Supporting Information

Title:

Small molecules targeting the flavivirus E protein with broad-spectrum activity and antiviral efficacy *in vivo*

Authors:

Pi-Chun Li[b,*], Jaebong Jang[b,*], Chih-Yun Hsia[a], Patrice V. Groomes[a], Wenlong Lian[a], Melissanne de Wispelaere[a], Jared D. Pitts, Jinhua Wang[b], Nicholas Kwiatkowski[b], Nathanael S. Gray[b], Priscilla L. Yang[a,+]

Affiliations:

[a] Department of Microbiology, Blavatnik Institute, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115.

[b] Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School and Department of Cancer Biology, Dana-Farber Cancer Institute, 360 Longwood Avenue, Boston, MA 02215.

* contributed equally

[+] to whom correspondence should be addressed: priscilla_yang@hms.harvard.edu

Supporting Information Table of Contents

Figure S1. The cyanohydrazones inhibit multiple flaviviruses.
Structures of compounds 3-149-3 and 3-149-15 are shown above their respective graphs. The antiviral activities of 3-149-3 and 3-149-15 against DENV2, ZIKV, and JEV were evaluated in the infectivity assay as outlined in Fig. 1A. Single-cycle viral yields were quantified by focus-forming assay for DENV2 and plaque-forming assays for ZIKV and JEV. Yields were normalized to the DMSO negative control. Representative data from one of $n \geq 2$ independent experiments are shown with error bars indicating standard deviation for two replicates within that experiment. The IC_{90} value presented on the graph is the average and standard deviation of the two independent experiments. Detailed description of cell lines and procedures are provided in Supporting Information.

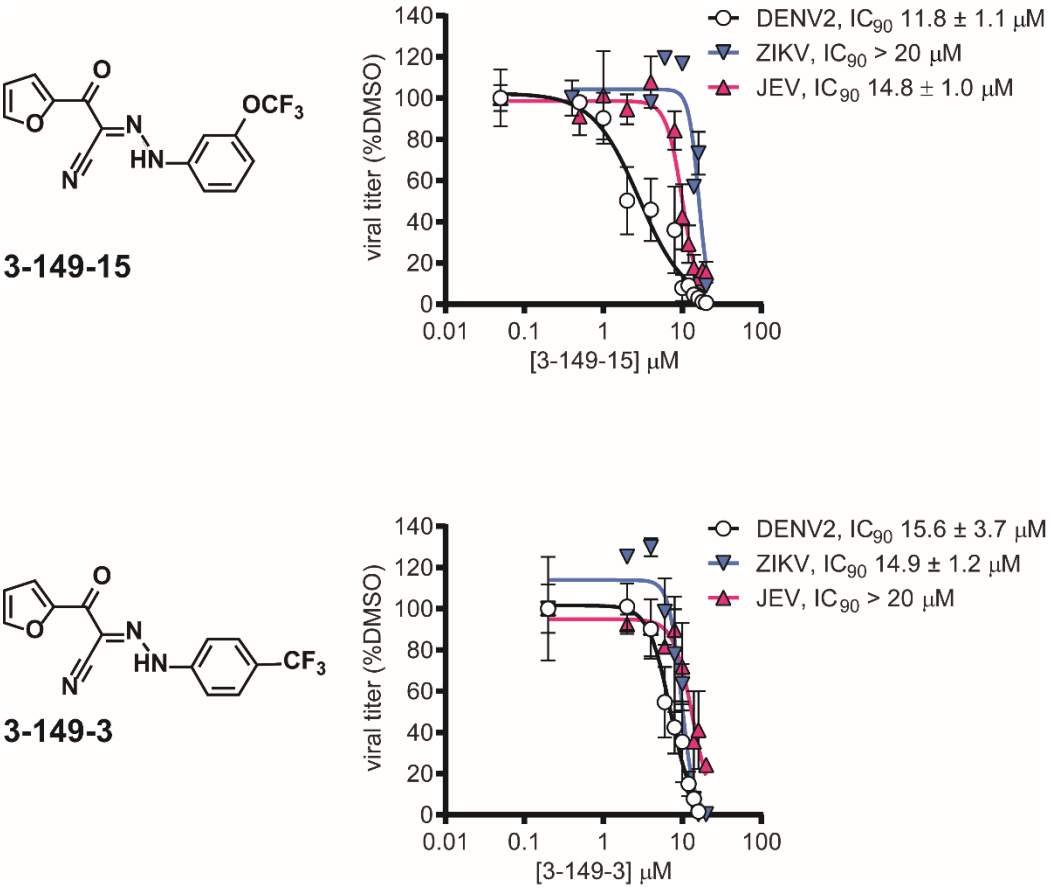


Figure S2. Examination of non-specific sources of antiviral activity

A. Inhibition of VSV-eGFP by 3-110-2 and 3-149-3. Inhibition of VSV-eGFP infectivity was assessed on BHK-21 cells as described in Fig. 1A. eGFP fluorescence was measured at 6 hours post-infection and plotted as a percentage normalized to the DMSO negative control ("relative VSV infection"). Representative data from one of n = 2 independent experiments are presented as means ± standard deviation of n = 2 biological replicates. Graphs showing representative data for 3-110-2 and 3-149-3 are provided in Fig. S2.

B. Malate dehydrogenase activity assay to detect PAINS activity. The effect of the compounds on malate dehydrogenase (MDH) activity was assessed in the presence and absence of Triton X-100. 3-110-2 exhibits significant detergent-sensitive inhibition of MDH (IC_{50} 8 ± 4 μM) at concentrations comparable to those that inhibit flavivirus infectivity. 3-149-3 also inhibits MDH in a detergent-sensitive manner but at significantly higher concentrations (IC_{50} 70 ± 26 μM). Representative data from one of n = 2 independent experiments are presented as means normalized to DMSO ± standard deviation of n = 2 experimental replicates.

