Supplementary Information for

Identification of small molecule inhibitors of Zika virus infection and induced neural cell death via a drug repurposing screen

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This document includes:

Supplementary Table 1. Protocol of caspase-3 activity assay.

Step	Parameter	Value		Description
		384-well	1536-well	
1	Cell plating	20 μl/well	3 μl/well	PDL-coated plates used for hNPCs/astrocytes
2	Incubation	overnight		at 37°C with 5% CO_2
3	Compound addition	6 μl/well	0.023 μl/well	in DMSO solution
4	Incubation	30 minutes		at 37°C with 5% CO_2
5	Zika virus addition	4 μl/well	2 μl/well	
6	Incubation	6 hours		at 37°C with 5% CO_2
7	Reagent addition	30 μl/well	3.5 μl/well	Caspase 3/7 assay mixture
8	Incubation	30 minutes		at room temperature
9	Plate reading	luminescence mode		ViewLux plate reader

Supplementary Table 2. Protocol of ATP cell viability assay for ZIKV-induced cell death.

Step	Parameter	Value		Description
		384-well	1536-well	
1	Cell plating	20 μl/well	3 μl/well	PDL-coated plates used for hNPCs/astrocytes
2	Incubation	overnight		at 37°C with 5% CO_2
3	Compound addition	6 μl/well	0.023 μl/well	in DMSO solution
4	Incubation	30 minutes		at 37°C with 5% CO_2
5	Zika virus addition	4 μl/well	2 μl/well	
6	Incubation	3 days		at 37°C with 5% CO_2
7	Reagent addition	30 μl/well	3.5 μl/well	ATP content assay mixture
8	Incubation	15 minutes		at room temperature
9	Plate reading	luminescence mode		ViewLux plate reader

Supplementary Table 3. Protocol of ATP content cell viability assay for compound cytotoxicity.

Step	Parameter	Value		Description
		384-well	1536-well	
1	Cell plating	24 μl/well	5 μl/well	PDL-coated plates used for NPCs/astrocytes
2	Incubation	overnight		at 37°C with 5% CO_2
3	Compound addition	6 μl/well	0.023 μl/well	in DMSO solution
4	Incubation	30 minutes		at 37°C with 5% CO_2
5	Incubation	6 hours		at 37°C with 5% CO_2
6	Reagent addition	30 μl/well	3.5 μl/well	ATP content assay mixture
7	Incubation	15 minutes		at room temperature
8	Plate reading	luminescence mode		ViewLux plate reader

Note: No ZIKV was added to assay plates in this experiment.

Supplementary Table 4. List of compounds confirmed in the caspase-3 assay with human astrocytes, SNB-19 cells, and hNPCs.

