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Discovery of Protease Inhibitors for New World Alphaviruses: Building in Broad Spectrum Activity Across Sequence Variants and Low Host Off-Target Binding

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PROJECT OVERVIEW

Strains of New World Alphaviruses (NWAs) Venezuelan (VEEV), eastern (EEEV), and western equine encephalitis virus (WEEV) can cause severe encephalitis in humans. NWAs are also important potential biothreats: They are stable in the environment and known to be weaponizable, and high titers achievable when growing NWAs in culture make production of large quantities feasible. There are currently no therapeutics against NWAs.

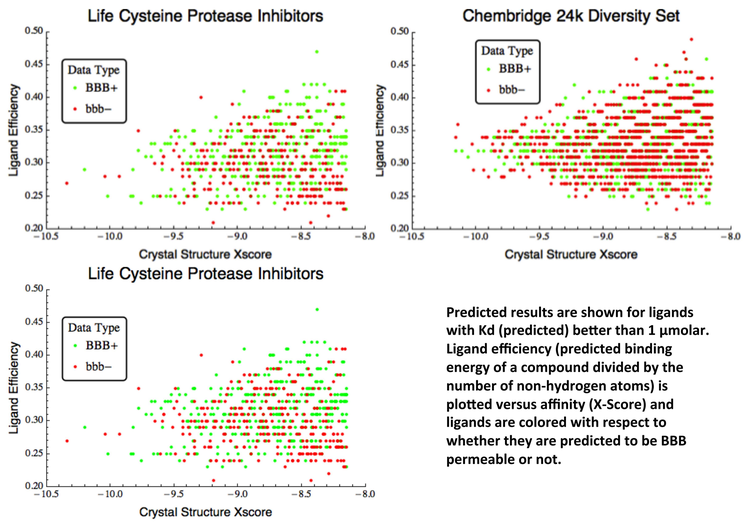
Most positive-strand RNA viruses encode a protease activity required during viral replication for processing viral preproteins into discrete structural and non-structural proteins. Protease inhibitors are highly effective antivirals against HIV and Hepatitis C, and could be useful NWA antivirals. Discovery of protease inhibitors using cell-based assays is challenging because of the high potential for inhibition of human proteases, which are often involved in viral pathogenesis, leading to false positive infectivity-inhibition assays. A high-resolution crystal structure is available for VEEV nsP2 protease domain (nsP2pro), making it a viable target for structure-based drug discovery.

We employed a two-pronged approach for discovery of nsP2pro inhibitors, both starting with biochemical screening of inhibitors of recombinant nsP2pro, which we have expressed and purified in mg quantities. In one prong, *in silico* screening of commercially-available chemical libraries identified drug-like compounds with high scores for binding to VEEV nsP2pro AND high predicted blood-brain barrier permeability, and selected compounds were purchased and screened for binding and inhibition of VEEV nsP2pro and off-target human proteases using mass spectrometry methods. In the other prong, we established an assay for nsP2pro inhibition, based on changes in substrate fluorescence resonance energy transfer (FRET) of a novel substrate, suitable for high-throughput screening (HTS). Short peptide substrates with fluorescein donor and rhodamine acceptor groups at either end are favored for screening HIV and HCV protease inhibitors, but do not work for NWA proteases, which require an extended peptide or protein substrate. Using fluorescent protein pairs with an unusually long FRET radius, we were able to construct a nsP2pro substrate that showed good cleavage kinetics and measureable dequenching of the donor fluorescent protein upon cleavage. We have used this novel assay system to perform high-throughput screening of ~45000 compounds at the UCLA screening facility.

In Silico Screening Methods and Results

We performed *in silico* docking (Autodock Vina, http://vina.scripps.edu/) of the crystal structure of VEEV nsP2pro (Watowich *et al*, Structure and Acta Cyrstall 2006) to three different commercially available libraries of drug-like compounds in order to select compounds predicted to bind to the nsP2pro active site: 1) 12 thousand highly diverse compounds from Life Chemicals; 2) ~ 20 thousand diverse compounds from Chembridge; and 3) 6615 cysteine protease inhibitor-like compounds from Life chemicals. Bound complexes were scored using X-Score (http://sw16.im.med.umich.edu/software/xtool/) and we selected compounds with predicted binding better than 10 μmolar Kd. Because we are seeking to treat encephalitic viruses, we also used a computational model to predict whether each of these compounds is blood brain barrier (BBB) permeable (http://b3pp.lasige.di.fc.ul.pt/).

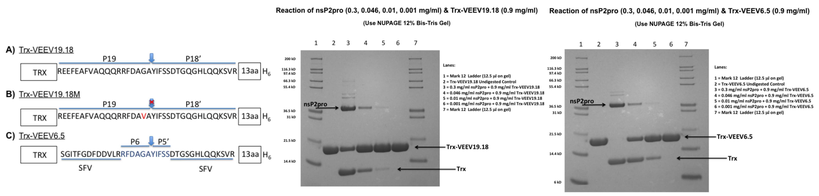
We identified 2690 BBB permeable high-activity compounds that had predicted wild-type VEEV DGbind ≤ –9.4 kcal/mol or ligand efficiency ≥ 0.30 kcal/mol/atom and were able to purchase 230 structurally diverse ligands from Life Chemicals and 189 from Chembridge .