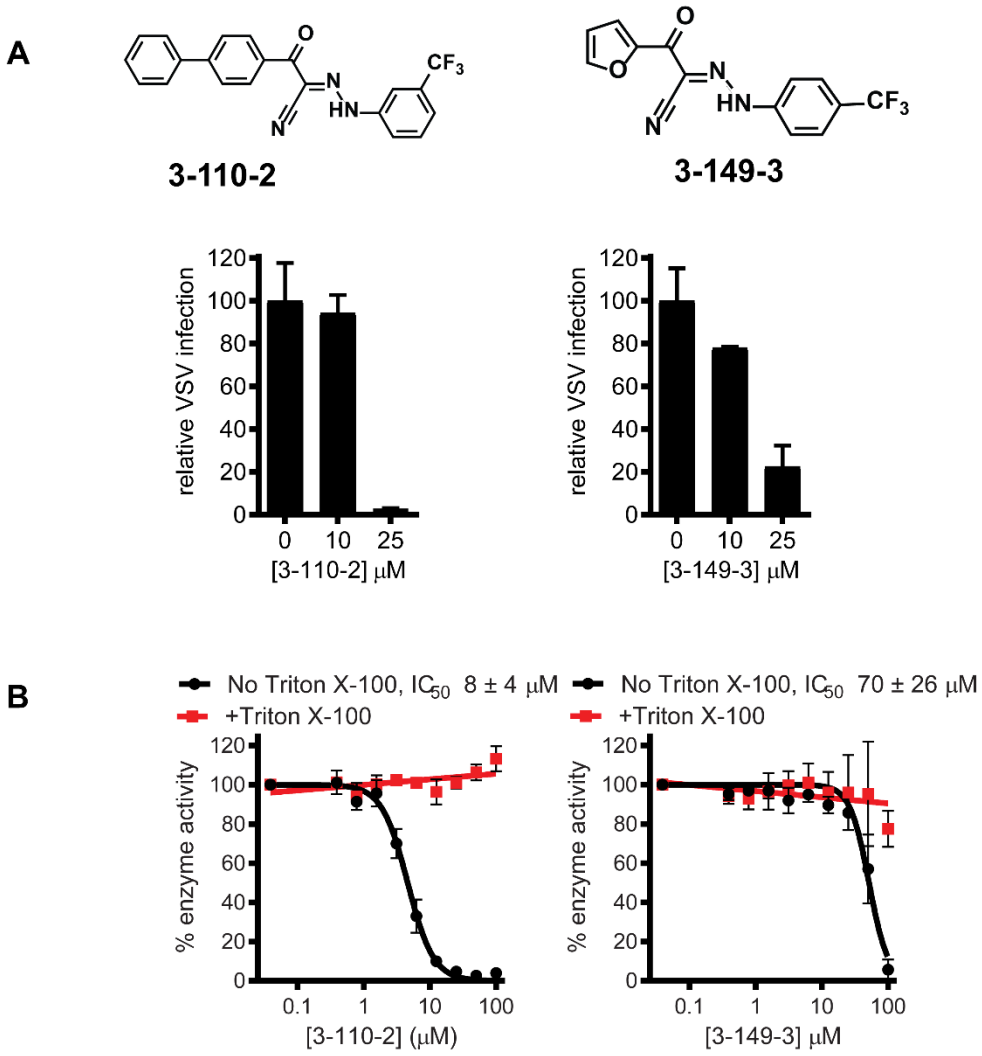


Figure S3. Characterization of JBJ series of compounds.
A. Antiviral activities against DENV2 NGC were measured in the infectivity assay as depicted in Fig. 1A. Virus titers were quantified by plaque-forming assay. The IC_{90} values of JBJ-01-162-02 and -03 were 8.9 ± 2.8 and 1.6 ± 0.6 μM, respectively (average of n ≥ 2 independent experiments). Representative data from one of n ≥ 2 independent experiments are shown with error bars indicating the standard deviation for 2 replicates within that experiment. BHK-21 cells were used for both infectivity and plaque-forming assays.
B. Assessment of cytotoxicity. BHK-21 cells were incubated with each of the indicated cyanohydrazones for twenty-four hours, and cell viability was assessed using a luminescence assay (see Materials and Methods).
C. Malate dehydrogenase activity assay to detect PAINS activity. The effect of the compounds on malate dehydrogenase (MDH) activity was assessed in the presence and absence of Triton X-100. JBJ-01-162-02 and -03 inhibit MDH activity in a detergent-sensitive manner; however, this requires compound concentrations significantly higher than those at which antiviral activity is observed. Representative data from one of $n \geq 2$ independent experiments are shown. Error bars represent the standard deviation of 2 replicates within the experiment.

