Testing USP5 ZnF-UBD analogues with a Displacement Assay and SPR

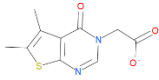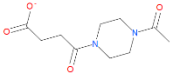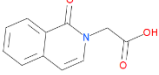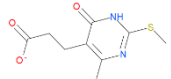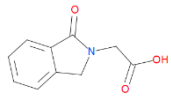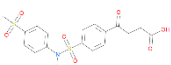Objective: To screen 20 commercial analogues from the Enamine REAL database against the USP5 zinc finger ubiquitin-binding domain (ZnF-UBD) using a displacement assay and to assess binding potency of best displacing compounds with a surface plasmon resonance (SPR) assay
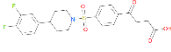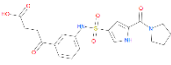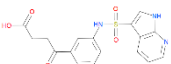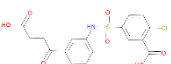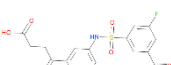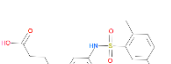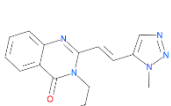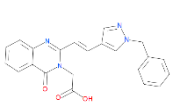
Methods and Results:
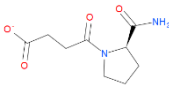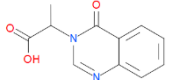
A. Displacement Screen

Experiments were completed in a 384-well black flat-bottomed streptavidin plate (Greiner). 20 µL of 1 µM protein (bio-USP5[171-290] TOC011B06 p28bioH-LIC) prepared in 20 mM Hepes pH 7.4, 150 mM NaCl, 1 mM TCEP, 0.005% Tween-20 (v/v), 0.5% DMSO buffer were incubated in wells for 1 hour at 4°C. The wells were then washed with 3x 50 µL buffer to remove excess unbound protein. 20 µL of a ligand at 100 µM (n=1) and 1 mM (n=1) with 0.2 µM N-terminally tagged FITC-ubiquitin (UBQ) (Boston Biochem) was incubated in wells for 1 hour at 4 °C. Wells were washed with 7x 50 µL of buffer to remove excess unbound FITC-UBQ. Plate was read using a Biotek plate reader with an emission and excitation of 528 nm and 485 nm respectively. Raw fluorescence data and displacement calculations can be found in the .xlsx file. The compound information can also be found in the .sdf file. Results are summarized in Table 1 and Figure 1.

Table 1. Displacement Screen of Enamine REAL analogues

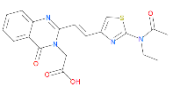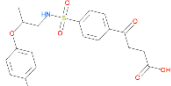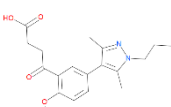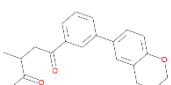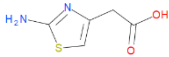| Experiment Compound #	Catalog #	Toronto Internal ID	Compound Structure	Compound SMILES	% Displacement at 1 mM (n=1)	% Displacement at 100 µM (n=1)
1	EN300-10660	UBTR012584a		Cc1c2C(N(CC([O-])=O)C=Nc2sc1C)=O	72	7
2	EN300-13338	UBTR012580a		CC(N1CCN(CC1)C(CCC([O-])=O)=O)=O	52	18
3	EN300-188561	UBTR012585a		C(C(O)=O)N1C=Cc2ccccc2C1=O	53	15
4	EN300-35451	UBTR012582a		CC1=C(CCC([O-])=O)C(NC(=N1)SC)=O	52	24
5	EN300-70527	PKTR013363b		C(C(O)=O)N1Cc2ccccc2C1=O	38	0
6	Z1359460504	UBTR012570a		CN(c1ccc(cc1)S(C)(=O)=O)S(c1ccc(cc1)C(CCC(O)=O)=O)(=O)=O	89	61

7	Z135946 3303	UBTR012574a		C1CN(CCC1c1ccc(c(c1)F)F)S(c1ccc(cc1)C(CCC(O)=O)=O)(=O)=O	85	73
8	Z137357 7229	UBTR012571a		C1CCN(C1)C(c1cc(c[nH]1)S(Nc1cccc(c1)C(CCC(O)=O)=O)(=O)=O)=O	78	50
9	Z137357 7255	UBTR012567a		C(CC(c1cccc(c1)NS(c1c[nH]c2c1cccn2)(=O)=O)=O)C(O)=O	85	40
10	Z141395 8135	UBTR012576a		C(CC(c1cccc(c1)NS(c1ccc(c(c1)C(O)=O)[Cl])(=O)=O)=O)C(O)=O	66	26
11	Z141395 8985	UBTR012572a		C(CC(c1cccc(c1)NS(c1cc(cc(c1)F)C(O)=O)(=O)=O)=O)C(O)=O	55	3
12	Z141395 9119	UBTR012568a		Cc1ccc(cc1S(Nc1cccc(c1)C(CCC(O)=O)=O)(=O)=O)C(O)=O	65	36
13	Z143675 7513	UBTR012578a		Cn1c(C=CC2=Nc3ccccc3C(N2CC(O)=O)=O)cnn1	18	12
14	Z143675 8306	UBTR012577a		C(C(O)=O)N1C(C=Cc2cnn(Cc3ccccc3)c2)=Nc2ccccc2C1=O	28	23
15	Z143675 8310	UBTR012579a		CCN(C(C)=O)c1nc(C=CC2=Nc3ccccc3C(N2CC(O)=O)=O)cs1	14	0
16	Z168215 2871	UBTR012575a		CC(CNS(c1ccc(cc1)C(CCC(O)=O)=O)(=O)=O)Oc1ccc(C)cc1	78	37
17	Z258155 8272	UBTR012573a		CCCn1c(C)c(c2ccc(c(c2)C(CCC(O)=O)=O)OC)c(C)n1	62	32
18	Z258156 4325	UBTR012569a		CC(CC(c1cccc(c1)c1ccc2c(CCCO2)c1)=O)C(O)=O	55	31