Name	Library	Caspase 3 (IC_{50}, μM)			Cell viability (IC_{50}, μM)			Maximal inhibition of Caspase 3 (%)			Cell viability (efficacy, %)		
		Astrocyte	*SNB-19*	*hNPCs*	*Astrocyte*	*SNB-19*	*hNPCs*	*Astrocyte*	*SNB-19*	*hNPCs*	*Astrocyte*	*SNB-19*	*hNPCs*
Emricasan	C	0.46	0.62	0.37	4.11	0.87	3.88	77.1	97.6	85.3	98.7	194	54.4
Teriflunomide	C	1.30	1.03	0.92	-	-	-	86.9	93.1	94.5	0.0	0.0	-86.9
Hydroxocobalamin	C	0.97	1.46	0.87	-	-	-	83.4	85.7	67.4	0.0	0.0	0.0
Ensulizole	B	1.95	1.84	1.38	-	-	-	82.7	73.3	80.6	0.0	0.0	0.0
Tenonitrozole	B	2.06	1.95	1.30	-	-	-	88.8	81.7	81.5	0.0	0.0	-47.2
Isoliquiritigenin	A	3.08	2.18	1.84	-	3.08	-	95.1	96.9	89.0	0.0	36.3	-46.1
Nitazoxanide	B	2.00	2.24	1.26	31.6	23.7	-	90.3	86.4	82.3	53.6	62.1	-68.9
Febuxostat	B	2.31	2.91	3.88	-	3.66	-	84.8	104	83.2	0.0	68.4	-43.8
Leflunomide	B	4.35	3.27	1.38	-	-	-	98.1	106	97.8	0.0	0.0	-55.6
Vidofludimus	C	2.91	3.66	2.45	-	-	-	101	101	87.8	0.0	0.0	0.0
SB-366791	A	4.11	3.66	2.91	-	-	-	93.9	93.3	89.9	0.0	0.0	-42.0
Emodin	A	3.27	4.35	2.59	-	-	-	86.4	83.3	82.1	0.0	0.0	0.0
Diphenyl isophthalate	B	6.68	4.73	4.47	-	-	-	104	92.8	89.4	0.0	0.0	0.0
Benzoylpas	B	5.81	6.90	4.89	-	-	-	94.9	92.8	85.4	0.0	0.0	0.0
Fenobam	B	9.20	7.31	5.48	-	1.73	-	97.2	110	108	0.0	60.5	0.0
Indobufen	B	6.52	7.74	6.15	-	-	-	86.3	85.8	81.3	0.0	0.0	0.0
2-(2H-Benzotriazol-2-yl)-4-methylphenol	B	9.20	8.69	4.89	-	-	-	68.5	80.1	77.6	0.0	0.0	0.0
PHA-690509	C	13.0	9.20	19.5	7.74	10.9	-	71.6	74.6	73.0	81.9	165	-60.6
Tiaprofenic acid	B	6.15	10.3	8.69	-	-	-	88.2	96.2	86.7	0.0	0.0	0.0
Flufenamic acid	B	8.69	10.9	17.3	-	-	-	71.9	61.5	68.1	0.0	0.0	0.0
Vitamin B12	B	8.69	11.6	6.90	-	-	-	77.1	101	94.7	0.0	0.0	0.0
Cinanserin	B	15.5	12.3	17.3	-	19.5	-	80.2	92.0	80.4	0.0	56.9	0.0
5-Nitro-2-(3-phenylpropylamino)benzoic acid	A	9.75	13.0	8.69	-	-	-	82.1	82.9	59.8	0.0	0.0	0.0
Veliflapon	C	9.20	13.8	9.75	-	-	-	65.9	70.0	74.3	0.0	0.0	-36.0
Thiabendazole	B	10.3	14.6	9.75	-	-	-	81.0	89.4	83.4	0.0	0.0	0.0
SIB 1893	A	12.3	15.5	8.69	-	-	-	96.2	105	95.2	0.0	0.0	0.0
Anethole trithione	B	13.8	15.5	13.0	-	-	-	62.7	85.5	70.3	0.0	0.0	0.0
Naringenin	C	17.3	15.5	15.5	-	-	-	65.2	66.3	63.4	0.0	0.0	-32.5
Phenazopyridine hydrochloride	B	15.5	16.4	11.6	-	27.5	-	69.9	73.7	72.5	0.0	35.3	-38.1
Fanetizole	B	10.9	17.3	14.6	-	-	-	72.9	78.1	81.8	0.0	0.0	0.0
Terazosin hydrochloride	B	12.3	17.3	10.9	-	-	-	88.9	77.3	84.4	0.0	0.0	0.0
Diacerein	B	15.5	17.3	15.5	30.8	-	-	72.9	63.2	56.5	35.2	0.0	0.0
CAY10505	C	16.4	17.3	10.9	-	-	-	80.1	91.2	83.5	0.0	0.0	0.0
Hesperetin	C	16.4	17.3	10.9	-	-	-	72.7	86.0	79.6	0.0	0.0	-52.8
Suprofen	B	19.5	17.3	15.5	-	-	-	64.5	65.6	59.1	0.0	0.0	0.0
Ketorolac tromethamine	B	21.8	17.3	10.3	-	-	-	61.7	84.5	75.0	0.0	0.0	0.0
Piperine	C	20.6	19.5	13.8	-	-	-	73.9	75.5	66.0	0.0	0.0	-44.4
Pirarubicin	B	13.8	20.6	-	-	-	-	59.8	63.9	51.5	0.0	0.0	-87.7
Piraxostat	C	8.91	21.1	15.8	63.1	5.01	-	70.5	63.5	67.1	34.7	60.3	-39.7
Albendazole oxide	B	15.5	21.8	12.3	-	-	-	78.8	86.0	88.1	0.0	0.0	-62.1
Tyrphostin AG 494	A	18.4	21.8	17.3	-	30.8	-	72.5	55.6	72.8	0.0	45.6	-62.9
Genistin	C	25.9	23.1	17.3	-	-	-	60.2	58.1	58.6	0.0	0.0	-80.6
Fenbufen	B	21.8	24.5	19.5	-	-	-	81.5	76.3	72.5	0.0	0.0	0.0
Apatinib	C	19.5	24.5	-	-	-	-	57.9	61.2	0.0	0.0	0.0	0.0
RITA	C	27.5	0.22	14.6	5.81	-	-	41.2	101	50.9	81.7	0.0	-91.4
Niclosamide	B	1.84	2.06	1.84	1.73	1.95	-	96.8	93.9	87.5	51.2	97.3	-77.4
BF-170 hydrochloride	A	4.61	6.15	4.11	6.15	-	-	118	104	92.7	65.8	0.0	-61.1
OSI-930	C	8.20	6.90	6.90	-	3.08	-	91.5	114	96.9	0.0	71.2	-56.2
Tribromsalan	B	6.90	7.74	13.8	-	-	-	102	107	108	0.0	0.0	-35.7
Pifexole	B	6.90	8.20	4.61	-	-	-	86.3	83.6	92.2	0.0	0.0	-35.3
Formononetin	C	14.6	12.3	10.9	-	-	-	94.7	104	88.3	0.0	0.0	-56.0
Ebselen	A	8.20	17.3	8.20	-	-	-	93.8	117	109	0.0	0.0	-82.0
Tranilast	C	17.3	17.3	19.5	-	-	-	71.6	70.3	107	0.0	0.0	-54.5
Benzylparaben	B	17.3	19.5	15.5	-	-	-	62.8	91.8	56.0	0.0	0.0	0.0
2-Ethoxylethyl-p-methoxycinnamate	B	-	27.5	18.4	-	-	-	0.0	53.0	56.0	0.0	0.0	0.0
Baicalein	C	-	29.1	18.4	-	-	-	0.0	50.6	57.5	0.0	0.0	-69.3
Nemorubicin	B	15.5	30.8	-	0.87	0.41	-	42.6	53.1	82.9	146	117	-57.2
Rutaecarpine	A	4.89	5.18	4.61	-	-	-	89.5	86.4	87.5	0.0	0.0	-44.7
MPEP	A	5.81	5.18	4.11	-	-	-	118	98.6	96.1	0.0	0.0	0.0
5,7-Dihydroxyflavone	C	15.5	8.69	5.48	-	-	-	55.1	42.7	51.0	0.0	0.0	-69.3
Vitamin B12	B	6.73	13.4	11.3	-	0.12	-	62.7	43.0	59.1	0.0	67.3	0.0
Pipofezine	B	11.3	15.1	12.0	-	-	-	56.3	31.5	47.7	0.0	0.0	0.0
Flurbiprofen axetil	B	21.8	19.5	-	-	-	-	65.5	45.7	0.0	0.0	0.0	0.0
2-Amino-6-nitrobenzothiazole	B	39.8	53.1	56.2	-	-	-	54.1	46.1	57.1	0.0	0.0	0.0
Malachite green oxalate	B	17.3	-	21.8	-	-	-	54.0	0.0	62.7	0.0	0.0	-89.7
Enfenamic acid	B	25.9	-	21.8	-	-	7.74	58.0	49.5	47.1	0.0	0.0	58.5
Fenaminosulf	B	35.5	-	-	-	15.8	-	58.5	0.0	0.0	0.0	36.7	-53.0
AS-252424	A	1.73	2.18	1.64	-	-	-	37.5	38.0	52.3	0.0	0.0	0.0
Phenserine	A	25.9	19.5	25.9	-	-	-	47.4	38.4	51.5	0.0	0.0	-105
Epalrestat	C	19.5	20.6	15.5	-	5.48	-	93.3	79.2	72.1	0.0	82.1	-47.9
Alizarin	B	30.8	21.8	21.8	-	-	-	47.9	40.9	58.5	0.0	0.0	0.0
Dalcetrapib	C	-	-	35.5	47.3	35.5	-	0.0	50.2	50.2	45.7	110	-43.6

