1. Compounds identified from a 19F NMR assay

Compound Name	Compound Structure
DAT180	
DAT183	

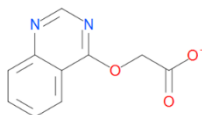
DAT187



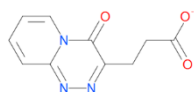
DAT190



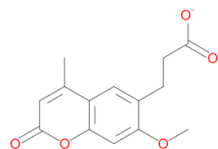
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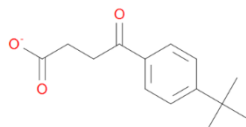
DAT196



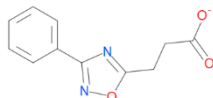
DAT198



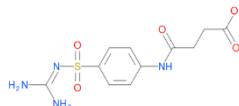
DAT201



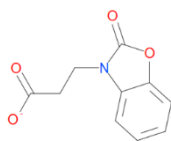
DAT202



DAT203



DAT208



DAT212

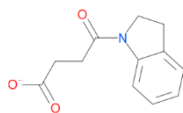


Table 2. Summary of SPR assay against USP5 Zf-UBD

Sample	Experiment 1 Average $K_d \pm$ SD (μ M) (n=3)	Experiment 2 Average $K_d \pm$ SD (μ M) (n=3)
Ubiquitin		6.1 ± 0.2
RLRGG	56.3 ± 1.3	
LRLRGG	20.0 ± 0.1	33.9 ± 0.6
DAT180	449 ± 19.3	142 ± 9.0
DAT183	929 ± 78.2	
DAT187	772 ± 49.2	
DAT190	$841. \pm 26.1$	
DAT194	233 ± 42.2	76.8 ± 2.4
DAT196	$1.5E+10 \pm$ $1.2E+09$	
DAT198	574 ± 72.1	89.7 ± 4.5
DAT201	592 ± 71.3	48.7 ± 1.9
DAT202	$13800 \pm$ 6470	
DAT203	6060 ± 545	
DAT208	$11100 \pm$ 2330	
DAT212	2560 ± 312	
DAT185		---
DAT188		---

Conclusions & Future Directions:

In a previous screening experiment, 19F NMR spectroscopy gave insight into structural perturbations of the USP5 binding pocket when small molecules bind. SPR was used as a secondary assay to confirm the compounds of interest as a more quantitative method.

Ubiquitin peptides, RLRGG and LRLRGG were used as positive controls. LRLRGG had the best binding affinity, with a K_d of 20 μ M. All 12 hit compounds from the ^{19}F NMR assay were included in the SPR assay, from which four of the compounds: DAT180, DAT194, DAT198, DAT201 showed weak binding affinities in the micromolar range, with DAT194 having the best binding affinity, with a K_d of approximately 230 μ M.

I then decided to repeat the SPR assay with the best binding compounds in order to validate the binding affinities. I also decided to include full length ubiquitin as a positive control and DAT185, DAT188 as negative controls. DAT185 and DAT188 showed no significant perturbation in the ^{19}F NMR assay, and as expected did not show binding in the SPR assay. I also expected to see tighter binding of USP5 Zf-UBD to ubiquitin than the LRLGG peptide. As expected, ubiquitin had a K_d of approximately 6 μ M whereas LRLRGG ranged from 20-40 μ M.

Surprisingly and interestingly I saw much tighter binding of the compounds in the second experiment, with a 3 to 10-fold increase in affinity! In the first experiment, compound stocks were dispensed in a 96-well plate and provided to me, whereas the second experiment I prepared my samples directly from the stock bottles. It is definitely concerning that I'm seeing such a difference in binding affinities from one experiment to the next. It is possible there may have been a mix-up/contamination in the plate provided to me; however, the UPLC/MS of the dispensed compounds in the plate are >95% pure. I cannot say with certainty why I'm seeing such variation in the binding affinities between the two experiments; however, I will definitely be repeating this experiment several more times to have more confidence in the binding affinities of these compounds.

In the future, I'll be repeating the SPR experiments and confirming the binding affinities with a second assay such as isothermal titration calorimetry (ITC).