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Identification of Small-Molecule Inhibitors of Chikungunya Virus Using High-Throughput Screening

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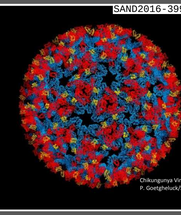
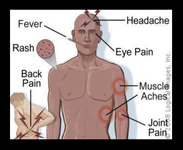


Abstract

Alphaviruses are arthropod borne (+)-strand RNA viruses that cause a number of human and animal diseases worldwide. Alphavirus infections may result in serious human morbidity and mortality, with symptoms including encephalitis, arthritis, rash, and fever. 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 debilitating, chronic arthritis that persists for several months or even years. There is a pressing need to rapidly develop therapeutic interventions, as there are no vaccines or treatment options available to combat CHIKV infection. The alphavirus nonstructural protein 2 (nsP2) is an attractive anti-viral target, as nsP2 proteolytically processes the nonstructural polyprotein into functional proteins, which is required for viral replication. The CHIKV nsP2 protease has a defined substrate sequence, and additionally, the availability of the protease's crystal structure allows for rational, structure-based drug design. To identify inhibitors of the viral nsP2 protease, we've developed a novel FRET-based high throughput assay to monitor substrate cleavage. This assay was used in March for an initial screen of 40,000 compounds at UCLA's Molecular Shared Screening Resource (MSSR) Center. We are currently awaiting production and shipment of small molecule compounds to be used for secondary assessment of the inhibitors' cell permeability, toxicity, and effectiveness against viral infection. Effective compounds will ultimately be transitioned to animal infection models for *in vivo* efficacy analyses. This screening system can be quickly adapted to multiple virus models, and offers a rapid and high-throughput way to find both specific and broad spectrum anti-viral compounds.

Chikungunya virus: impact and geographic range



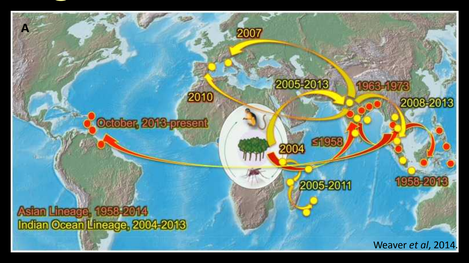
- CHIKV Infection: Rapid onset
- Transmitted by *Aedes aegypti* and *Aedes albopictus*
- Category C biodefense priority
- No licensed vaccines
- No current therapeutic treatments
- Rapidly emerging threat

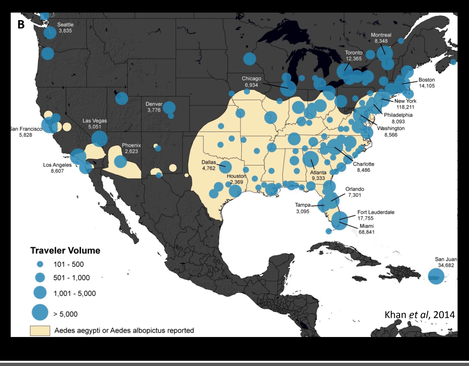
CDC: Countries and territories where cases have been reported *(through October 20, 2015)*

- There have been several recent outbreaks (Fig. A)
- **More than 1.7 million suspected cases** in 45 American countries or territories since the 2013 outbreak.
- **253 CHIKV related deaths** reported in the Americas

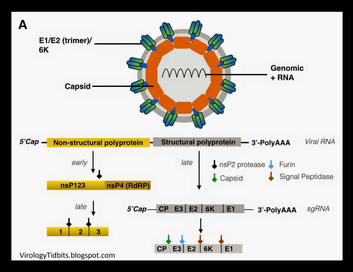
In the United States *(up through January 12, 2016)*

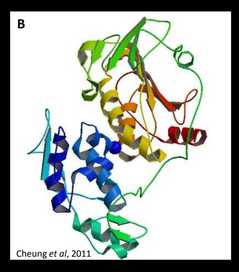
- 679 disease cases in 44 US states from travelers.
- 202 locally-transmitted cases (Puerto Rico and the US Virgin Islands)
- High volume travel (over one million people annually, Fig. B) to newly endemic regions greatly increase the risk of Chikungunya becoming endemic to the United States

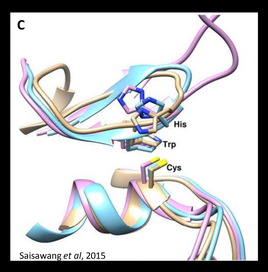




The alphavirus non-structural protein 2 (nsP2) is a critical protease





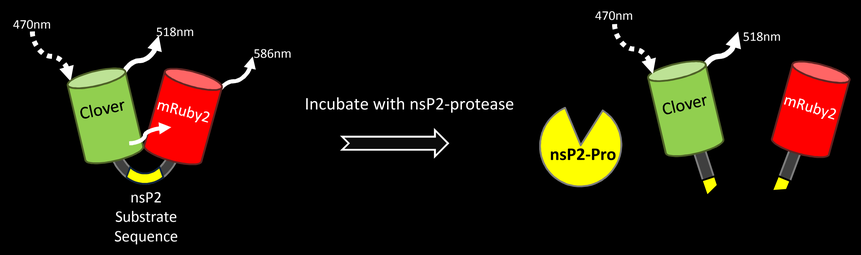


(A) Simplified representation of an Alphavirus virion, genome, and proteins. The virus must process the non-structural polyprotein into mature proteins using the nsP2 protease.

