INHIBITORS OF USP9X IDENTIFIED BY HIGH THROUGHPUT SELECTIONS OF DNA ENCODED LIBRARIES.

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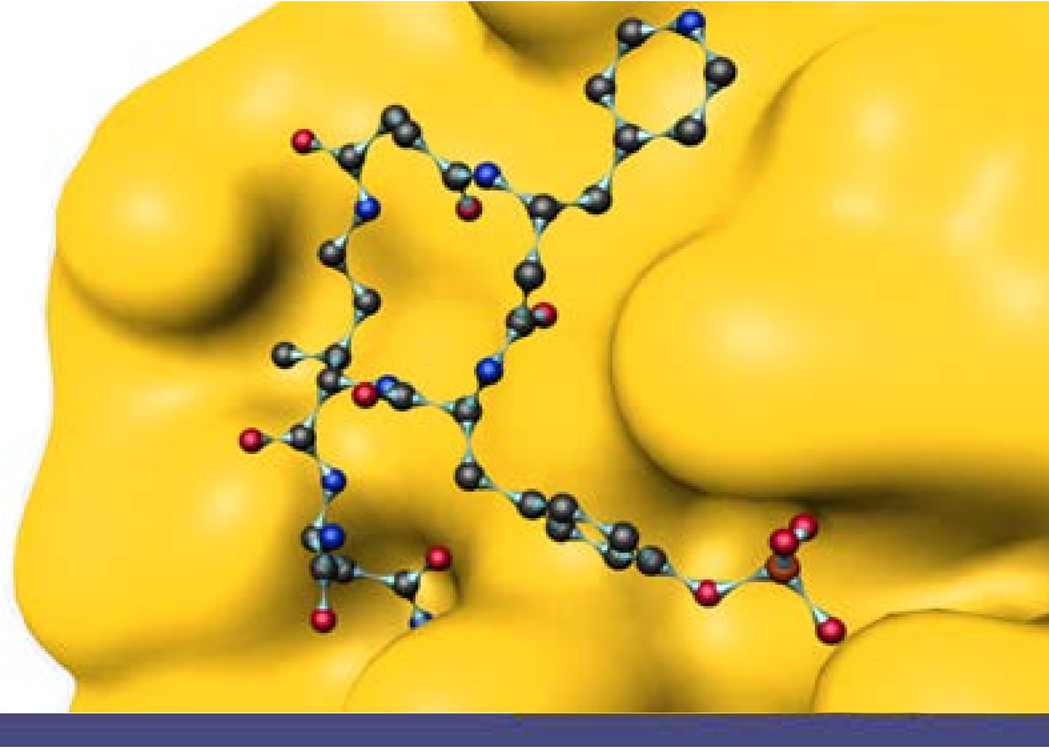


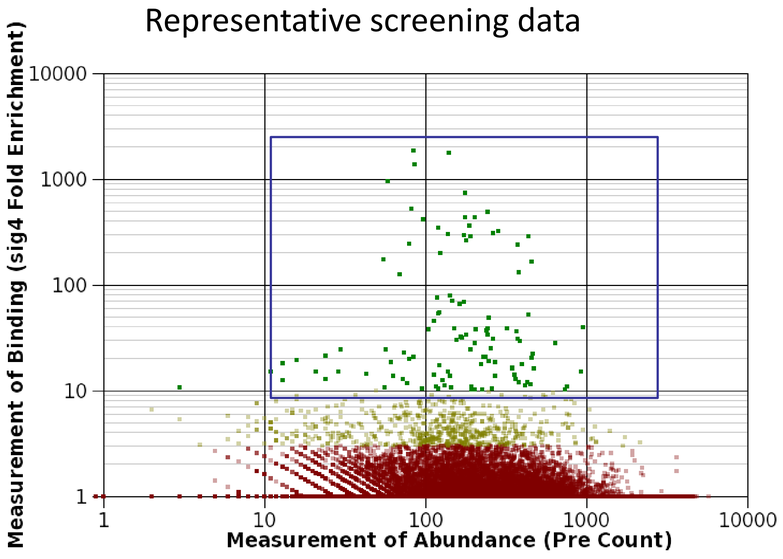
Recently, Ubiquitin Specific Peptidases (USPs), a family of deubiquitinating enzymes (DUB) have been shown to have a rich biology. They are responsible for the deubiquitination of proteins destined for proteasomal degradation. USPs play a part in developmental disorders, Parkinson's and Alzheimer's disease, and in human cancers. Since many cancers exploit normal cellular functions to escape apoptosis, targeting functions that interfere with these processes is an attractive therapeutic strategy.

The ubiquitin specific protease 9X (USP9x) is a substrate specific DUB. Mcl-1, an anti-apoptotic Bcl-2 protein, has been identified as a substrate for USP9x and both are overexpressed in many cancers, especially in those cancers that have developed resistance to chemotherapies. USP9x deubiquitinates Mcl-1, thus rescuing it from proteasomal degradation and the cancer cell from programed cell death. Knockdown of this DUB with shRNAi or blocking it with a USP9x inhibitor, reduced Mcl-1 levels and sensitized cells to chemotherapeutics.

