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Do ZnF-UBD ligands bind full length USP5?

Objective: Use a surface plasmon resonance (SPR) assay to determine the binding affinities of USP5 zinc finger ubiquitin-binding domain (ZnF-UBD) ligands to full length (FL) $USP5^{1-835}$

Method & Results:

A. Chip Preparation

For more details on the growth and purification of the protein constructs used in this assay visit: **10.5281/zenodo.3403804**

Experiment 1: An SA chip was used in a Biacore T-200 system at 20°C. The chip was equilibrated with 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v) and then primed with 3x60 s injections of 50 mM NaOH to all chip channels. 0.05 mg/mL of biotinylated USP5 ZnF-UBD WT (construct: TOC011B06, AA: 171-290) was injected onto channel 2 for 200 s. 0.05 mg/mL of biotinylated USP5 ZnF-UBD R221A (construct: TOC023B01, AA: 171-290) and biotinylated FL USP5 WT (construct: TOC023A01, AA: 1-835) was injected to channel 3 and 4 for 300 s respectively. Protein capture was completed at a flow rate of 10 µL/min. Approximately 7000 RU, 5000 RU, and 4000 RU of protein was captured to channel 2, 3, and 4 respectively. 5x10 s of 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v) was injected to all chip channels. Channel 1 was left blank as a reference channel.

Experiment 2: An SA chip was used in a Biacore T-200 system at 20°C. The chip was equilibrated with 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v) and then primed with 3x60 s injections of 50 mM NaOH to all chip channels. 0.05 mg/mL of biotinylated FL USP5 WT(construct: TOC023A06, AA: 1-835) and biotinylated FL USP5 R221A (construct: TOC023A06, AA: 1-835) was injected to channel 2 and 3 for 300 s respectively. 0.05 mg/mL of biotinylated USP5 ZnF-UBD WT (construct: TOC011B06, AA: 171-290) was injected onto channel 4 for 300 s. Protein capture was completed at a flow rate of 10 µL/min. Approximately 4000 RU, 5000 RU, and 7000 RU of protein was captured to channel 2, 3, and 4 respectively. 5x10 s of 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v) was injected to all chip channels. Channel 1 was left blank as a reference channel.

Experiment 3: An SA chip was used in a Biacore T-200 system at 20°C. The chip was equilibrated with 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v) and then primed with 3x60 s injections of 50 mM NaOH to all chip channels. 0.05 mg/mL of biotinylated FL USP5 WT (construct: TOC023A06, AA: 1-835) and biotinylated FL USP5 R221A (construct: TOC023A06, AA: 1-835) was injected to channel 2 and 3 for 300 s respectively. 0.05 mg/mL of biotinylated USP5 ZnF-UBD WT (construct: TOC011B06, AA: 171-290) was injected onto channel 4 for 300 s. Protein capture was completed at a flow rate of 10 µL/min. Approximately 5000 RU, 4000 RU, and 5000 RU of protein was captured to channel 2, 3, and 4 respectively. 5x10 s of 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v) was injected to all chip channels. Channel 1 was left blank as a reference channel.

B. Plate Preparation

Ligands were prepared in 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% DMSO (v/v) buffer. In all experiments, ligands were diluted 1:2 in a 12-point concentration series starting at 2 mM for compounds and 100 µM for ubiquitin (AA: 1-76) in a 96-well plate. The plates were sealed and centrifuged at 1000 RPM for 1 minute.

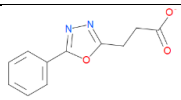
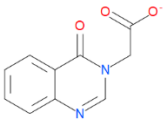
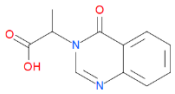
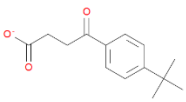
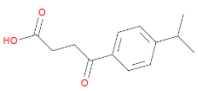
C. Assay

For all experiments a multi-cycle kinetics method was run for the sample plates with the following parameters:

- Contact time: 60 s
- Dissociation time: 120 s
- Flow Rate: 30 µL/min
- Temperature: 20°C
- Running buffer: 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 1% 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 1-2. Please see attached Biacore result files (.bme) for fitted data.