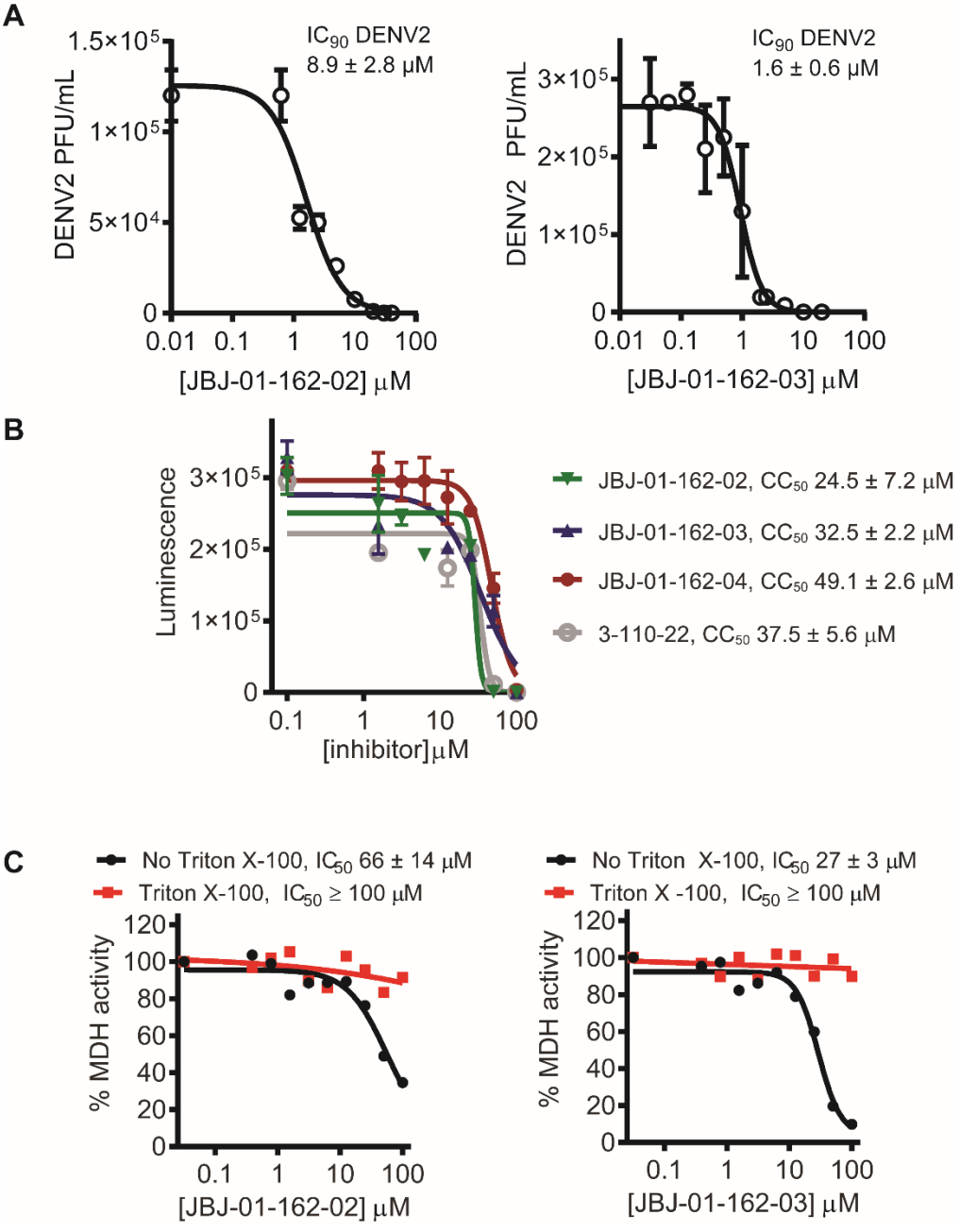

A
IC90 DENV2
8.9 ± 2.8 μM
DENV2 PFU/mL
1.5×10^5
1×10^5
5×10^4
0
0.01 0.1 1 10 100
[JBJ-01-162-02] μM
IC90 DENV2
1.6 ± 0.6 μM
DENV2 PFU/mL
3×10^5
2×10^5
1×10^5
0
0.01 0.1 1 10 100
[JBJ-01-162-03] μM
B
Luminescence
3×10^5
2×10^5
1×10^5
0
0.1 1 10 100
[inhibitor]μM
JBJ-01-162-02, CC50 24.5 ± 7.2 μM
JBJ-01-162-03, CC50 32.5 ± 2.2 μM
JBJ-01-162-04, CC50 49.1 ± 2.6 μM
3-110-22, CC50 37.5 ± 5.6 μM
C
No Triton X-100, IC50 66 ± 14 μM
Triton X-100, IC50 ≥ 100 μM
% MDH activity
120
100
80
60
40
20
0
0.1 1 10 100
[JBJ-01-162-02] μM
No Triton X-100, IC50 27 ± 3 μM
Triton X -100, IC50 ≥ 100 μM
% MDH activity
120
100
80
60
40
20
0
0.1 1 10 100
[JBJ-01-162-03] μM

Table S1. Pharmacokinetics data for JBJ-01-162-04. Pharmacokinetics parameters were determined in *n* = 3 male C57BL/6 mice. Compounds were administrated at indicated doses via intravenous tail vein injection or by oral gavage. Blood was collected at 5min, 15min, 30min, 1h, 2h, 4h, 6h, and 8h.

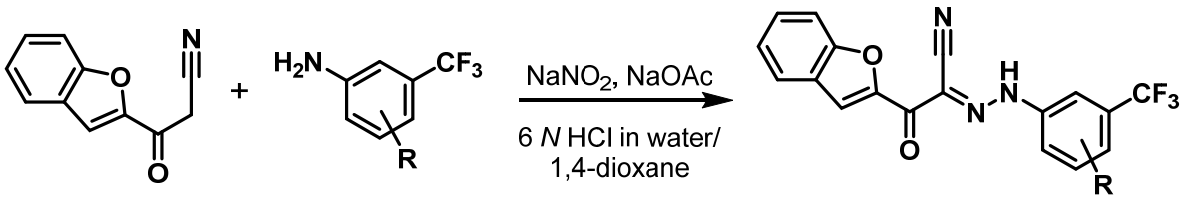
Route	Dose (mg/kg)	$T_{1/2}$ (hr)	T_{max} (hr)	C_{max} (μM)	AUC_{last} (min*ng/mL)	AUC_{last} (hr*μM)	AUC_{INF_obs} (min*ng/mL)	AUC %Extrap	CL obs (mL/min/kg)	MRT_{INF_obs} (hr)	Vss_obs L/kg	F (%)
IV	1	1.89	0.08	38.37	572996	21.85	585191	2.04	1.74	1.17	0.12	
PO	10	2.99	2.33	6.02	797724	30.42	1077940	15.61	13.00			14