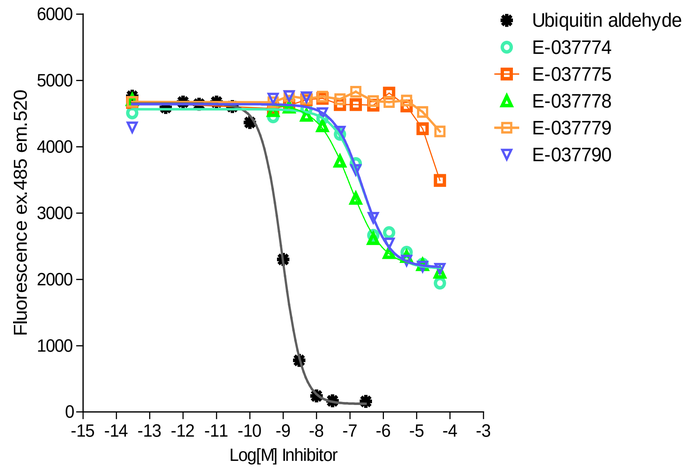
Libraries consisting of greater than 10 million macrocyclic compounds have been created using DNA-programed chemistry. These libraries are screened using *in-vitro* selection assays to identify compounds that bind to specific targets of interest. The Ensemble Therapeutics platform has been used successfully for the discovery of compounds that interact with a number of targets such as the oncology targets Bcl-xL and XIAP, and the inflammation target IL-17.

Screening our libraries against USP9x yielded many hits consisting of families of compounds that were structurally related, and specific to USP9x compared to other previously screened targets. Compounds from two of these families were synthesized and tested in a number of *in-vitro* assays such as ELISA, the DUB enzymatic assay, and SPR to determine potency, specificity and selectivity. Many of these compounds were found to bind specifically to USP9x in SPR, have sub-micromolar IC_{50}s in the USP9x enzymatic assay, and are up to 30 fold selective over the DUB, USP7. A representative compound was subsequently studied kinetically in the enzyme assay and determined to be a hyperbolic mixed type inhibitor.

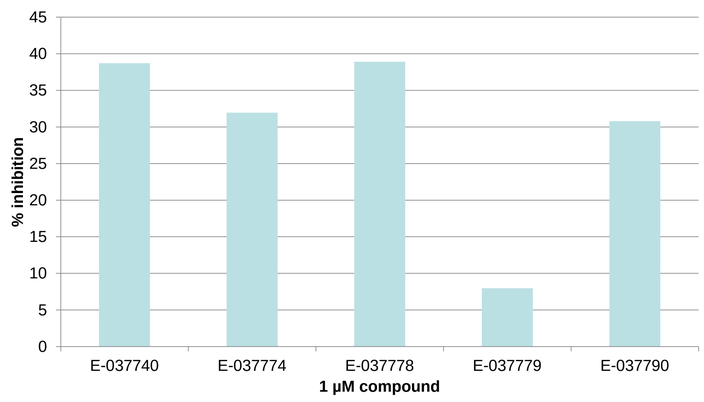
Representative screening data



- Libraries are designed to contain an full distribution of DNA tagged compounds.
- Sequencing results of selected libraries identified 'outliers' that are several fold more prevalent in the post selection sample than in the original library.
- Two families of compounds, AVVCB and BKXOC were chosen from separate selections, synthesized and tested in a number of *in-vitro* assays.

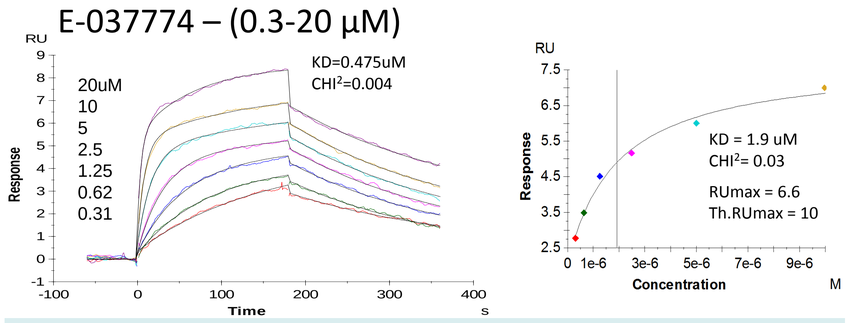


- Discrete compounds were found to inhibit USP9x hydrolysis of Ubiquitin-Rhodamine110.
- The AVVCB series demonstrated sub-µM IC_{50}s.
- The BKXOC series were less active and not studied further.
- Solubility and assay interference were ruled out as the reason for incomplete inhibition by the AVVCB series.