Table 1. Summary of SPR Results of FL USP5 WT and R221A

Compound SGC Global ID	Compound Toronto ID	Compound Structure	Experiment 1 K_D (µM)		Experiment 2 K_D (µM)		Experiment 3 K_D (µM)		Avg K_D (µM)	
			FL USP5 WT	FL USP5 R221A	FL USP5 WT	FL USP5 R221A	FL USP5 WT	FL USP5 R221A	FL USP5 WT	FL USP5 R221A
DAT00000180a	DAT00000239a		NB (n=1)		331 (n=1)	NB			331	NB
DAT00000194a	DAT00000097b		514 (n=1)		670 (n=1)	NB			592 ± 110	NB
	XST00090960c		58 ± 4 (n=2)		68 ± 3 (n=2)	NB	127 ± 14 (n=3)	NB	84 ± 37	NB
DAT00000201a	DAT00000231a		118 (n=1)		199 (n=1)	NB			159 ± 57	NB
	UBT00008295a		253 ± 9 (n=2)		278 ± 23 (n=2)	NB			265 ± 20	NB
Ubiquitin (1-76)			0.3 (n=2)		0.2 (n=1)	36 (n=1)	0.4 ± 0.005 (n=3)	40 ± 0.6 (n=3)	0.3 ± 0.1	38 ± 3

NB: no binding (K_D > 1 mM)

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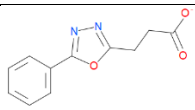
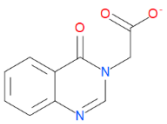
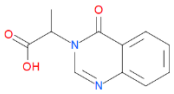
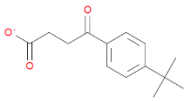
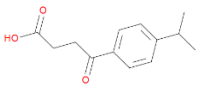
Table 2. Summary of SPR Results of USP5 ZnF-UBD WT and R221A

Compound SGC Global ID	Compound Toronto ID	Compound Structure	Experiment 1 K_D (µM)		Experiment 2 K_D (µM)		Experiment 3 K_D (µM)		Avg K_D (µM)	
			USP5 ZnF-UBD WT	USP5 ZnF-UBD R221A	USP5 ZnF-UBD WT	USP5 ZnF-UBD R221A	USP5 ZnF-UBD WT	USP5 ZnF-UBD R221A	USP5 ZnF-UBD WT	USP5 ZnF-UBD R221A
DAT00000180a	DAT00000239a		653 (n=1)	NB	473 (n=1)				563 ± 127	NB
DAT00000194a	DAT00000097b		296 (n=1)	NB	219 (n=1)				258 ± 54	NB
	XST00090960c		58 ± 0.7 (n=2)	NB	57 ± 2 (n=2)			NB	58 ± 1	NB
DAT00000201a	DAT00000231a		179 (n=1)	NB	147 (n=1)				163 ± 23	NB
	UBT00008295a		157 ± 0.7 (n=2)	NB	149 ± 1 (n=2)				153 ± 5	NB
Ubiquitin (1-76)			4.3 (n=1)	NB	4.2			NB	4.3 ± 0.1	NB

NB: no binding (K_D > 1 mM)

Conclusions

USP5 ZnF-UBD ligands were tested against FL USP5 WT and R221A as well as the ZnF-UBD WT and R221A. Ligand binding to the WT ZnF-UBD and the FL protein are comparable. For example, in the above experiments, compound XST00090960c binds to the ZnF-UBD with a K_D of 58 ± 1 µM and to FL USP5 with a KD of 84 ± 37 µM. R221 is an important residue in the binding pocket of the ZnF-UBD that co-ordinates ubiquitin binding. A mutation of arginine (R) to alanine (A) should prevent binding of ubiquitin and the inhibitors to the ZnF-UBD. There was no ligand binding to the ZnF-UBD R221A and FL USP5 R221A confirming ligands are indeed binding to the ZnF-UBD in the context of the FL protein. These results corroborate previous displacement assay findings. Interestingly, ubiquitin binds to FL USP5 R221A (K_D=38 ± 3 µM) 100-fold weaker than the FL USP5 WT (K_D=0.3 ± 0.1 µM). This is likely because the FL USP5 contains multiple ubiquitin associated domains that still bind ubiquitin.