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**USP5 Zf-UBD Co-Crystal Structure with Compound XSR00035795a & Testing Selectivity**

Objective: To grow well-diffracting co-crystals of USP5 zinc finger ubiquitin binding domain (Zf-UBD) to solve the co-crystal structures to determine ligand interactions in the binding pocket and determine if methyl group on carboxylic chain of compound XSR00035795a confers selectivity to USP5 versus HDAC6 using a surface plasmon resonance assay (SPR).

Experiment & Results:

A. Co-crystal structure determination

Based on previous SPR assay results, compound AE-641/11456811 ($K_D$= 65 ± 5 µM; n=2), now given an SGC global ID of XSR00035795a, an analogue of DAT194 ($K_D$=215 ± 23 µM; n=9) was used to set up co-crystallization screen with USP5$^{171-290}$. 80 µL of 12 mg/mL USP5 and 5 mM compound XSR00035795a solution was prepared in 50 mM Tris pH 8, 150 mM NaCl, 1 mM TCEP.

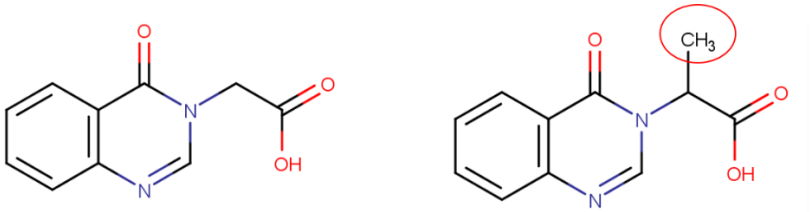


Figure 1. DAT194 and compound XSR00035795a, a DAT194 analogue

A 96-well optimization block of the crystallization condition SGC-G03 (1.8 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 5.5), a condition which grows apo USP5 Zf-UBD crystals well, was prepared. The pH and ammonium sulfate concentrations were titrated across the optimization block. Please see the attached excel file for optimization block preparation calculations (optimizationblock.xls).

The optimization block was used to prepare a 96-well Intelli-plate (Art Robbins Instruments). 70 µL of each condition was dispensed into the well of the plate and then 0.5 µL of the well solution was dispensed to the drop in the crystal plate by the liquid handling robot (Phoenix) followed by 0.5 µL of protein:compound solution. Crystal plates were sealed and stored at 18ºC.

Two days after preparing the crystal plates, co-crystals had formed in the following conditions: H08 (1.75 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.80), H09 (1.81 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.80).

The crystals were mounted using a nylon loop then transferred to a 2 µL drop of well solution supplemented with 25% ethylene glycol (v/v) and submerged for a few seconds, and then cryo-cooled in liquid nitrogen. The crystal was screened using our in-house diffractometer, RIGAKU FR-E SUPERBRIGHT at 1.54178 Å. 2 images at 90 degrees with a 0.5 degrees oscillation, 20 s exposure and 100 mm crystal-detector distance were collected with a RIGAKU SATURN A200 CCD detector at 100 K. Figures 2 and 3 outline the best diffracting crystals from each condition.