Experimental Section

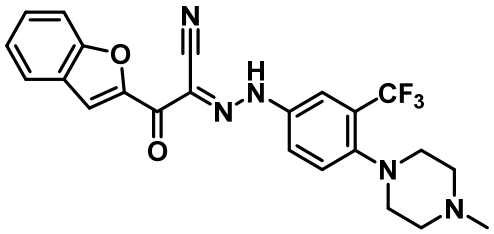
Compound synthesis and characterization

Starting materials, reagents and solvents were purchased from commercial suppliers and were used without further purification unless otherwise noted. All reactions were monitored using a Waters Acquity UPLC/MS system (Waters PDA eλ Detector, QDa Detector, Sample manager - FL, Binary Solvent Manager) using Acquity UPLC® BEH C18 column (2.1 x 50 mm, 1.7 µm particle size): solvent gradient = 85 % A at 0 min, 1 % A at 1.7 min; solvent A = 0.1 % formic acid in Water; solvent B = 0.1 % formic acid in Acetonitrile; flow rate : 0.6 mL/min. Reaction products were purified by flash column chromatography using CombiFlash®Rf with Teledyne Isco Redi*Sep*® normal-phase silica flash columns (4 g, 12 g, 24 g, 40 g or 80 g) and Waters HPLC system using SunFire™ Prep C18 column (19 x 100 mm, 5 µm particle size): solvent gradient = 80 % A at 0 min, 10 % A at 25 min; solvent A = 0.035 % TFA in Water; solvent B = 0.035 % TFA in MeOH; flow rate : 25 mL/min. ^{1}H NMR spectra were recorded on 500 MHz Bruker Avance III spectrometers and ^{13}C NMR spectra were recorded on 125 MHz Bruker Avance III spectrometer. Chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Spin multiplicities are described as br (broad), s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet).

Scheme 1. General synthetic route[1].



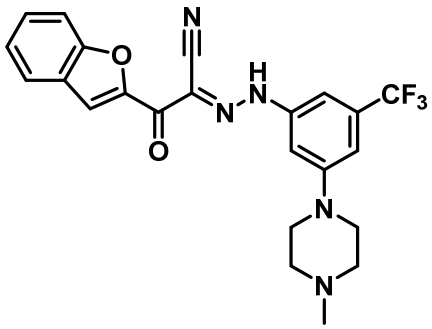
1. Representative Procedure: Synthesis of JBJ-01-162-01 [(*E*)-2-(benzofuran-2-yl)-*N*-(4-(4-methylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide].



To a solution of 4-(4-methylpiperazin-1-yl)-3-(trifluoromethyl)aniline (154 mg, 0.51 mmol) in 6 *N* hydrochloric acid (1 mL) was added dropwise a solution of sodium nitrite (41 mg, 0.59 mmol) in water (1 mL) at 0 °C under icebath. After stirring at room temperature for 30 min, a mixture of sodium acetate (49 mg) and 3-(benzofuran-2-yl)-3-oxopropanenitrile (100 mg, 0.54 mmol) in 1,4-dioxane (2 ml) was added to the reaction mixture. After stirring for 6 hr, the reaction mixture was filtered through celite, and the filtrate was diluted with EtOAc and washed with sat. $NaHCO_3$ and brine. The organic layer was dried over sodium sulfate, filtered and concentrated under reduced pressure. The resulting residue was purified by prepHPLC to give JBJ-01-162-01 as a yellowish solid (103 mg, 42 %).

^{1}H NMR (500 MHz, DMSO-d_6) δ 9.79 (br s, 1H), 7.97 (s, 1H), 7.95 - 7.93 (m, 1H), 7.90 - 7.83 (m, 2H), 7.75 (d, *J* = 8.5 Hz, 1H), 7.70 (d, *J* = 8.9 Hz, 1H), 7.58 (t, *J* = 7.8 Hz, 1H), 7.41 (d, *J* = 7.5 Hz, 1H), 3.56 - 3.40 (m, 4H), 3.20 - 3.07 (m, 4H), 2.90 (s, 3H); LC/MS (ESI) calcd. $C_{23}H_{21}F_3N_5O_2$ $[M+H]^+$ *m/z* 456.16, found 456.22; retention time 0.99 min.

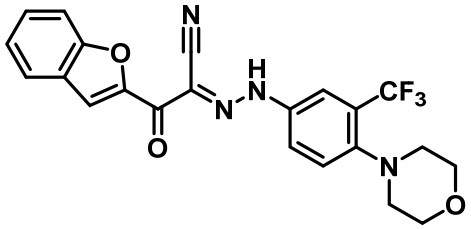
JBJ-01-162-02 [(*E*)-2-(benzofuran-2-yl)-*N*-(3-(4-methylpiperazin-1-yl)-5-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide].



^{1}H NMR (500 MHz, DMSO-d_6) δ 9.85 (br s, 1H), 7.97 (s, 1H), 7.84 (d, *J* = 7.6 Hz, 1H), 7.75 (d, *J* = 8.2 Hz, 1H), 7.58 (t, *J* = 7.6 Hz, 1H), 7.42 (t, *J* = 7.6 Hz, 1H), 7.36 (br s, 2H), 7.19 (s, 1H), 4.00 (br s, 2H), 3.53

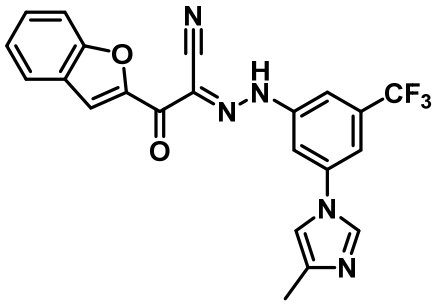
(br s, 2H), 3.27 - 3.03 (m, 4H), 2.89 (s, 3H); LC/MS (ESI) calcd. $C_{23}H_{21}F_3N_5O_2$ $[M+H]^+$ *m/z* 456.16, found 456.28; retention time 1.1 min.

JBJ-01-162-03 [(*E*)-2-(benzofuran-2-yl)-*N*-(4-morpholino-3-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide].



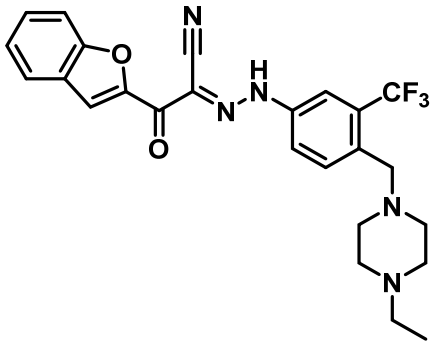
^{1}H NMR (500 MHz, DMSO-d_6) δ 9.85 (br s, 1H), 7.98 (s, 1H), 7.92 (d, *J* = 1.8 Hz, 1H), 7.88 (d, *J* = 7.9 Hz, 1H), 7.85 - 7.81 (m, 1H), 7.75 (d, *J* = 8.2 Hz, 1H), 7.70 (d, *J* = 8.9 Hz, 1H), 7.57 (t, *J* = 7.6 Hz, 1H), 7.40 (t, *J* = 7.5 Hz, 1H), 3.74 - 3.68 (m, 4H), 2.89 - 2.82 (m, 4H); LC/MS (ESI) calcd. $C_{22}H_{18}F_3N_4O_3$ $[M+H]^+$ *m/z* 443.13, found 443.19; retention time 1.6 min.