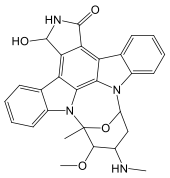
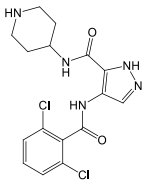
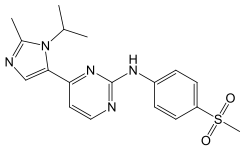
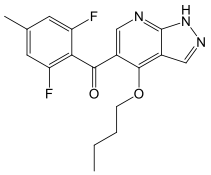
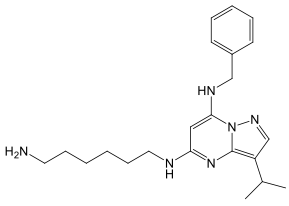
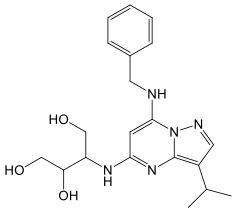
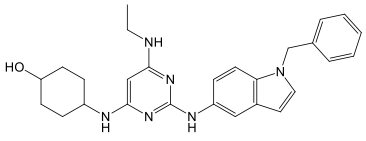
Note: Full compound names can be found at the Pubchem website (https://pubchem.ncbi.nlm.nih.gov/) using the Sample ID number in the first column. A: LOPAC library (Sigma-Aldrich). B: Approved drug library. C: Clinical drug candidate library. For Cell viability (efficacy, %), if a compound does not protect the cells from death caused by ZIKV infection, the efficacy is 0. A positive number indicates a cytoprotective effect – 100% represents full protection. A negative number indicates additional cell death caused by compounds alone. In the last column, the negative number indicates the compound cytotoxicity to hNPCs at the highest compound concentration of 47 μM.