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

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

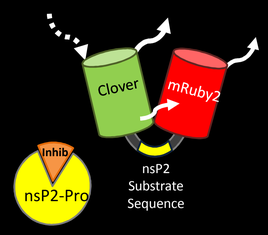
FRET as a biosensor for nsP2 protease activity



A Clover and mRuby2 FRET (fluorescence resonance energy transfer) protein was designed using the CHIKV nsP2-substrate sequence as the protein linker. Excitation of Clover results in FRET-excitation of both Clover and mRuby2 (left). CHIKV nsP2 protease activity cleaves the linker between the fluorescent proteins, resulting in loss of FRET-induced excitation of mRuby2. Relative emission ratios of Clover and mRuby2 are used to quantify the level of nsP2-mediated cleavage.

High-throughput screening of small molecule libraries

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



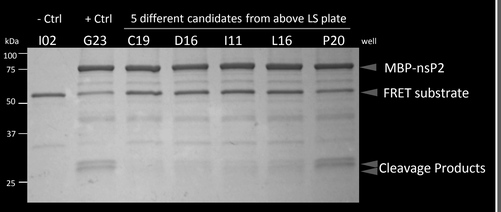
UCLA's MSSR has libraries of small-molecule inhibitors and robotics for automated, high-throughput screening

Representative results from high-throughput screening

<>	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

(above) Screening for CHIKV nsP2-Protease inhibitors was done in 384-well plate formats, allowing the testing of 320 small-molecule compounds per plate. 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.

(right) A major advantage of our FRET assay is that the readout is not transient, but instead very stable *in vitro,* and can be used to assess relative cleavage independently of fluorescence measurements. The above plate was re-read after 10 days, and the indicated wells were run on a protein gel and visualized by Coomassie staining to assess relative abundance of cleavage products.



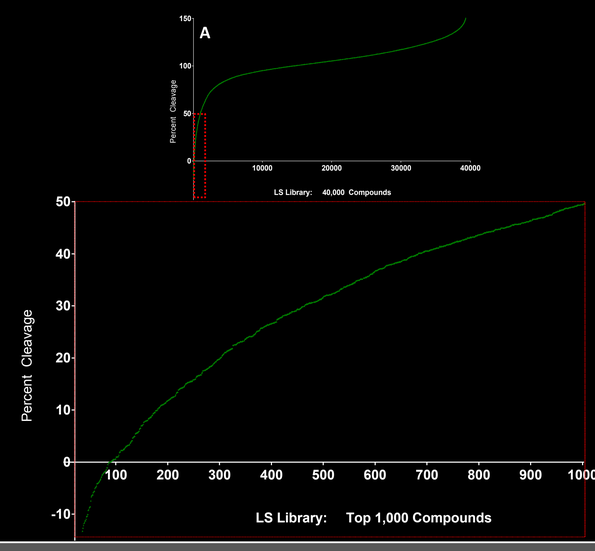
Cleavage percentage as calculated by fluorescence ratios (at day = 10)

0% 100% 6% 19% 27% 12% 95%

HTS identifies over 300 strong inhibitors of CHIKV nsP2-Protease

(A) The vast majority of the 40,000 compounds show no obvious inhibition (top). The top 1,000 compounds (red box) show greater than 50% inhibition of FRET cleavage. Compounds scoring greater than 80% inhibition were chosen for synthesis and secondary screening.

(B) The Z' scoring of the screen, compromised of 125 384-well plates. The white 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.



Summary and Future Directions

- Created a FRET-based assay for measuring activity of CHIKV nsP2-Protease
- High-throughput screening of 40,000 compounds
- Identified nearly 300 high-confidence inhibitors of nsP2-mediated cleavage

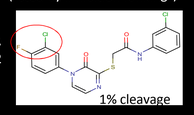
- We previously did a similar screen against another alphavirus, VEEV (Venezuelan Equine Encephalitis Virus). Many inhibitors identified were in common to both (right).

- In parallel efforts, we are using the nsP2 crystal structures to explore structure-activity relationships via computational analyses. These analyses can highlight key molecular features, and may be used to help with rational drug design and modifications to current compound candidates, especially when data for closely-related compound analogues are available (right, red circles)

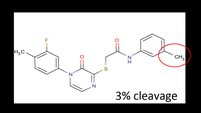
- Currently, we are getting compounds synthesized for follow up studies. This will include assaying compounds for cytotoxicity and cell permeability, effectiveness against virus infections in cell culture, and ultimately move to animal-infection models.

Three example compounds
(listed by inhibition rankings)

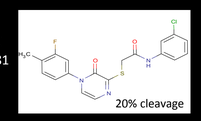
Candidate A
- CHIKV # 72
- VEEV # 7



Candidate B
- CHIKV # 91
- VEEV # 71



Candidate C
- CHIKV # 281
- VEEV # 11



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