JBJ-01-162-04 [(*E*)-2-(benzofuran-2-yl)-*N*-(3-(4-methyl-1H-imidazol-1-yl)-5-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide].



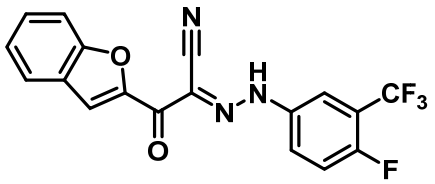
^{1}H NMR (500 MHz, DMSO-d_6) δ 8.31 (s, 1H), 7.83 (br s, 1H), 7.73 (d, *J* = 7.6 Hz, 1H), 7.66 (d, *J* = 8.2 Hz, 1H), 7.63 (s, 1H), 7.62 - 7.59 (m, 3H), 7.44 (t, *J* = 7.6 Hz, 1H), 7.33 (t, *J* = 7.5 Hz, 1H), 2.18 (s, 3H); LC/MS (ESI) calcd. $C_{22}H_{15}F_3N_5O_2$ $[M+H]^+$ *m/z* 438.12, found 438.17; retention time 1.1 min.

JBJ-01-162-05 [(*E*)-2-(benzofuran-2-yl)-*N*-(4-((4-ethylpiperazin-1-yl)methyl)-3-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide].



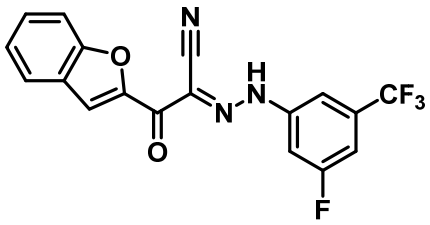
^{1}H NMR (500 MHz, DMSO-d_6) δ 9.38 (br s, 1H), 8.01 (s, 1H), 7.97 (s, 1H), 7.89 (d, *J* = 7.9 Hz, 1H), 7.85 (s, 2H), 7.77 (d, *J* = 8.2 Hz, 1H), 7.59 (t, *J* = 7.8 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 1H), 3.72 (s, 2H), 3.15 (q, *J* = 7.3 Hz, 2H), 3.05 - 2.90 (m, 4H), 2.42 (t, *J* = 11.9 Hz, 2H), 1.22 (t, *J* = 11.9 Hz, 3H); LC/MS (ESI) calcd. $C_{25}H_{25}F_3N_5O_2$ $[M+H]^+$ *m/z* 484.20, found 484.29; retention time 0.98.

JBJ-03-019-01 [(*E*)-2-(benzofuran-2-yl)-*N*-(4-fluoro-3-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide].



^{1}H NMR (500 MHz, DMSO-d_6) δ 7.97 (s, 1H), 7.95 - 7.90 (m, 1H), 7.87 (d, *J* = 7.9 Hz, 1H), 7.74 (d, *J* = 8.5 Hz, 1H), 7.63 (t, *J* = 9.6 Hz, 1H), 7.56 (t, *J* = 7.8 Hz, 1H), 7.40 (t, *J* = 7.5 Hz, 1H); LC/MS (ESI) calcd. $C_{18}H_{10}F_4N_3O_2$ $[M+H]^+$ *m/z* 376.07, found 376.07; retention time 1.6 min.

JBJ-03-019-02 [(*E*)-2-(benzofuran-2-yl)-*N*-(3-fluoro-5-(trifluoromethyl)phenyl)-2-oxoacetohydrazonoyl cyanide].



^{1}H NMR (500 MHz, DMSO-d_6) δ 8.00 (s, 1H), 7.89 (d, J = 7.6 Hz, 1H), 7.76 (s,1H), 7.73 (d, J = 8.5 Hz, 1H), 7.62 - 7.57 (m, 2H), 7.50 (t, J = 8.2 Hz, 1H), 7.41 (t, J = 7.5 Hz, 1H); LC/MS (ESI) calcd. $C_{18}H_{10}F_4N_3O_2$ $[M+H]^+$ m/z 376.07, found 376.07; retention time 1.6 min.

Synthesis of **3-110-22**, **3-110-2**, **3-149-3** and **3-149-15** were previously described[1-2].

Cell lines

African green monkey kidney-derived Vero cells (ATCC, RRID:CVCL_0059) were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Baby hamster kidney-derived BHK-21 cells (laboratory of Eva Harris, University of California, Berkeley) were maintained in Eagle minimum essential medium (MEM-α) supplemented with 5% FBS at 37 °C with 5% CO_2. C6/36 cells, a continuous mosquito cell line derived from *Aedes albopictus* (Diptera: *Culicidae*) embryonic tissue (ATCC, RRID:CVCL_Z230), were maintained in Leibovitz medium (L-15) containing 10% FBS at 28 °C. *Spodoptera frugiperda cells* (Sf9) (laboratory of Stephen C. Harrison, Cat # B825-01 Invitrogen/Thermo Fisher Scientific) were cultured in Sf-900™ II SFM media (Thermo Fisher Scientific) at 27 °C. *Trichoplusia ni* cells (High Five™, laboratory of Stephen C. Harrison Thermo Fisher Scientific) were maintained at 27 °C in EX-CELL® 405 Serum-Free Medium (Sigma).

Viruses

All work with infectious virus was performed in a biosafety level 2 (BSL2) laboratory using additional safety practices as approved by the Harvard Committee on Microbiological Safety.