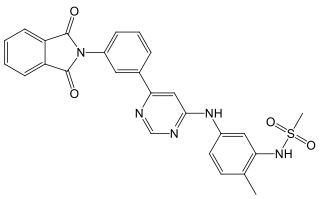
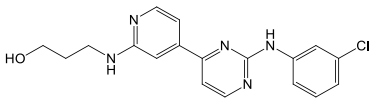
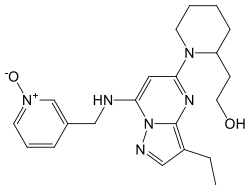
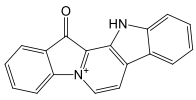
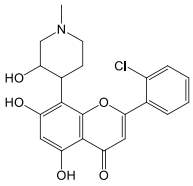
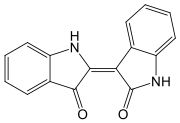
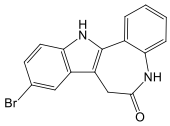
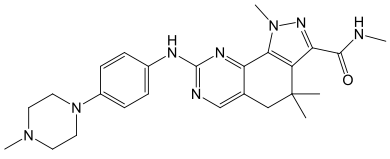
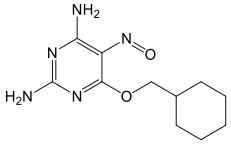
Supplementary Table 5. IC_{50} values of selected compounds for improving cell viability in hNPCs, astrocytes and SNB-19 cells following ZIKV infection.

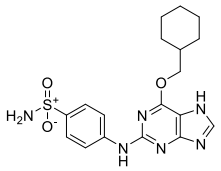
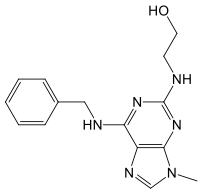
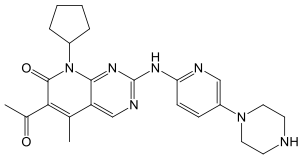
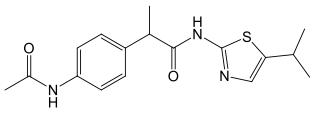
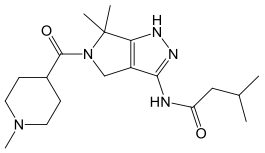
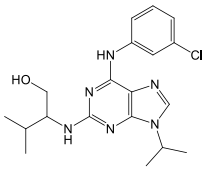
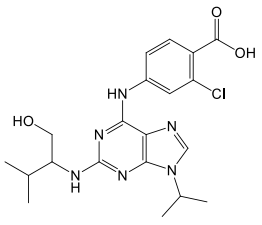
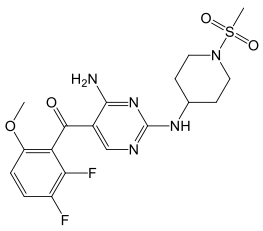
Compound ID	Compound name	Collection*	IC_{50} (μM)		
			hNPCs	Astrocytes	SNB-19
NCGC00346477	Emricasan	C	3.88	4.11	0.87
NCGC00263191	PHA-690509	C	-	7.47	10.1
NCGC00015735	Niclosamide	B	-	1.73	1.95

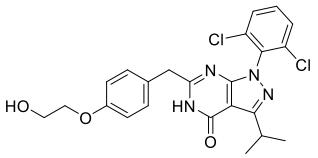
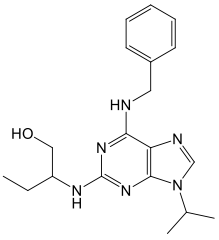
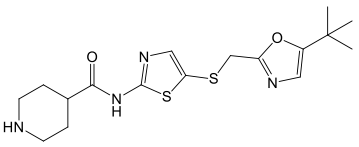
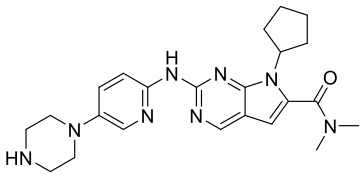
Note: * compound is from “A” - LOPAC library, “B” – approved drug library, and “C” – clinical drug candidate library. “-“: n.a.