Predicted results are shown for ligands with Kd (predicted) better than 1 μmolar. Ligand efficiency (predicted binding energy of a compound divided by the number of non-hydrogen atoms) is plotted versus affinity (X-Score) and ligands are colored with respect to whether they are predicted to be BBB permeable or not.

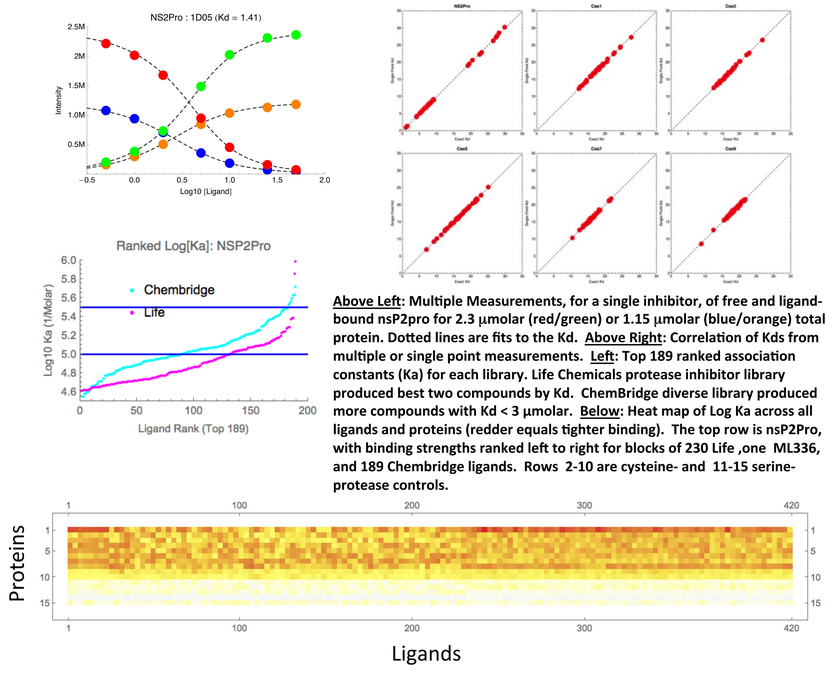
Enzyme and Substrate Production

The gene sequence of VEEV nsP2pro used in the crystal structure (Watowich et al, Structure and Acta Cyrstall 2006) was synthesized by a commercial vendor (Epoch Life Science) and expression for nsP2 in E. Coli performed after by Zhang et al (Protein Expres Purif. 2009). Multiple constructs were produced containing the nsP2pro target cleavage sequence in order to find a substrate that would cleave efficiently and also prove our expressed VEEV was active. Below left is a schematic diagram of Thioredoxin (TRX) fusion protein substrates for VEEV nsP2pro. (A) 50 amino acids following the TRX domain, 37 amino acids of which are derived from the VEEV p3/p4 processing site (P19-P18'). B) A noncleavable version of (A) with Glycine to Valine mutation at P2 (red). C) Construct using the P19-P18' of Simliki Forest virus (SFV) p3/p4 site with VEEV p3/p4 residues at positions P6-P5'. Gels at right show that the hybrid SFV/VEEV construct is completely cleaved by nsP2pro.



Mass Spectrometry Assays for Ligand-Enzyme Binding and Inhibition

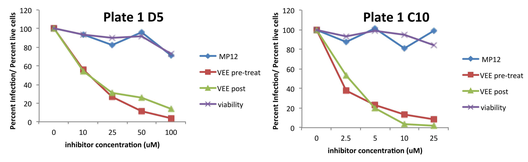
After experimentation with buffer conditions and ionization conditions, good native ESI/MS conditions were found for our purified nsP2pro and other off-target proteases, and these conditions worked as well for observing nsP2pro-substrate and protease-compound noncovalent complexes . (A Bruker APEX 7 Tesla Quadrupole-Fourier Transform Ion Cyclotron Resonance with Apollo Ion Source was used for all experiments.) Michaelis-Menten modeling of observed rates of product formation confirmed that the nsP2pro was enzymatically active (Km=36 μmolar) in our buffer conditions. Using ESI/MS of free and ligand-bound enzyme, we screening of solutions of nsP2pro with each of the 230 Life Chemicals compounds identified compounds at different concentrations of protein and ligand and calculated precise Kds. Subsequently we determined that dissociation constants (Kds) could be measured from only a single ligand: protein ratio (10 μmolar: 2.3 μmolar) within 1% accuracy (compared to multipoint Kd), and we measured the Kd of 420 compounds (230 Life Chemicals and 189 Chembridge compounds plus one published nsP2Pro ligand ML336 (Schroeder, *et. al., J. Med Chem.* 2014)) against nsP2pro as well as 9 off-target human cysteine proteases (Cas1, Cas3, Cas5, Cas7, Cas9, CatB, CatD, CatF, CatH) and 5 serine proteases (Lactoferin, PRSS3, PRSS8, GranzymeB, PRS45) purchased from Sigma. Compounds identified to bind strongly to nsP2pro (better than ~10 μmolar) were always found to preferentially bind it over the other kinases, showing that *in silico* screening did not produce non-selective/promiscuous binders.