Dengue virus serotype 2 strain New Guinea C (DENV2 NGC) was a kind gift from Lee Gehrke (Massachusetts Institute of Technology). Dengue virus serotype 2 strain S221 (DENV2 S221) was a kind gift from Sujan Shresta (La Jolla Institute for Allergy and Immunology). Virus stocks were propagated in C6/36 cells.

The Zika virus (ZIKV) strain PF-251013-18 was obtained from Didier Musso (Institut Louis Malardé, Tahiti Island, French Polynesia), and was kindly provided by Nathalie Pardigon (Institut Pasteur, France). The ZIKV strain PF-251013-18[3] was isolated from a viremic patient in French Polynesia in 2013, and was propagated three times on Vero cells. The viral stocks used in the current study were produced in C6/36 cells and were not propagated on cells more than six times.

The Japanese encephalitis virus (JEV) vaccine strain SA14-14-2 was kindly provided by Gregory Gromowski (Walter Reed Army Institute of Research). The viral stocks used in the current study were produced in C6/36 cells, and were not propagated more than twice.

VSV-eGFP was kindly shared by Sean Whelan (Harvard Medical School) and was propagated in Vero cells.

Antibodies

Monoclonal antibody 4G2 against the DENV E protein was produced from culture supernatants of hybridoma D1-4G2-4-15 (ATCC HB-112, RRID:CVCL_J890). Mouse hybridoma producing monoclonal antibody 6F3.1 against DENV2 core protein were generously provided by John Aaskov (Queensland University of Technology)[4]. Rabbit polyclonal antibodies against the ZIKV core protein, rabbit polyclonal antibodies against the JEV E protein, and mouse monoclonal antibodies against the JEV core protein were purchased from GeneTex (GTX133317, GTX125867 and GTX634153, respectively). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG antibodies were obtained from Bio-Rad Laboratories (170-6516 and 170-6515, respectively).

Cytotoxicity assay

BHK-21 cells were seeded at a density of 10,000 cells/well in a 96-well white format. Small molecules were serially diluted in MEM-α supplemented with 2% FBS (final DMSO was 1%) and added to plates incubated at 37°C for 24 hours. The cytotoxicity, was measured by quantitation of the ATP present using a CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions. The values for the concentrations that lead to 50% cytotoxicity (CC_{50}) were calculated using the nonlinear fit variable slope model (GraphPad Software).

Plaque-forming assay (PFA)

DENV2 and JEV titers were quantified by plaque-forming assay (PFA) on BHK-21 cells. ZIKV titers were quantified by PFA on Vero cells. Cells were seeded in 24-well plates and incubated overnight at 37 °C until formation of confluent monolayers. Aliquots from viral stocks or infections were thawed at room temperature. Ten-fold dilutions in Earle's balanced salt solution (EBSS) were prepared in duplicate, and 100 μL of each dilution was added to the cells. The plates were incubated for 1 hour at 37 °C and rocked every 15 minutes. Non-adsorbed virus was removed, after which 1 mL of DMEM (Vero cells) or MEMα (BHK-21 cells) supplemented with 1.05% carboxymethyl cellulose (CMC), 44 mM sodium bicarbonate, and 2% FBS was added to each well, followed by incubation at 37 °C for 2.5 days (ZIKV), 3 days (JEV), or 4.5 days (DENV2). The CMC overlay was aspirated, the cells were washed with 1X PBS and stained with crystal violet, and viral plaques were counted.

Focus-forming assay (FFA)

Where indicated, DENV2 titers were quantified by focus-forming assay (FFA) on BHK-21 cells. The described above for PFA was followed except that after incubation at 37°C for 3 days, the cells were fixed with methanol for 15 min at −20°C. After fixation, the cells were washed with PBS and incubated for 1 hour at room temperature with monoclonal antibody 6F3.1 against DENV2 core protein. The cells were then washed and incubated with HRP-conjugated anti-mouse IgG antibody (Bio-Rad laboratories). The plates were developed with the Vector VIP peroxidase substrate kit (Vector Laboratories) according to the

manufacturer's instructions. The number of focus-forming units (FFU) per milliliter was determined by counting the number of foci.

Viral infectivity assays for IC_{90} determination

Virus inocula were diluted in EBSS to achieve an MOI of 1 and were preincubated with the given small molecule at varying concentrations for 45 min at 37 °C. The mixture was then added to cells for 1 hour at 37 °C to allow infection, after which the inoculum was removed, and the cells were washed with 1X PBS to remove unbound virus and compound. Cells were overlaid with medium lacking inhibitor and incubated at 37 °C for 24 hours, corresponding to a single cycle of infection. Unless stated otherwise, culture supernatants were harvested at this time, stored at -80 °C, and the yield of infectious particles produced was quantified by PFA or FFA. The values for the concentrations that result in 90% inhibition (IC_{90}) were calculated using the nonlinear fit variable slope model (GraphPad Software).

Vesicular stomatitis virus-eGFP (VSV-eGFP) infectivity assay

Virus inocula were diluted in EBSS to achieve a MOI of 1 and were preincubated with the given small molecule at varying concentrations for 45 min at 37 °C. The virus-inhibitor mixture was then added to cells for 1 h at 37 °C to allow infection, after which the inoculum was removed, and the cells were washed with 1X PBS to remove unbound virus and compound. Cells were overlaid with medium lacking inhibitor and incubated at 37 °C for 6 h. Following removal of the supernatants, the cells were washed with 1X PBS and overlaid with PBS and then imaged. Fluorescence (excitation 473 nm, emission 650 nm) was measured using a Typhoon FLA 9500 (GE Healthcare Life Sciences) and quantified using ImageJ software (http://imagej.nih.gov/ij/).