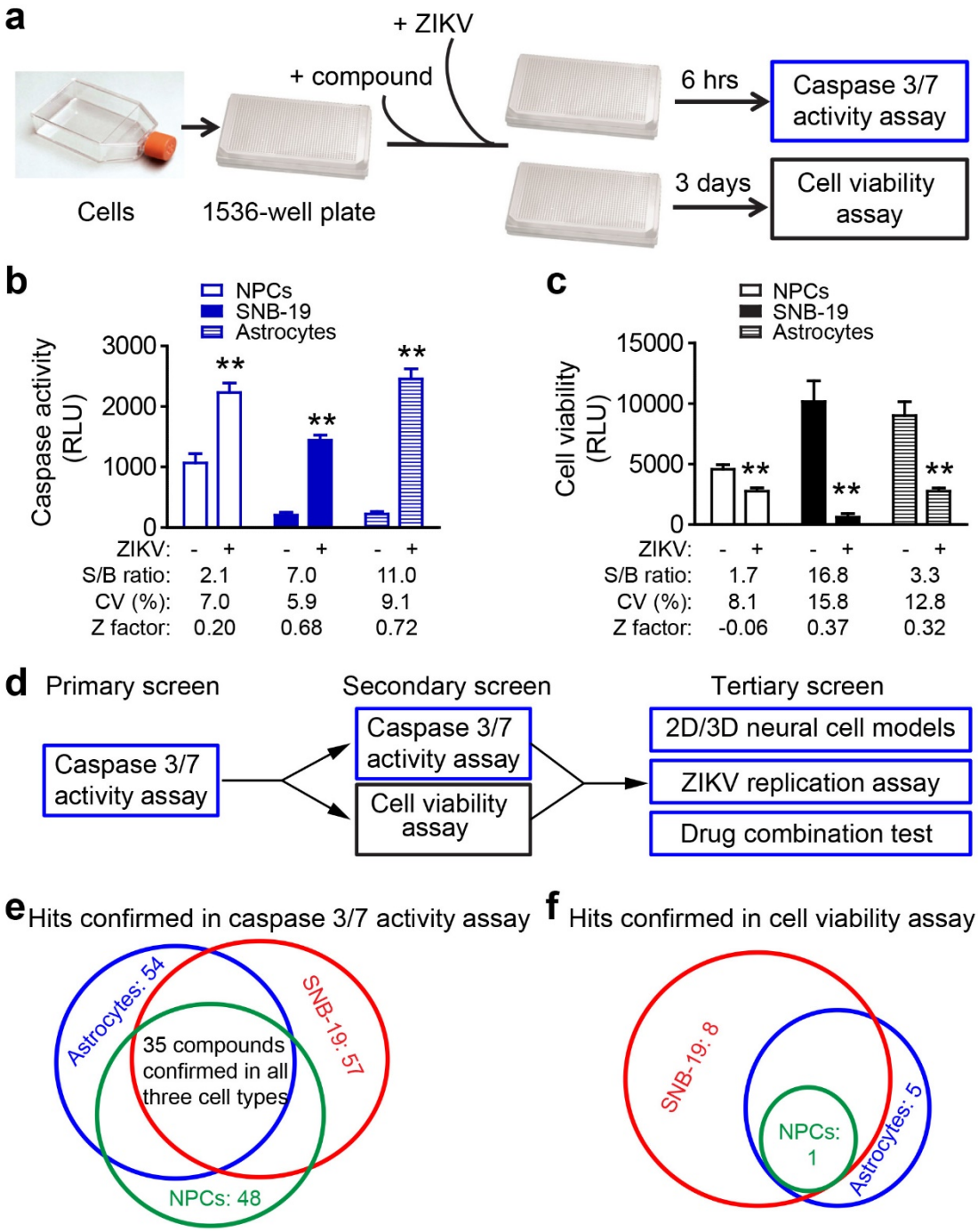
Supplementary Table 6. Chemical Structures of 28 CDKis that were tested for inhibition of ZIKV replication.

Compound ID	Compound name	Structure
NCGC00346827-01	7-Hydroxystaurosporine	
NCGC00263091-01	AT7519	
NCGC00346617-01	AZD-5438	
NCGC00346693-01	BMS-265246	
NCGC00346553-01	BS-181	
NCGC00345852-01	BS-194	
NCGC00346952-01	Cdk4/6 Inhibitor IV	