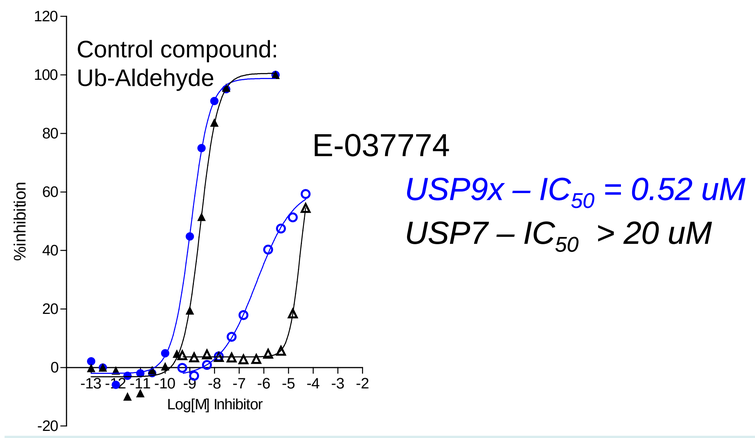


- Compounds inhibited USP9x binding to captured ubiquitin aldehyde in ELISA.
- As in the enzymatic assay, complete inhibition was not achieved and order of potency was maintained.

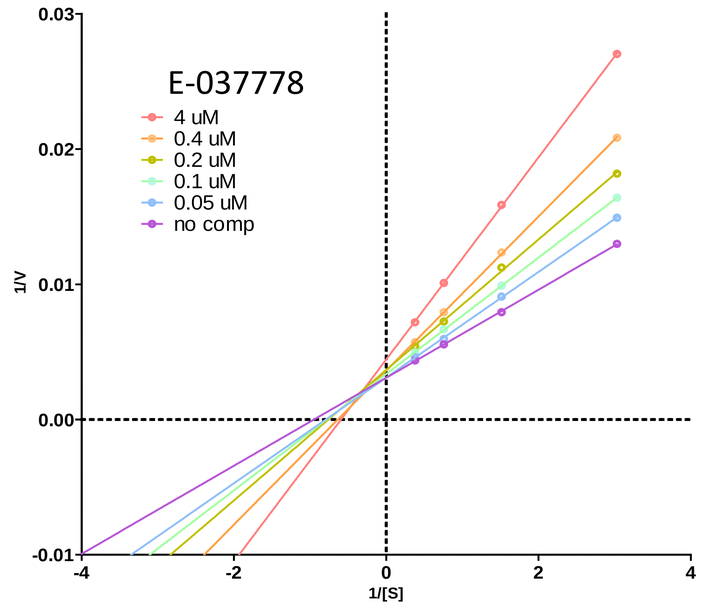
E-037774 – (0.3-20 µM)



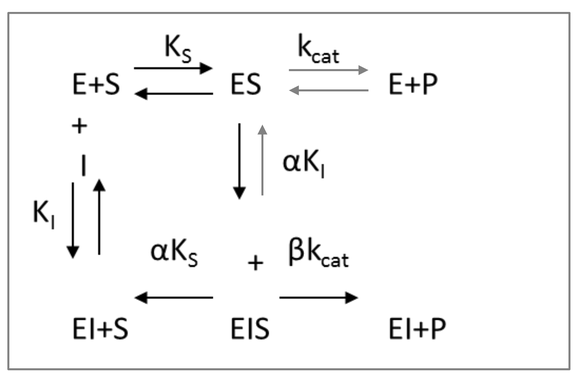
- Direct binding was determined by measuring surface plasmon resonance using a Biacore T200.
- E-037774, which has sub-µM IC_{50} in the enzymatic assay, demonstrates ideal binding and kinetics.
- Binding saturates close to the theoretical maximum and did not bind to a negative control protein.



- Hydrolysis of Ubiquitin-Rhodamine110
- E-037774 exhibits 40x selectivity for USP9x over USP7 when tested using the substrate K_m for each enzyme.



- To determine if the incomplete inhibition could be explained with a mechanistic model, substrate titrations, time course and inhibitor titrations were performed.
- Assuming rapid equilibrium, values for K_I, α and β were calculated from the data.



- Mechanism
  - If α =1 and β = 0, pure noncompetitive inhibition
  - If α = ∞, pure competitive inhibition
  - If 1 ≤ α ≤ ∞ and 0 ≤ β ≤ 1, hyperbolic mixed type inhibition
- Calculated parameters from kinetic data
  - β = 0.71, from the y intercept of the Δ intercept plot
  - α = 1.73, from the y intercept of the Δ slope plot
- The calculated values of the parameters α and β fit the definition of hyperbolic mixed inhibition
- K_I = 0.29 µM, determined from the x intercept of the Δ intercept plot
- K_I = 0.12 µM, determined from the x intercept of the Δ slope plot

Summary

Screening of our DNA-encoded libraries against USP9x yielded many hits consisting of families of compounds that were structurally related. One of these compounds, E-037774 was found to inhibit USP9x enzymatic activity with an IC_{50} of 210 nM, with incomplete inhibition. Biacore analysis revealed specific binding properties which correlate with enzymatic activity. Kinetic analysis showed that the compound is not directly competitive with ubiquitin and thus may offer more selectivity between DUBs.

ENSEMBLE THERAPEUTICS