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Identification of Broad Spectrum Inhibitors of Alphaviruses Using High-Throughput Screening

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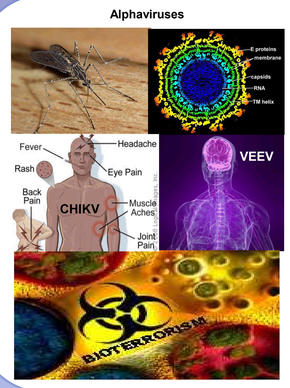
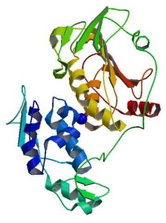
Introduction



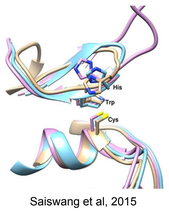
Alphaviruses are arthropod borne (+)-strand RNA viruses that cause a number of human and animal diseases worldwide. Infection with alphaviruses may result in serious human morbidity and mortality, with symptoms including encephalitis, arthritis, rash, and fever. New World alphaviruses (NWA), are distributed across the Americas, cause debilitative, acute and sometimes fatal encephalitis and include: Eastern equine encephalitis virus, Western equine encephalitis virus and Venezuelan equine encephalitis virus (VEEV). VEEV is considered a potential biological weapon and is identified as a Category B pathogen by the National Institute of Allergy and Infectious Disease (NIAD). The Old World alphavirus, Chikungunya Virus (CHIKV), first identified in West Africa in 1952, has been responsible for a number of disease outbreaks in Africa, Asia, Europe, and the Indian and Pacific Oceans. In 2013, the first locally-acquired case of CHIKV in the Americas was reported in the Caribbean. Since then, there have been more than 1.7 million suspected cases in the Americas, and the outbreak is still spreading. CHIKV infection has a rapid onset, characterized by fever and severe arthralgia, which in many cases develops into a debilitating, chronic arthritis that persists for several months or even years. There are no vaccines or available treatment options to combat VEEV or CHIKV infection, and there is a pressing need to develop rapid, therapeutic intervention.

The alphavirus non-structural protein 2 (nsP2) is a critical protease

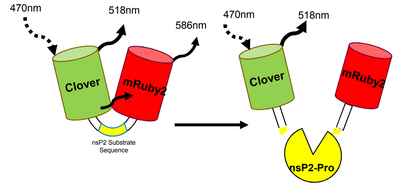
Crystal structure of the Chikungunya virus nsP2 protease (PDB ID: 3TRK).



Superposition of the 3 currently available alphavirus nsP2 protease active sites. Tan is CHIKV nsP2 (PDB ID: 3TRK), blue is VEEV nsP2 (PDB ID: 2HWK) and purple is Sindbis virus (SINV) nsP2 (PDB ID: 4GUA).



A Clover and mRuby2 fluorescence resonance energy transfer (FRET) protein designed using the nsP2-substrate sequence as the protein linker. Excitation of Clover results in FRET-excitation of both Clover and mRuby2 (left). nsP2 protease activity cleaves the linker between the fluorescent proteins, resulting in loss of FRET-induced excitation of mRuby2 (right).

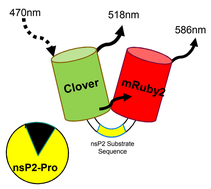


Saiswang et al, 2015

The alphavirus nonstructural protein 2 (nsP2pro) is an attractive anti-viral target, since its proteolytic processing of the nonstructural polyprotein into functional proteins, is required for viral replication. In addition, the nsP2pro substrate has a defined sequence and availability of the nsP2pro crystal structure for VEEV and CHIKV allows rational structure-based drug design. Moreover, protease inhibitors have played a key role in combating the HIV pandemic, and more recently, in treatment advances for hepatitis C virus.

FRET as a biosensor for nsP2 protease activity

nsP2-specific inhibitors result in continued FRET-excitation of mRuby2



UCLA's MSSR has libraries of small-molecule inhibitors and robotics for automated, high-throughput screening.