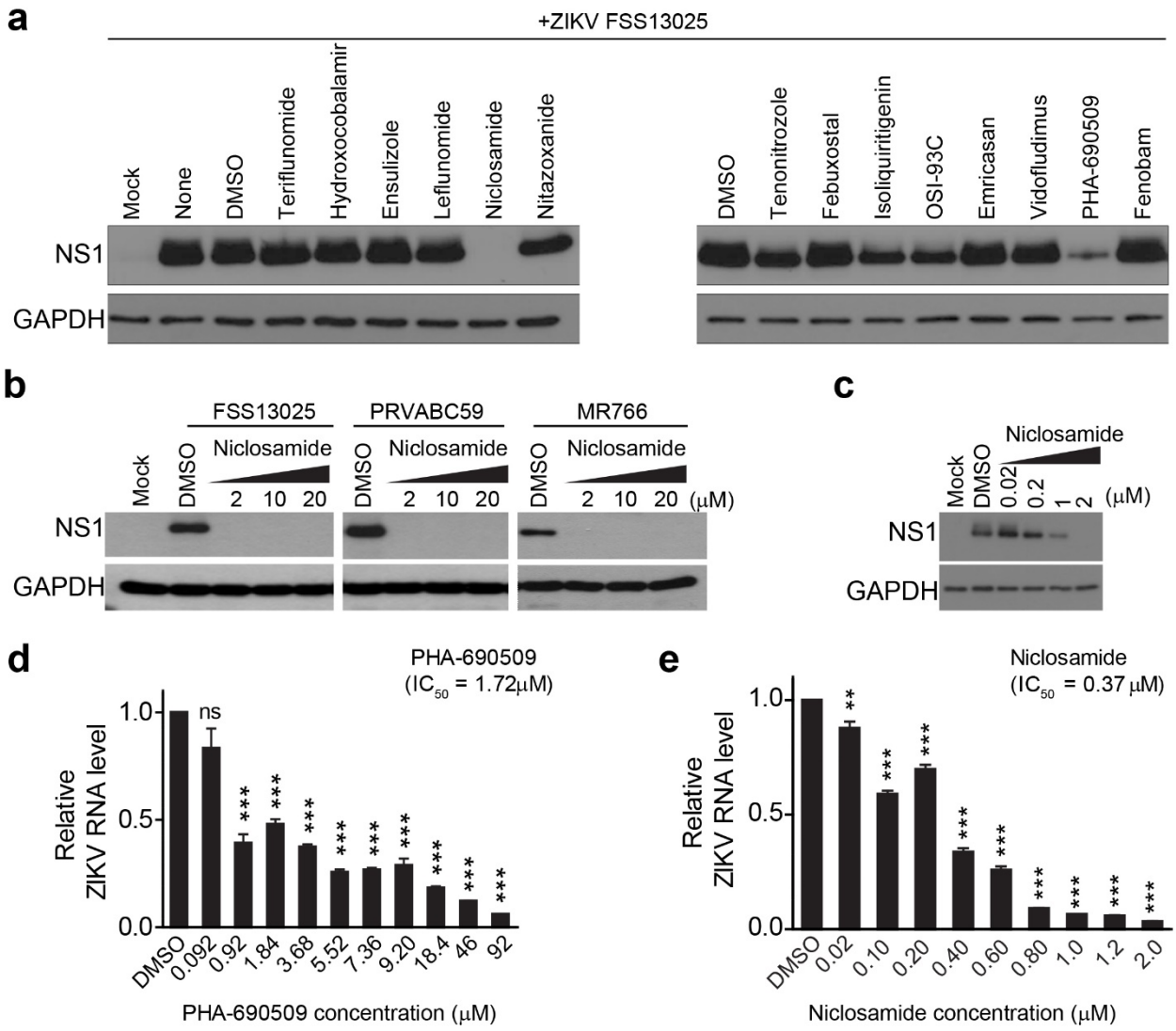
NCGC00346946-01	CDK9 inhibitor	
NCGC00346940-01	CGP-60474	
NCGC00346656-01	Dinaciclib	
NCGC00346951-01	Fascaplysin	
NCGC00250401-01	Flavopiridol (Alvocidib)	
NCGC00179302-02	Indirubin	
NCGC00015582-06	Kenpaullone	
NCGC00346673-01	Milciclib	
NCGC00165844-02	NU-6027	

NCGC00346950-01	NU-6102	
NCGC00015763-09	Olomoucine	
NCGC00263129-01	PD-0332991	
NCGC00263191-01	PHA-690509	
NCGC00263168-01	PHA-793887	
NCGC00025219-04	Purvalanol A	
NCGC00025220-03	Purvalanol B	
NCGC00346632-01	R-547	

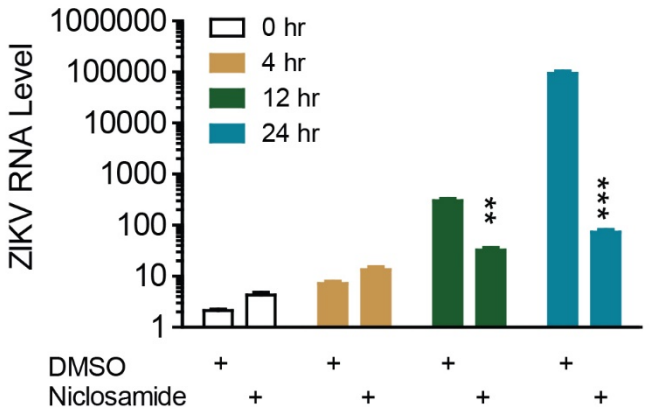
NCGC00345809-01	RGB-286147	
NCGC00094374-05	Seliciclib	
NCGC00263167-02	SNS-032	
NCGC00386317-01	Lee011	