19	A0954	UBTR012583a		C(C(O)=O)c1csc(N)n1	3	4
20	CDS0143 72	UBTR012581a		C1C[C@H](C(N)=O)N(C 1)C(CCC([O-])=O)=O	81	45
21: Positive Control	AE-641/114 56811	XSTR090960c		CC(C(O)=O)N1C=Nc2ccc cc2C1=O	70	40
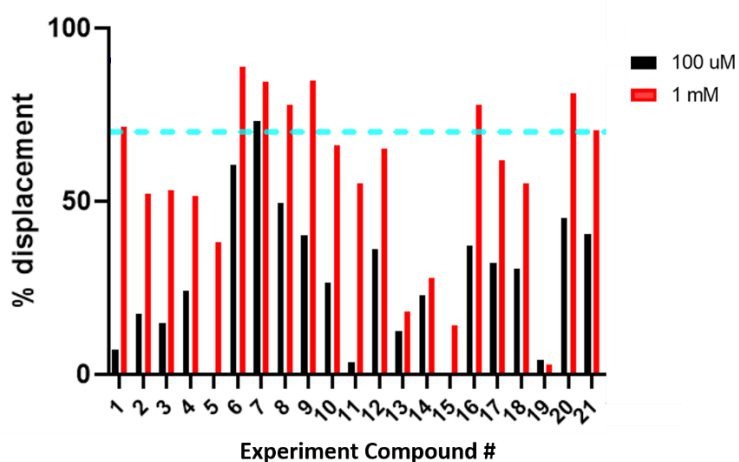
Figure 1. Summary of displacement screen

Compound displacement was compared to the control (experiment compound #21) which has been shown to have a K_D of approximately 60 μM in previous SPR experiments. Compounds with comparable displacement at 1 mM to compound 21 were selected to test with SPR. This included compounds: 1, 6, 7, 8, 9, 10, 12, 16, 17, 18, 20.

B. SPR Assay

1. Chip Preparation

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 TCPE, 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 (construct: TOC011B06, AA: 171-290) was injected onto channel 2 and 3 for 150 s and 300 s respectively. 0.05 mg/mL HDAC6 ZnF-UBD (construct: TOC004A01, AA: 1109-1215) was injected onto channel 4 for 300 s. Protein capture was completed at a flow rate of 10 μL/min. Approximately 6500, 7000, 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.

2. 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. Ligands were diluted 1:4 in a 8-point concentration series starting at 2 mM for all compounds in a 96-well plate. The plates were sealed and centrifuged at 1000 RPM for 1 minute.
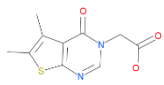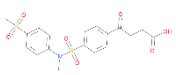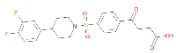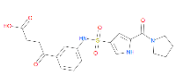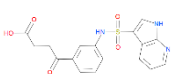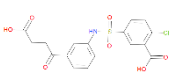
3. Assay
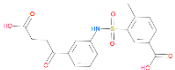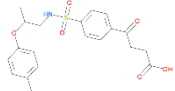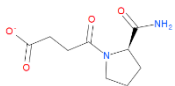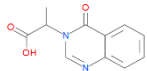
A multi-cycle kinetic method was run for the sample plates with the following parameters:

- Contact time: 60 s
- Dissociation time: 120 s
- Flow Rate: 30 µL/min
- Temperature: 20⁰C
- 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 2. Please see attached Biacore result file (.bme) for fitted data.