A single representative plate

<>	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0.964	1.008	1.537	1.582	1.523	1.360	1.543	1.524	1.511	1.585	1.523	1.399	1.509	1.517	1.580	1.500	1.504	1.537	1.502	1.551	1.485	1.536	1.570	1.595
B	0.978	0.982	1.567	1.233	1.573	1.677	1.582	1.412	1.607	1.597	1.766	1.431	1.587	1.526	1.501	1.414	1.491	1.558	1.539	1.619	1.361	1.653	1.602	1.583
C	0.973	0.989	1.607	1.580	1.657	1.599	1.598	1.523	1.613	1.586	1.553	1.502	1.504	1.552	1.534	1.577	1.624	1.598	1.034	1.569	1.605	1.626	1.583	1.564
D	0.977	1.017	1.590	1.325	1.635	1.489	1.610	1.417	1.572	1.592	1.339	1.406	1.537	1.435	1.553	1.110	1.432	1.629	1.578	1.612	1.454	1.601	1.568	1.609
E	0.975	0.999	1.628	1.602	1.566	1.572	1.633	1.622	1.630	1.596	1.377	1.662	1.600	1.636	1.540	1.578	1.585	1.593	1.651	1.625	1.621	1.633	1.588	1.609
F	1.028	1.012	1.639	1.469	1.595	1.537	1.609	1.601	1.598	1.292	1.480	1.540	1.654	1.480	1.215	1.613	1.510	1.601	1.217	1.652	1.444	1.693	1.626	1.615
G	0.986	1.004	1.583	1.602	1.570	1.604	1.585	1.627	1.597	1.602	1.313	1.620	1.515	1.615	1.468	1.584	1.608	1.600	1.617	1.616	1.545	1.602	1.655	1.601
H	0.996	1.031	1.596	1.486	1.637	1.429	1.583	1.421	1.558	1.602	1.621	1.514	1.237	1.605	1.399	1.617	1.510	1.622	1.576	1.568	1.397	1.705	1.585	1.615
I	1.007	0.996	1.591	1.559	1.479	1.633	1.618	1.575	1.611	1.571	1.096	1.554	1.618	1.634	1.538	1.564	1.604	1.567	1.620	1.646	1.700	1.643	1.608	1.633
J	0.980	1.030	1.563	1.124	1.625	1.376	1.650	1.407	1.546	1.575	1.536	1.564	1.647	1.574	1.702	1.657	1.462	1.646	1.524	1.554	1.484	1.640	1.635	1.585
K	1.011	1.002	1.581	1.589	1.583	1.591	1.646	1.568	1.591	1.575	1.229	1.583	1.669	1.582	1.590	1.534	1.575	1.622	1.603	1.595	1.639	1.577	1.627	1.615
L	0.992	1.029	1.576	1.431	1.596	1.323	1.634	1.606	1.346	1.563	1.419	1.630	1.430	1.582	1.582	1.069	1.382	1.605	1.390	1.651	1.443	1.530	1.564	1.602
M	0.998	1.005	1.599	1.579	1.588	1.557	1.451	1.509	1.597	1.557	1.578	1.588	1.387	1.594	1.588	1.582	1.557	1.608	1.623	1.609	1.612	1.600	1.625	1.595
N	1.000	1.030	1.697	1.440	1.571	1.455	1.596	1.579	1.549	1.602	1.542	1.623	1.509	1.411	1.167	1.410	1.204	1.598	1.218	1.613	1.442	1.511	1.561	1.579
O	0.976	1.005	1.556	1.544	1.553	1.525	1.567	1.577	1.549	1.503	1.568	1.573	1.548	1.483	1.556	1.532	1.580	1.562	1.559	1.583	1.560	1.543	1.571	1.572
P	1.008	1.013	1.554	1.317	1.476	1.334	1.461	1.395	1.513	1.512	1.530	1.464	1.313	1.441	1.437	1.381	1.224	1.602	1.274	1.590	1.404	1.538	1.598	1.603