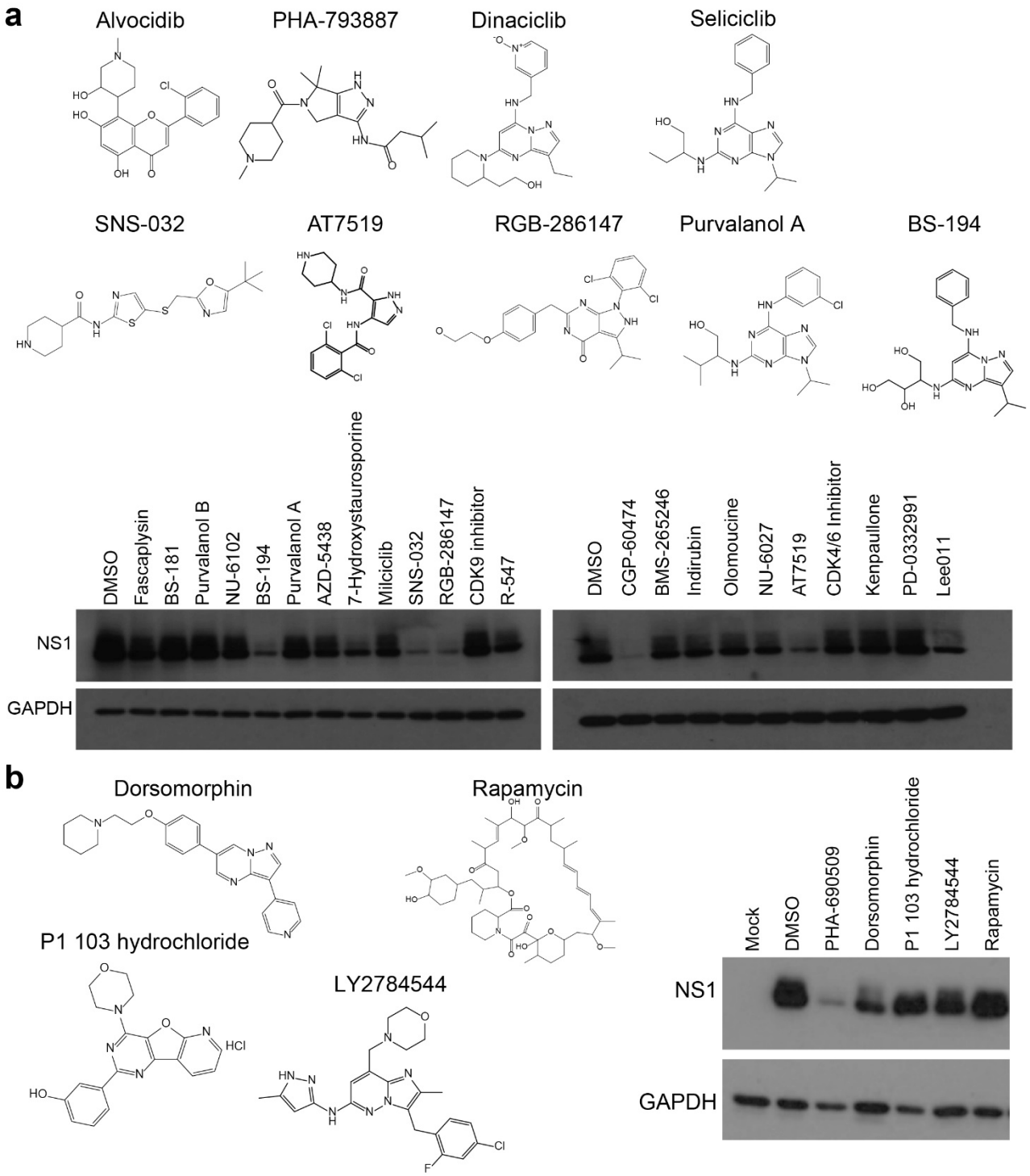
Supplementary Figure 1. Two high-throughput assays that measure increased caspase-3 activity and reduced cell viability of ZIKV infected cells. MR766 was used. (**a**) Flowchart of caspase-3 activity and cell viability assays for compound screening. (**b**) Increased caspase-3 activity in the ZIKV infected hNPCs, SNB-19 cells, and human astrocytes. Values represent mean ± s.d. (n = 3; $^{**}P < 0.01$; One-way ANOVA for comparison with no ZIKV group). RLU: relative luminescence units. (**c**) Decreased cell viability after ZIKV infection for three days. Values represent mean ± s.d. (n = 3; $^{*}P < 0.01$; One-way ANOVA for comparison with no ZIKV group). (**d**) Schematic diagram of compound screening and hit validation process. (**e**) Summary of numbers of confirmed compounds in the caspase-3 activity assay. Thirty-five compounds were confirmed in all three cell types. (**f**) Summary of confirmed compounds in the cell viability assay that improved cell viability in cells infected with ZIKV.