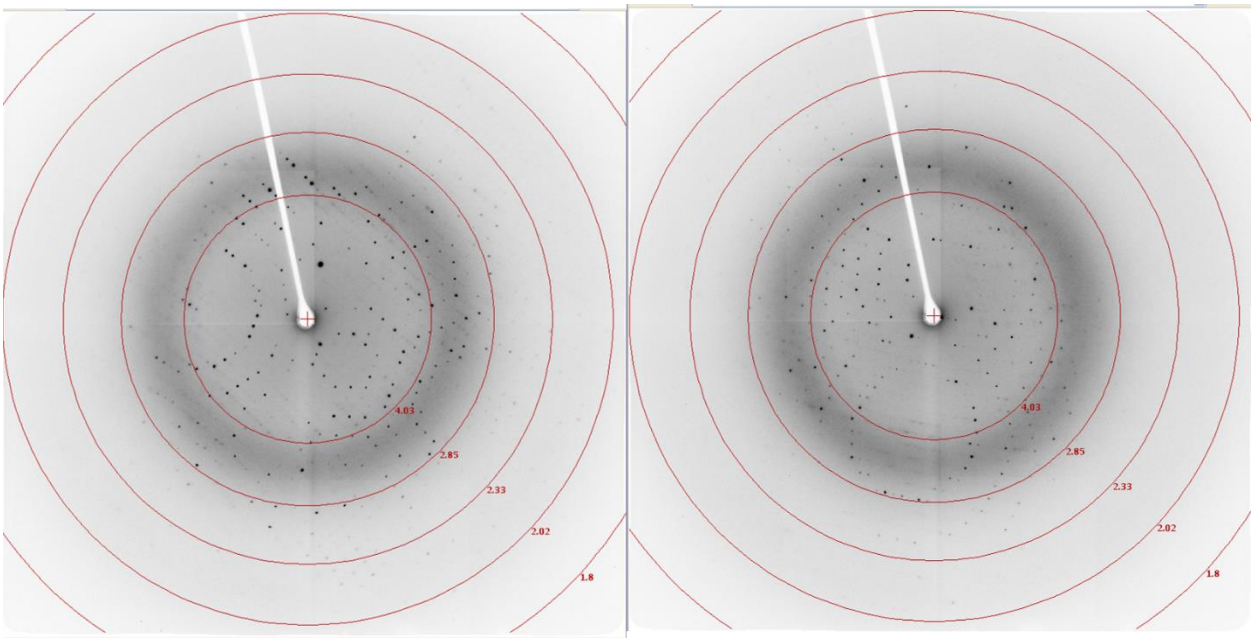


Figure 2. Diffraction images with USP5$^{171-290}$ and XSR00035795a in optimization condition H08: 1.75 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.80, 1.1% DMSO (v/v), 25% ethylene glycol (v/v)

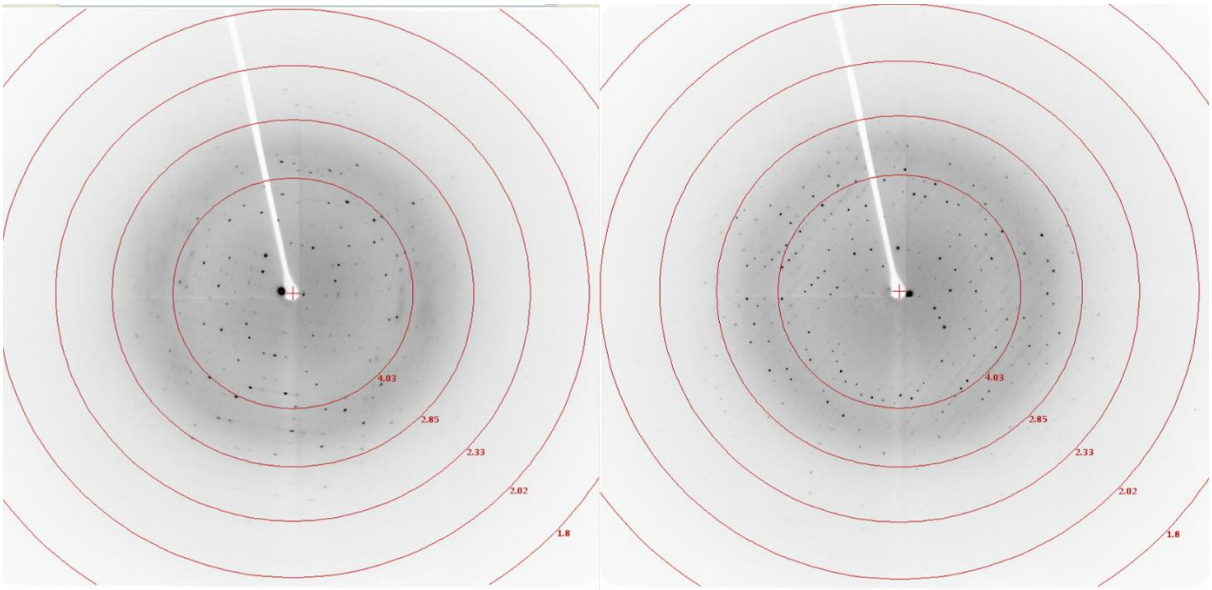


Figure 3. Diffraction images with USP5$^{171-290}$ and XSR00035795a in optimization condition H09: 1.81 M ammonium sulfate, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.80, 1.1% DMSO (v/v), 25% ethylene glycol (v/v)

Large scale data collection of the crystals from Figure 2 were collected in house using the following conditions: crystal detector distance: 60 mm, 180 images, 1 degree oscillation and an exposure time of 10 sec.