To discover inhibitors of nsP2pro with broad spectrum efficacy and low affinity for human proteases we developed novel FRET-based high throughput assays to monitor substrate cleavage. The assay was initially developed for VEEV nsP2pro for DTRA CBM under CB4037, CB-SEED-SEED09-2-0050. and was adapted for CHIKV nsP2pro. The assays for VEEV and CHIKV were used to screen 50,000 and 40,000 compounds, respectively, at the UCLA molecular shared screening center. Screening for CHIKV nsP2pro inhibitors was done in 384-well plate formats, allowing the testing of 320 small-molecule compounds per plate. Relative emission ratios of Clover and mRuby2 are used to quantify the level of nsP2-mediated cleavage. Fluorescence ratios of Clover to mRuby2 are measured after 20 hours, and are shown in a color-coded heat-map. Values were normalized to the negative controls (red, columns 1 and 2). In this image, compounds resulting in greater than 90% inhibition of cleavage (relative to the positive controls, columns 23 and 24) are shown bolded and boxed. Down-selected inhibitors were then tested for cell permeability, toxicity, specificity and effectiveness against viral infection. From these screens we identified six compounds, two that specifically block VEEV infection (figure 1), four that block CHIKV (figure 3-5), three of which are also effective against VEEV (figure 5), and one compound that was effective against a diverse set of viruses, including bunyaviruses, flaviviruses and alphaviruses without being cytotoxic (figure 5). This compound screening pipeline can be quickly adapted to new natural or synthetic threats, and offers a rapid and high-throughput way to find both specific and broad spectrum therapeutic compounds.

Inhibitors that Decrease VEEV Infection with Minimal Cell Toxicity*

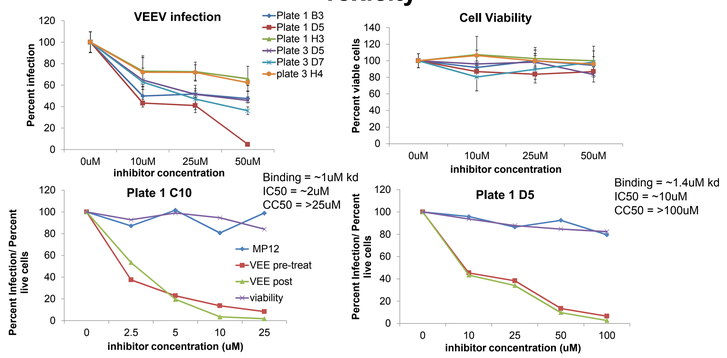


Figure 1. (A) HeLa cells were pretreated with indicated inhibitors for 1 h prior to and during infection with VEEV TC-83 (MOI 1). 24 hours after infections cell viability was measured (upper right panel) then cells were fixed and stained with anti-VEEV antibodies. DMSO treated and infected samples were treated as 100% infected (upper left panel). (B) Two Inhibitors Specifically and Effectively reduce VEEV Infection by Blocking Activity of nsP2. For pretreatment samples (pre-treat) inhibitors were added at indicated concentrations for 1 h prior to and during 3 h infection, then virus and inhibitors were removed for overnight incubation. Alternatively, untreated cells were incubated with indicated viruses (MOI 1) for 1 h, washed with PBS to remove unbound virus, then incubated with inhibitors in complete medium overnight. RVFV-GFP was included as a control for specificity. To account for differences in cell number from well to well, GFP/488 fluorescence was normalized to DAPI fluorescence on a well-to-well basis and the background value (uninfected cells) was subtracted. The percentage was determined by dividing the average of the treated and infected samples by the average of untreated or DMSO treated and infected samples. Shown are the means for 3 independent experiments performed in triplicate.

*Funding was provided by DTRA CBM under CB4037, CB-SEED-SEED09-2-0050.

320 Inhibitors Decrease CHIKV Substrate Cleavage by Greater than 80%

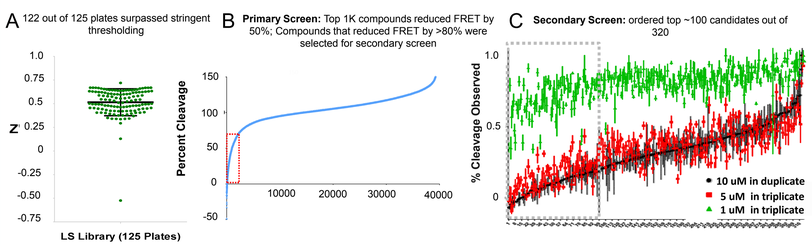
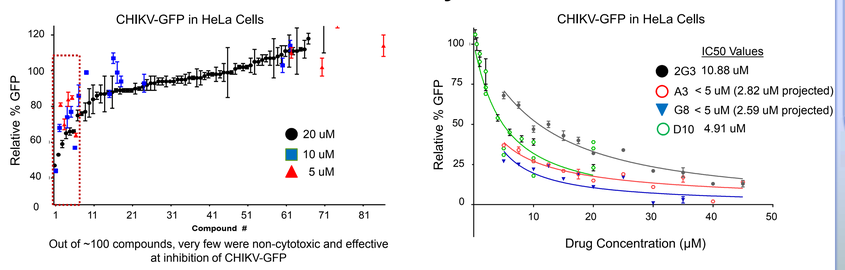