Above Left: Multiple Measurements, for a single inhibitor, of free and ligand-bound nsP2pro for 2.3 μmolar (red/green) or 1.15 μmolar (blue/orange) total protein. Dotted lines are fits to the Kd. Above Right: Correlation of Kds from multiple or single point measurements. Left: Top 189 ranked association constants (Ka) for each library. Life Chemicals protease inhibitor library produced best two compounds by Kd. ChemBridge diverse library produced more compounds with Kd < 3 μmolar. Below: Heat map of Log Ka across all ligands and proteins (redder equals tighter binding). The top row is nsP2Pro, with binding strengths ranked left to right for blocks of 230 Life ,one ML336, and 189 Chembridge ligands. Rows 2-10 are cysteine- and 11-15 serine-protease controls.

Toxicity and Infectivity Assays

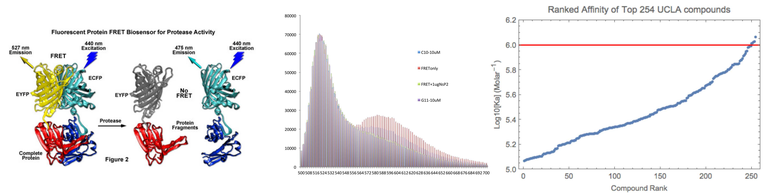
We obtained Venezuelan Equine Encephalitis Virus, TC-83, NR-63 from BEI resources and implemented viral infectivity assays in Hela cells. As a control for non-specific replication inhibition, we used the vaccine strain MP12 for Rift Valley fever Virus. We performed dose dependence studies on 33 compounds of inhibition of infection as well as cell viability assays using vital dye uptake. Compounds were added either before or after viral infection in order to screen for external blockade of viral entry.



Inhibition of infection and toxicity results from the two best compounds from *in silico* screening. Compounds 1C10and 1D05 had, respectively, Kds of 1 and 1.4 μmolar , Kis of 1.4 and 1.7 μmolar, IC50 of 3 and 10 μmolar , and 80% viability at 25 and 100 μmolar. Inhibition of MP12 replication was absent.

High-Throughput Screening Assay Using A Novel Fluorigenic Substrate

High throughput screening of large libraries in academic or industrial screening centers requires assays suitable for robotic handling and common methods of concentration measurement such as fluorescence. We designed several substrates for nsP2pro where an extended peptide sequence connects a fluorescent resonance energy transfer (FRET) donor and acceptor pair. After testing FRET pairs with a large Forster radius and optimizing the substrate sequence, we were able to create a substrate construct that showed good donor fluorescence increase and reduced acceptor FRET upon cleavage, and could be expressed well. Using the UCLA-DOE Protein Expression Technology Center we were able to express >1 gram of FRET substrate and sufficient nsP2pro to screen 43838 compounds selected from the UCLA molecular screening shared resource (MSSR) chemical libraries. ESI/MS methods were used to measure the Kds for the 254 compounds that showed the largest reduction in nsP2pro activity (smallest fluorescence ratio changes). Five Kds were <1 μmolar and 48 < 2 μmolar.



Above Left: Notional structure of fluorigenic protease substrate. Above Middle: Fluorescence spectrum (490 nm Ex) of nsP2pro substrate in the presence of no inhibitor or enzyme (red), enzyme (green), 10 μmolar of a 1 μmolar Kd inhibitor (blue), or ineffective inhibitor (purple). Effective inhibitors reduce the loss of FRET signal. Above Right: Top 254 ranked association constants (Ka) for HTS hits, based on ESI/MS measurements.

CONCLUSIONS AND FUTURE DIRECTIONS

We have implemented a novel set of technologies for discovery of small-molecule inhibitors of alphavirus proteases. In combination, our methods enable

- High throughput screening of very large libraries of compounds
- Precise measurements of compound Kd at moderate throughput
- Screening for off-target binding early in discovery

In silico methods yielded only one compound (out of 419 screened experimentally) with 1 μmolar Kd, while HTS yielded 5 with Kd < 1 μmolar (out of 43838 screened experimentally), an enrichment of about 20-fold, though *in silico* screening did have high selectivity and hits with promising toxicity and in vivo (cell-based) activities.

We have recently implemented a HTS assay for WEEV based on our VEEV design. Further funding is needed to characterize our VEEV hits and perform a comprehensive structure-activity analysis, and to determine their spectrum of activity against other NWAs, or to perform additional HTS screens.

ACKNOWLEDGEMENTS

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