The images were processed with Xia2, scaled with AIMLESS and phased using Phaser with chain A of PDB: 6DXH as a starting model by Dr. Wolfram Tempel. The structure was then refined with iterative builds in Coot and refinement in refmac. Clear electron density of the compound was seen in the binding pocket. The resulting pdb and mtz files are attached (XSR35795.pdb/XSR35795.mtz). It is to be noted that the structure is not yet processed or refined to the point of being ready for PDB deposition. Table 1 summarizes the co-crystal structure.

Table 1. Co-crystal structure summary of USP5 Zf-UBD and compound XSR00035795a

| **Compound name** | **$R_{factor}/R_{free}$** | **Symmetry: space group** | **Unit Cell** | | **Resolution (Å)** |
|---|---|---|---|---|---|
| | | | **Length (Å)** | **Angle (°)** | |
| XSR00035795a | 0.22/0.24 | I 2 2 2 | a=47.85<br>b=82.41<br>c=99.82 | α=90.00<br>β=90.00<br>γ=90.00 | 2.1 |

The addition of the methyl group on compound XSR00035795a leads to increased hydrophobic interactions in the binding pocket with W209; the side chain of R221 also rotates out of the pocket. In Figure 4, the methyl group of XSR00035795a fits into a small groove in the pocket. It may be possible to further extend the aliphatic group on the carboxylic chain to increase potency further.

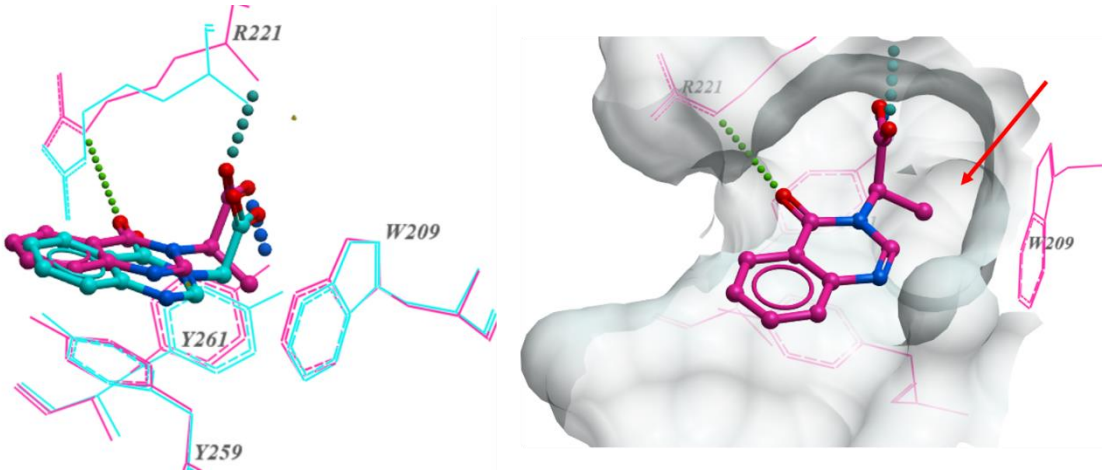


**Figure 4**. a) Superimposed USP5 structures of compound DAT194 (PDB: 6NFT; cyan) and compound XSR00035795a (magenta) b) Binding pocket of co-crystal structure of USP5 Zf-UBD and XSR00035795a

B. Selectivity: USP5 Zf-UBD vs. HDAC6 Zf-UBD

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% Twee-20 (v/v), 0.5% DMSO (v/v) and then primed with 3x60 s injections of 50 mM NaOH to all chip channels. 0.05 mg/mL of biotinylated

HDAC61109-1215 was injected to channel 2 and 3 for 500 and 300 seconds respectively and biotinylated USP5171-290 was injected to channel 4 for 500 seconds. 5x10 s injections of buffer followed. Channel 1 was left blank as a reference channel.

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. Samples were diluted 1:4 in a 12-point dilution series starting at 1 mM in a 96-well plate. The plates were sealed and centrifuged at 1000 RPM for 1 min.

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

Contact time: 35 s

Dissociation time: 120 s

Flow Rate: 30 uL/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 from lowest to highest concentration. Data was fitted with a steady state affinity model. Representative binding curves and sensograms are shown in Figure 5.

