Mandeep Mann
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USP5 Ubiquitin-Rhodamine110 Catalytic Activity Assay

Objective: to determine if ZnF-UBD ligands antagonize USP5 deubiquitinase (DUB) activity using a ubiquitin-rhodamine110 assay

Method & Results:

A. DUB activity of WT USP5, R221A ZnF-UBD mutant and C335A catalytic mutant

Experiments were performed in a total volume of 60 µL in a 384-well black polypropylene microplate (Greiner). Fluorescence was measured using a Biotek Synergy H1 microplate reader (Biotek) at excitation and emission wavelengths of 485 and 528 nm, respectively. 0.2 µM UbRho110 (UBPBio) and 1 nM USP5 (wild-type (WT): SDC075B03, residues 1-835; R221A: TOC023A06, residues 1-835; C335A: TOC023A02, residues 1-835) were prepared in 30 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.01 % (v/v) Triton X-100, 1 mM DTT, 1% DMSO (v/v). Following a 1-minute centrifugation at 250 g, fluorescence readings were immediately taken. The data was analyzed with GraphPad Prism. Results are summarized in Figure 1.

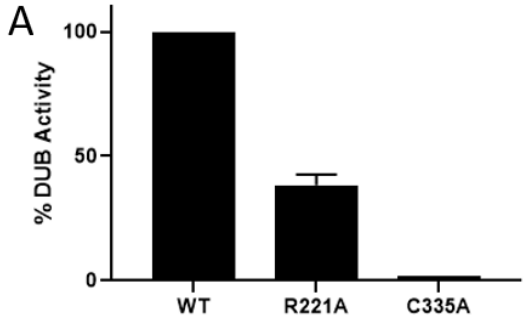

Figure 1. DUB activity of USP5 constructs (n=2)

B. DUB activity of WT USP5 with titration of XST00090960c

Experiments were performed in a total volume of 60 µL in a 384-well black polypropylene microplate (Greiner). Fluorescence was measured using a Biotek Synergy H1 microplate reader (Biotek) at excitation and emission wavelengths of 485 and 528 nm, respectively. 0.2 µM UbRho110 (UBPBio) and 1 nM USP5 (SDC075B03, residues 1-835) were prepared in 30 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.01 % (v/v) Triton X-100, 1 mM DTT, 1% DMSO (v/v). A 1:2- 11 point concentration series of XST00090960c was tested (N=3). Following a 1-minute centrifugation at 250 g, fluorescence readings were immediately taken. The data was analyzed with GraphPad Prism. DUB activity is derived from the slope of fluorescence readings taken over time for each concentration in the dilution series against the control (no inhibitor, 0 µM). Results are summarized in Figure 2.

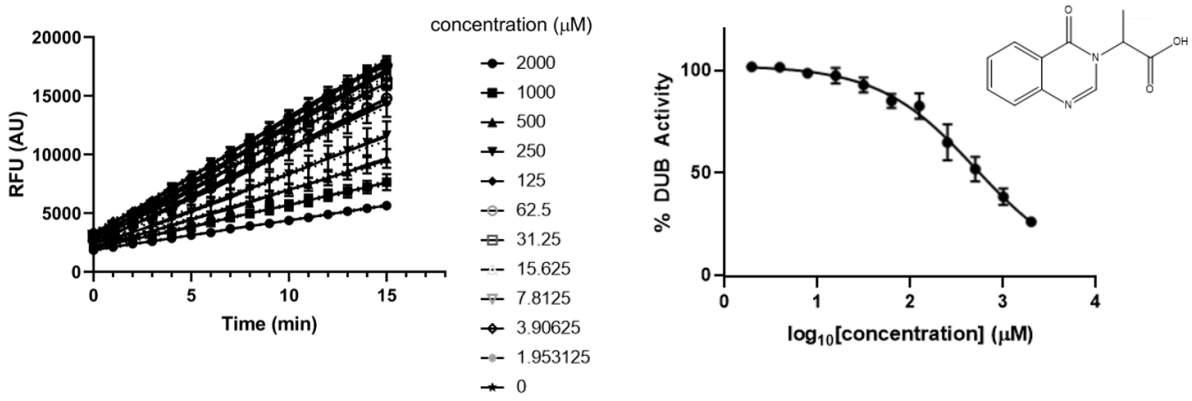

Figure 2. Representative activity data of compound XST00090960c

Conclusions & Future Directions

The final optimized conditions for the UbRho110 assay are as follows: 20 mM Tris-HCl (pH 7.5), 30 mM NaCl, 0.01 % Triton X-100, 1 mM DTT; 1 nM $USP5^{1-835}$ and 0.2 μM UbRho110 (development and optimization completed by Leanna Smith at SGC Toronto). Introduction of a ZnF-UBD mutant, R221A results in approximately 40% DUB activity compared to the WT USP5. A C335A catalytic mutant results in no activity. To determine if ZnF-UBD ligands antagonize USP5 activity in the UbRho110 assay, XST00090960c was tested. XST00090960c inhibits approximately 75% of USP5 activity. Therefore, ZnF-UBD ligands are able to antagonize the catalytic cleavage of the UbRho110 substrate. It should be noted, UbRho110 is not a native substrate, and further validation with native polyubiquitin species in an orthogonal assay is required. In the future I plan to use a western blot assay to determine whether ZnF-UBD ligands antagonize native polyubiquitin species.