Figure 2 (A) 125 plates containing 40,000 unique compounds were used in high-throughput screening. Screen quality was assessed by Z' scoring of the 125 plates (right). The black bars represent mean and standard deviation. A Z' factor above 0.5 is considered "excellent" and represents very-high confidence. Compounds from assay plates scoring below a 0.3 threshold (dotted line) were filtered out. (B) Changes in the Clover:mRuby2 fluorescence ratios were normalized against DMSO-treated controls, and plotted as a percentage of relative cleavage. The top 1,000 compounds (red box, lower) reduced FRET emission to below 50% of controls, indicating inhibition of nsP2pro cleavage activity. (C) 320 candidate compounds from the initial screen were chosen for secondary screening and arrayed into a single plate. These compounds were then assessed at three different concentrations: 10uM in duplicate, 5uM in triplicate, or 1uM in triplicate. Results are plotted as percentage cleaved relative to DMSO-treated controls based on FRET fluorescence ratios. The top compounds (gray square) were chosen for subsequent analyses.

Inhibitors Decrease CHIKV-GFP Infection with Minimal Cell Toxicity



Out of ~100 compounds, very few were non-cytotoxic and effective at inhibition of CHIKV-GFP

Figure 3. To facilitate initial screening of compounds for anti-viral efficacy in cells we generated a CHIKV 181/25 reporter virus that expresses GFP upon entry and replication (A) Top 100 compounds were screened against CHIKV-GFP reporter virus, at 5, 10, or 20uM concentrations. Compounds inhibiting CHIKV-GFP to below DMSO-treated controls at 20uM are plotted relative to inhibitory effect. (B) Four compounds, designated A3, G8, D10, and 2G3, significantly and reproducibly reduced virus-induced GFP fluorescence in HeLa cells. The anti-viral efficacy at a variety of concentrations was measured for these four compounds in HeLa cells to generate an IC50 dose response curve for each inhibitor. The IC50 of each compound was determined using a nonlinear regression curve fitting, using a [inhib] vs normalized response w/variable slope analysis. Compound efficacy was assessed in triplicates per dosage, GFP values were normalized against DMSO-treated controls, and a curve was fit to calculate IC50 values.

Four Compounds Significantly Decrease CHIKV Infection in Multiple Cell Types as Measured by GFP Expression and Protein Expression

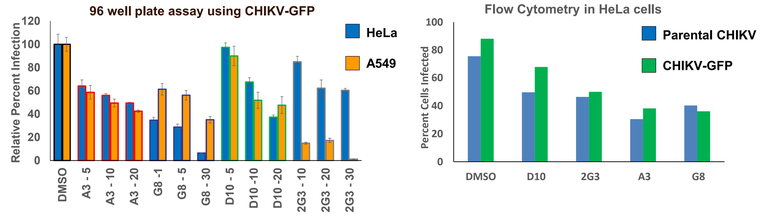


Figure 4. (A) HeLa (blue bars) or A549 cells (orange bars) were treated with indicated concentrations of inhibitors for 1 h prior to infection, during 3 h incubation with CHIKV-GFP (MOI of 1), and during overnight incubation. The relative percent infection was determined by taking DMSO-treated and infected samples as 100%. Three independent experiments were performed in triplicate. Data are presented as means ±SD. (B) HeLa cells were treated with 20μM DMSO, 20μM D10, 20μM 2G3, 20μM A3, or 10uM G8 for 1 h prior to and during infection with MOI = 3 of parental CHIKV (blue bars) or CHIKV-GFP (green bars). The percent cells infected was measured by flow cytometry using mouse anti-CHIKV monoclonal antibody (blue bars) or GFP expression (green bars). The data shown are representative results from three similar experiments.