Capsid protection assay to monitor fusion of virions with liposomes

The composition of liposomes was designed to mimic late endosomal membranes as previously described[1-2, 5-6]. Liposomes were made with (1,2-dioleoyl-sn-glycero-3-phosphocholine) (DOPC) (Avanti

Polar Lipids), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Avanti Polar Lipids), L-α-phosphatidylinositol, Soy (PI), bis(monooleoylglycero)phosphate, S,R Isomer (BMP) and cholesterol (Avanti Polar Lipids) at 4/1/1/2/4 molar ratio in TAN buffer (20 mM triethanolamine, 100 mM NaCl, pH 8.0). Lipids were dried down and resuspended thoroughly by vortexing and sonication. Liposomes were prepared by extrusion through a 0.2 micron filter after five freeze/thaw cycles. For trypsin-containing liposomes, 3.8 mg of trypsin was added to 0.38 mL of lipids (2.8 mg each) after the third freeze/thaw cycle, prior to extrusion. Liposomes were separated from unincorporated trypsin by size-exclusion chromatography using a 10/300 Increase Superdex 200 column (GE Healthcare) on an Akta fast performance liquid chromatography (FPLC) system.

50 µL of DENV2, strain NGC ($5x10^5$ PFU); ZIKV, strain PF13 ($9.5x10^5$ PFU); and JEV, strain SA14-14-2 ($9.5x10^5$ PFU) were incubated with various concentrations of compounds (0, 10 µM, 50 µM) for 45 min at 37 °C in TAN buffer (pH 8.0) prior to addition of 20 µL liposomes for 5 minutes. This represents > 10-fold increase in virus and hence target abundance relative to the viral infectivity assays. After incubation with liposomes, 2.5 µL 1M sodium acetate (pH 5.0) was added to drop the pH to 5.3 for 5 minutes. Samples were back-neutralized with 2.5 µL 2M triethanolamine (pH 8.1) and incubated for 20 minutes at 37°C to allow trypsin digestion. SDS sample buffer was added, and samples were boiled for 20 minutes before separation by SDS-PAGE on a 10% gel. Proteins were transferred to a PVDF membrane using a semi-dry transfer apparatus. The following antibodies were used for detection of envelope and capsid protein respectively: 4G2 (1:100) and 6F3.1 antibodies for DENV, 4G2 (1:100) and ZIKV capsid protein (1:100) antibodies for ZIKV, and JEV envelope protein (1:1000), and JEV core (1:500) antibodies for JEV. Membranes were developed with enhanced chemiluminescence reagents (Pierce), and the signal was captured using the Amersham Imager 600.

Malate dehydrogenase (MDH) assay

Small molecule inhibitors were serially diluted (2-fold dilution series from 100 µM) and were mixed with 200 µM oxaloacetic acid (VWR) and 200 µM NADH (VWR) in working buffer (100 mM potassium

phosphate, pH 7.0). Malate dehydrogenase (EMD Millipore) was added to a final concentration of 0.7 nM, and absorbance was immediately monitored at 340 nm for 5 min. The final concentration of DMSO was 1% for all samples. All assays were repeated in the presence of 0.01% Triton X-100. The values for the concentrations that result in 50% inhibition (IC_{50}) were calculated using the nonlinear fit variable slope model (GraphPad Software).

Expression, purification, and biotinylation of DENV2 soluble envelope protein (sE_2)

Expression, purification, and biotinylation of recombinant, soluble DENV2 prefusion E dimer (sE_2) followed published procedures[7-8]. The coding sequence for the soluble portion of the DENV2 NGC envelope protein (sE), fused to a N-terminal signal peptide was codon optimized for expression in insect cells and was synthesized and generously donated by Daryl Klein and Stephen C. Harrison. The coding sequences of the soluble domains of the DENV2 envelope protein were cloned into a pFastBacTM/CT-TOPO® vector (Thermo Fisher Scientific) to enable expression of sE - with a TEV-His6 tag at its C terminus. The plasmid was modified to introduce a C-terminal AviTag^{TdeM4} to allow production of AviTag^{TM} DENV2 sE_2 protein for use in biolayer interferometry experiments. Protein expression and purification were performed as previously described[7-8].

Biotinylation of AviTag^{TM} sE proteins

Biotinylation of DENV2 sE_2 protein was conducted as previously described[7]. The purified AviTag^{TM} DENV2 sE_2 proteins were biotinylated using the BirA-500 kit (Avidity). Briefly, the protein was concentrated to around 400 μM using a 30 kDa Amicon filter (Millipore) and was then resuspended in 50 mM Biocine pH 8.5. The proteins were incubated for 60 minutes at 30 °C in the presence of 2.5 μg BirA per 10 nmol of purified protein, according to the manufacturer's instructions. The biotinylated protein was then purified from the reaction using a Superdex 200 grade 10/300 GL column (GE Healthcare) pre-equilibrated with 20 mM triethanolamine hydrochloride (pH 8.0) and 100 mM NaCl.

-

Determination of dissociation constant (K_D) values by bio-layer interferometry (BLI)

Experiments were performed as previously described[7] using a Octet RED384 system (FortéBio). BLI mixtures (80 µL) were prepared in wells of a 384-well black tilted-bottom plate (FortéBio).

Super streptavidin (SSA) biosensor reaction for determination of binding of small molecule inhibitors to biotinylated DENV2 sE_2 protein: 1.6 µg of the biotinylated protein was loaded on an SSA biosensor tip (FortéBio) for 600 seconds and then quenched with 0.8 µg biocytin for 120 seconds. The SSA biosensors were then equilibrated in reaction buffer [1X Kinetic buffer (FortéBio), 1X PBS, 2% DMSO] for 180 seconds prior to baseline collection. Association with small molecules was monitored for 120 seconds with inhibitor concentrations that ranged from 50 nM to 20 µM; dissociation was performed in reaction buffer and monitored for 120 seconds.

Equilibrium dissociation constants (K_D) values were determined by plotting the local fit maximum response (nm) as a function of small molecule concentrations (µM) using Octet Data Analaysis Software (FortéBio) and Prism (GraphPad Software). Titration curves were fit to the following steady-state analysis equation:

$$\text{Response} = (R_{max}*\text{Conc})/K_D + \text{Conc}$$

where R_{max} is the local fit response maximum; Conc is the concentration of small molecule; and K_D is the equilibrium dissociation constant.