Table 2. Summary of SPR Results

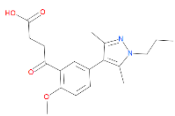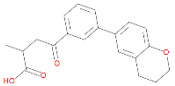| Toronto Internal ID	Compound Structure	USP5 ZnF-UBD K_D (n=2) (µM)	HDAC6 ZnF-UBD K_D (n=1) (µM)
UBTR012584a		128 ± 6	130
UBTR012570a		36 ± 1	11
UBTR012574a		10 ± 0.2	17
UBTR12571a		92 ± 1	259
UBTR012567a		76 ± 1	117
UBTR012576a		552 ± 13	642

UBTR012568a		363 ± 8	382
UBTR012575a		172 ± 1	58
UBTR012573a		488 ± 4	NB
UBTR012569a		770 ± 41	96
UBTR012581a		325 ± 4	701
XSTR090960f		138 ± 3	45

One of the most promising compounds, UBTR012574a has a K_D of approximately 10 μM, a significant increase in potency from the preliminary hits (Figure 2). Compounds UBTR012574a, UBTR012571a, UBTR12567a, and UBTR012581a have a slightly better potency for the USP5 ZnF-UBD over the HDAC6 ZnF-UBD.

AC-907/2501427
K_D= 680 ± 62 μM

DAT00000201a
K_D=170 ± 50 μM

Compound 7 (displacement screen)
UBTR012574a
K_D=10 ± 0.2 μM
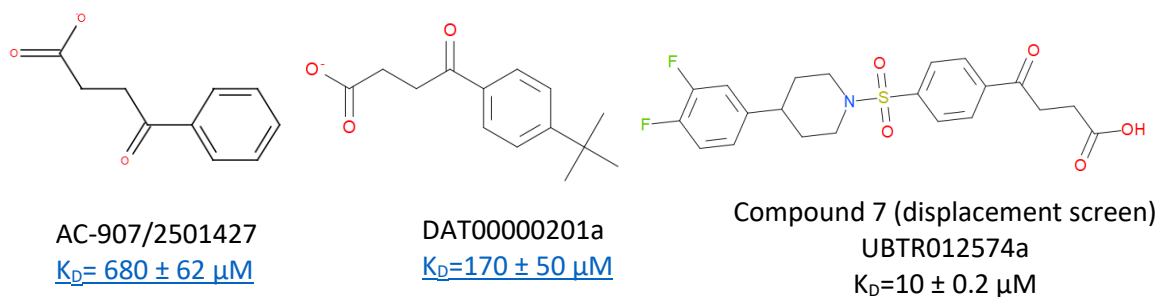
Figure 2. SAR of chemical series

There was a trend when comparing the displacement screen and the SPR assay. The higher the % displacement the lower the K_D of the compound (Figure 3). It should be noted however, the displacement screen was done with n=1.
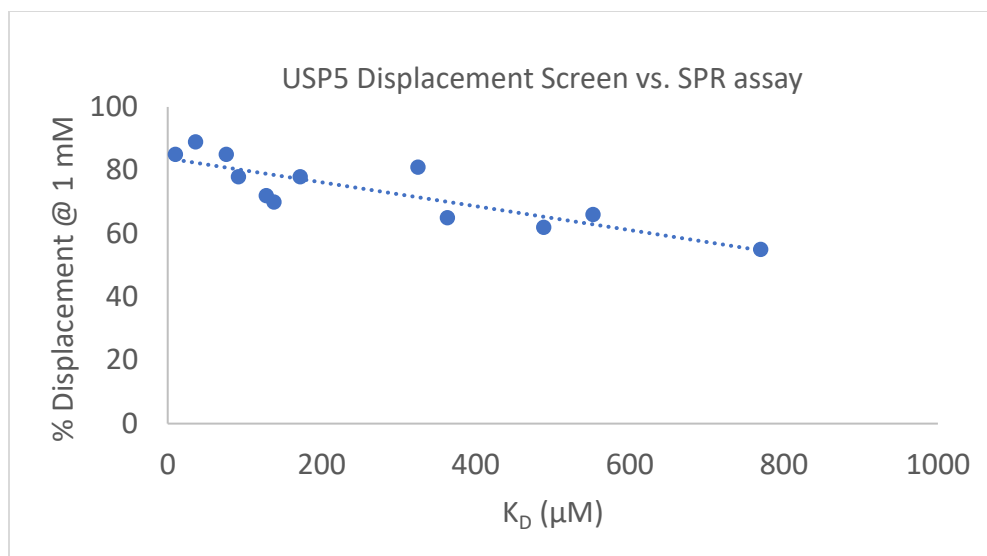
Figure 3. Trend between displacement at 1 mM and SPR binding affinities

Conclusions and Future Directions

Analogues ordered from Enamine's REAL database were tested in a displacement screen at two concentrations relative to a control compound with known K_D of approximately 60 μM. Compounds with comparable displacement were then tested in an SPR assay. The most potent compound was UBTR12574a, which had a K_D of approximately 10 μM; almost a 20-fold increase from the parent compound which was one of my preliminary hits! Focusing on UBTR012574a, I will try to solve the co-crystal structure of USP5 ZnF-UBD in complex with UBTR012574a to determine if the predicted binding pose is similar to the experimental binding pose. This will shed some insight into what chemical moieties of the compound are lending to the increased potency. I am also going back to Enamine's REAL database and looking for compounds similar to UBTR012574a. I'll be ordering some more analogues to get a better understanding of the structure activity relationship of the extended sulfonamide groups at the para position of the benzene ring. Stay tuned!