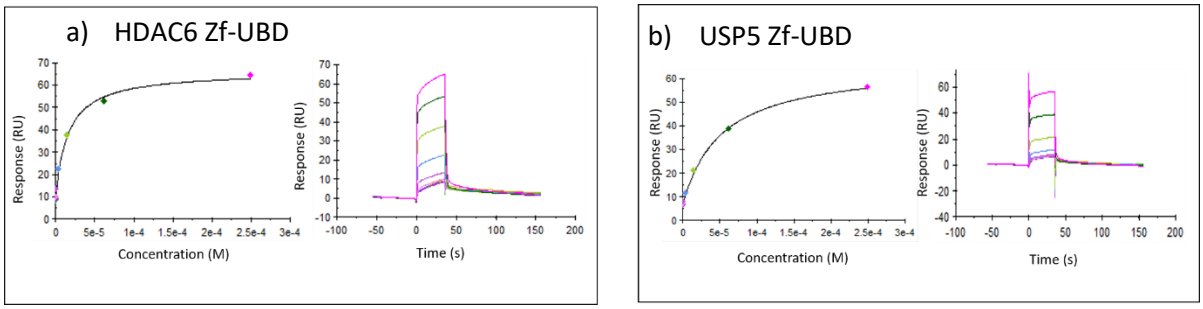


**Figure 5**. Representative SPR binding curves and sensograms of compound XSR00035795a with a) HDAC6 Zf-UBD ($K_D$= 15 ± 0.4 µM; n=2) and b) USP5 Zf-UBD ($K_D$= 60 ± 8 µM; n=3)

Compound XSR00035795a (LE=0.36) was not selective for USP5 Zf-UBD. XSR00035795a and HDAC6 Zf-UBD has a $K_D$ of 15 µM whereas USP5 Zf-UBD has an average $K_D$ of 60 ± 8 µM, including previous replicates (n=3).

When co-crystal structures of HDAC6 Zf-UBD and USP5 Zf-UBD are superimposed, binding of XSR00035795a to HDAC6 Zf-UBD makes sense. Figure 6 shows the binding pocket of HDAC6 Zf-UBD is able to accommodate the methyl group of compound XSR00035795a. Replacing a valine residue in the primary pocket of HDAC6 with an alanine in USP5 generates a small cavity that may be exploited by extending the aliphatic group on the carboxylic chain to confer selectivity towards USP5.

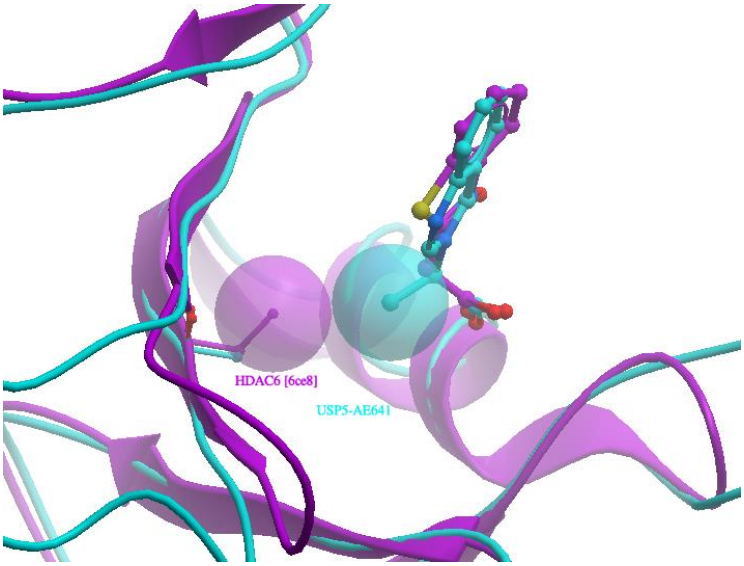


**Figure 6**. Superimposed CPK representation of HDAC6 Zf-UBD (purple PDB: 6CE8) and USP5 Zf-UBD and compound XSR00035795a co-crystal structure (cyan)

Conclusions & Future Directions

The co-crystal structure of USP5 Zf-UBD and compound XSR00035795a was solved to a resolution of 2.1 Å and has an I 2 2 2 crystal space group, a similar space group to previously solved co-crystal structure PDB: 6DXH. The methyl group on the carboxylic chain of XSR00035795a leads to increased hydrophobic interactions in the binding pocket with W209; the side chain of R221 also rotates out of the pocket. Compound XSR00035795a is not selective to USP5, and is able to bind to HDAC6 Zf-UBD with a binding affinity of approximately 15 µM. Extending the aliphatic group on the carboxylic chain may increase potency further and confer selectivity to USP5 Zf-UBD over HDAC6 Zf-UBD.