Pharmacokinetic study in mice

Pharmacokinetic studies *in vivo* were performed by the Drug Metabolism and Pharmacokinetics Core at Scripps Florida, following protocols reviewed and approved by the Institutional Animal Care and Use Committee. Briefly, C57BL/6 mice were administered JBJ-01-162-04 formulated in 0.1 and 1 mg/mL solution in DMSO:Tween-80:water (10:10:80) for 1 mg/Kg by intravenous (i.v.) and 10 mg/Kg by oral gavage (n = 3 mice per group), respectively. Blood samples were collected at 9 time points over the course of 500 min post-dosing for determination of PK parameters. Due to limited oral bioavailability and

clearance issue by the i.v. route, we further tested intraperitoneal (i.p.) administration of JBJ-01-162-04 with the same formulation at 20 mg/kg twice daily for 3 days. Blood and liver samples were collected and compound concentrations were quantified at 4, 8, 12 h on days 1 and day 3.

Hepatic microsomal stability

Microsome stability assays were performed by the Drug Metabolism and Pharmacokinetics Core at Scripps Florida. 1 μM test compound was incubated with 1 mg/mL hepatic microsomes in 100 mM potassium phosphate buffer, pH 7.4. The reaction was initiated by adding NADPH (1 mM final concentration). Aliquots were removed at 0, 5, 10, 20, 40, and 60 minutes and added to acetonitrile (5X v:v) to stop the reaction and precipitate the protein. NADPH dependence of the reaction was evaluated with -NADPH samples. At the end of the assay, the samples were centrifuged through a Millipore Multiscreen Solvinter 0.45 micron low binding PTFE hydrophilic filter plate and analyzed by LC-MS/MS. Data were log-transformed and represented as half-life and intrinsic clearance.

Antiviral efficacy *in vivo.* All experiments performed in mice followed protocols and procedures reviewed and approved by the Institutional Animal Care and Use Committee of Harvard University. Breeding pairs of AG129 (with knockout INF-α/β and INF-γ receptors) mice were generously donated by Sujan Shresta (LJIA) and bred in the specific-pathogen-free animal facility at the Harvard Medicial School. 6-8 week old AG129 mice were challenged with 1×10^3 PFU of DENV-2, mouse-adapted strain S221 by retro-orbital injection in 100 μL of DPBS with 0.2% BSA. For prophylaxis experiments, mice were treated with either JBJ-01-162-04 at 40 mg/kg formulated in DMSO:Tween-80:water (10:10:80) or vehicle (10 mice per group for both sexes) via the intraperitoneal route twice daily starting 1 day prior to inoculation with DENV until day 3 post-infection. Blood was collected on day 3 post-infection by the retro-orbital route, as this reproducibly coincides with the peak of viremia under these experimental conditions. The serum was separated and harvested after centrifugation in a microvette blood collection tube (Sarstedt), and then stored at -80 ℃ until processed. Serum viremia was assessed by viral plaque forming assay.

The *in vivo* experiments were performed in two independent studies. Statistical analysis was performed by paired t test for two-tailed analysis.

Statistical Analysis

Statistical tests and the associated error bars are identified in the corresponding figure legends. ‘‘Independent’’ experiments describe the number of biological replicates, which were repeat experiments performed on different days. Statistical analysis was performed using Prism (GraphPad Software).

REFERENCES for Supporting Information

1. Schmidt, A. G.; Lee, K.; Yang, P. L.; Harrison, S. C., Small-Molecule Inhibitors of Dengue-Virus Entry. *PLOS Pathogens* **2012,** *8*, e1002627. DOI 10.1371/journal.ppat.1002627.

2. Clark, Margaret J.; Miduturu, C.; Schmidt, Aaron G.; Zhu, X.; Pitts, Jared D.; Wang, J.; Potisopon, S.; Zhang, J.; Wojciechowski, A.; Hann Chu, Justin J.; Gray, Nathanael S.; Yang, Priscilla L., GNF-2 Inhibits Dengue Virus by Targeting Abl Kinases and the Viral E Protein. *Cell Chemical Biology* **2016,** *23*, 443-452. DOI 10.1016/j.chembiol.2016.03.010.

3. Aubry, M.; Richard, V.; Green, J.; Broult, J.; Musso, D., Inactivation of Zika virus in plasma with amotosalen and ultraviolet A illumination. *Transfusion* **2016,** *56* (1), 33-40. DOI 10.1111/trf.13271.

4. Bulich, R.; Aaskov, J. G., Nuclear localization of dengue 2 virus core protein detected with monoclonal antibodies. *J Gen Virol* **1992,** *73 (Pt 11)*, 2999-3003. DOI 10.1099/0022-1317-73-11-2999.

5. van Meer, G.; Voelker, D. R.; Feigenson, G. W., Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* **2008,** *9* (2), 112-24. DOI 10.1038/nrm2330.

6. Zaitseva, E.; Yang, S. T.; Melikov, K.; Pourmal, S.; Chernomordik, L. V., Dengue virus ensures its fusion in late endosomes using compartment-specific lipids. *PLoS Pathog* **2010,** *6* (10), e1001131. DOI 10.1371/journal.ppat.1001131.

7. de Wispelaere, M.; Lian, W.; Potisopon, S.; Li, P. C.; Jang, J.; Ficarro, S. B.; Clark, M. J.; Zhu, X.; Kaplan, J. B.; Pitts, J. D.; Wales, T. E.; Wang, J.; Engen, J. R.; Marto, J. A.; Gray, N. S.; Yang, P. L., Inhibition of Flaviviruses by Targeting a Conserved Pocket on the Viral Envelope Protein. *Cell Chem Biol* **2018,** *25* (8), 1006-1016 e8. DOI 10.1016/j.chembiol.2018.05.011.

8. Lian, W.; Jang, J.; Potisopon, S.; Li, P. C.; Rahmeh, A.; Wang, J.; Kwiatkowski, N. P.; Gray, N. S.; Yang, P. L., Discovery of Immunologically Inspired Small Molecules That Target the Viral Envelope Protein. *ACS Infect Dis* **2018,** *4* (9), 1395-1406. DOI 10.1021/acsinfecdis.8b00127.