Three Inhibitors Decrease CHIKV and VEEV titers and One Compound, G8, was Effective Against a Diverse Set of Viruses

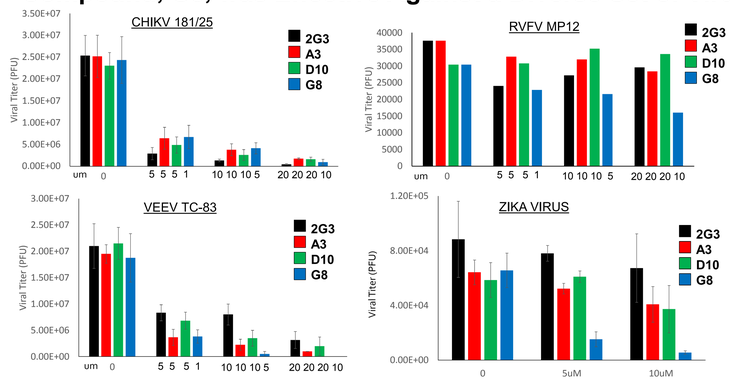


Figure 5. HeLa cells were incubated indicated inhibitors for 1 h prior to and during viral infection (MOI 1). 2G3, A3, and D10 were used at 5, 10 and 20 μM, while G8 was used at 1, 5, and 10 μM for infections with CHIKV 181/25, VEEV TC-83, and Rift Valley fever virus (RVFV) strain MP12 (RVFV-MP12). For Zika virus infections, all compounds were used at 5 and 10 μM. Supernatants were collected at 24 hpi, and viral titers were measured by plaque assay on Vero cells. Zika virus and RVFV were included to characterize the spectrum of inhibition of these compounds. Zika virus belongs to the Flaviviridae family, and alphaviruses, Zika virus has a positive strand genome that contains a protease for post-translational processing of the polyprotein. RVFV is a member of the Bunyaviradae family with a negative strand RNA segmented genome that does not encode a protease.

Summary and Conclusions

In conclusion, we've developed an in vitro screening system that's readily amenable for large scale high-throughput screening of anti-viral compounds. Using the alphavirus nsP2pro, we screened 50,000 compounds against VEEV nsP2pro and 40,000 compounds against CHIKV nsP2pro and identified dozens of candidate anti-viral compounds that show some efficacy in cell culture. Four of these compounds were characterized in a variety of assays, showing reduction in viral protein levels, reduced cellular effects, and a pronounced reduction of viral titers. Although CHIKV nsP2 has a longer substrate sequence than VEEV nsP2, the crystal structure for both protease domains has been resolved and superposition of the two indicates that these proteases have a highly conserved tertiary structure despite only having an amino acid sequence identity of 42%. Thus, it is not surprising that the compounds that strongly inhibit CHIKV nsP2 also appear to have efficacy against VEEV in cell culture. The finding that one compound, G8, worked on additional viruses such as Zika, and particularly Rift Valley Fever Virus is unexpected, owing to the distance between Bunyaviruses and Alphaviruses and the lack of a viral protease. As G8 showed varied efficacy amongst different cell types (data not shown), it's possible that the anti-viral properties being observed are due to some off-target effects or interactions. In the time of addition assay (data not shown), G8 was the only compound to show consecutively reduced levels of efficacy at later timepoints, suggesting a role in either early viral replication or cellular entry. Further, detailed characterization is required to better understand whether G8 acts in a host-directed manner, thus enabling a broader anti-viral effect. As compounds were screened against nsP2 protease, resistance mutation mapping should occur to better assess the suitability of these compounds for long-term therapeutic treatment options. Additionally, combinatorial treatments should be assessed, to determine whether any of these compounds can act in concert with each other to synergistically enhance anti-viral efficacy. Compounds that are additively synergistic to can be administered at lower dosages, which may enhance suitability for therapeutic application and reduce off-target effects in vivo. Future studies are aimed at utilizing animal models of both CHIKV and VEEV, in parallel with *in silico* lead compound optimization studies, to assess anti-alphavirus efficacy *in vivo*.

Acknowledgments

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