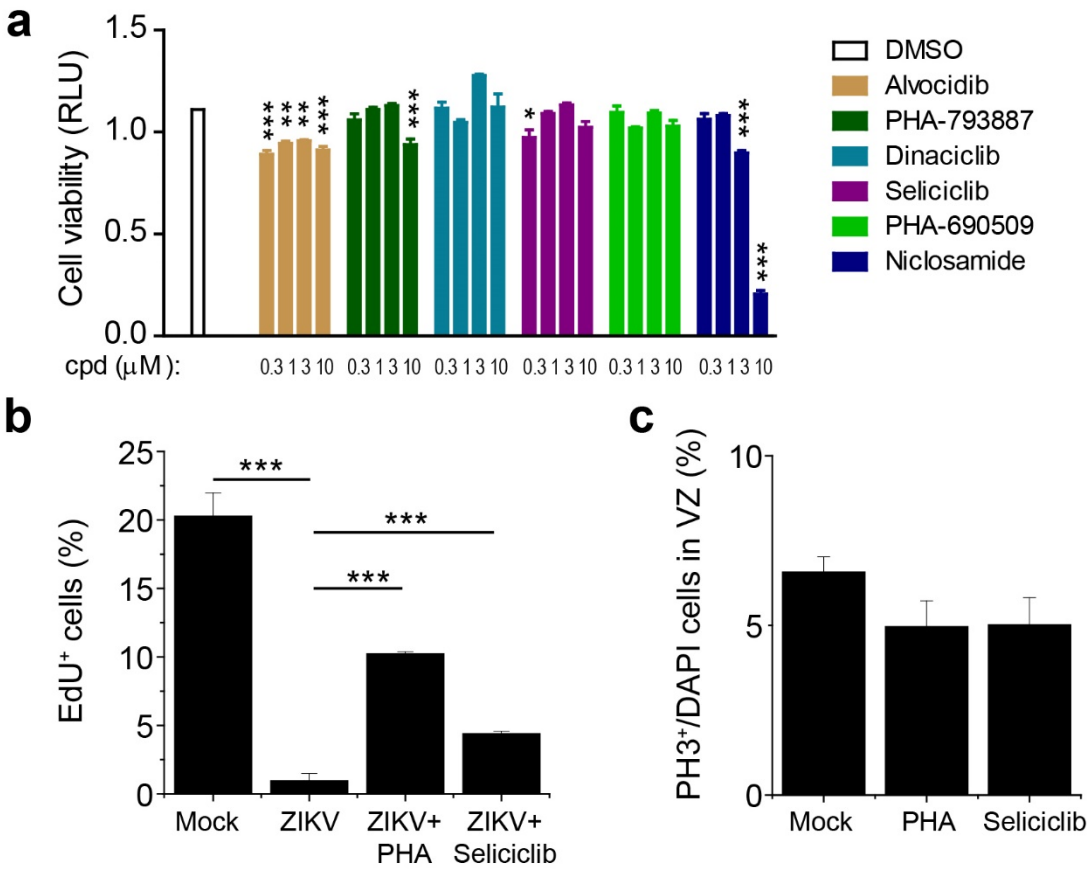
Supplementary Figure 2. Niclosamide and PHA-690509 inhibit ZIKV infection in a dose-dependent manner. (**a**) Representative western blot images of SNB-19 cells treated with compounds at 10x IC_{50} concentration (determined in primary caspase-3 screen) for 1 hour prior to infection with ZIKV-FSS13025 and harvested 24 hours later for NS1 detection. (**b**) Representative western blot images of SNB-19 cells treated with 2, 10, or 20 μM Niclosamide 1 hour prior to infection with indicated ZIKV strains and harvested 24 hours later and analyzed as in (**a**). (**c**) Dose-dependence of Niclosamide on ZIKV-NS1 protein levels. Cells were treated with 0.02 – 2 μM Niclosamide, infected with PRVABC59, and analyzed as in (**b**). (**d-e**) Dose-dependence of Niclosamide (**d**) or PHA-690509 (**e**) on intracellular ZIKV-RNA levels. Cells were treated with compounds at indicted concentrations for one hour then infected with ZIKV-FSS13025, and harvested 48 hours later for RNA purification. ZIKV RNA levels were measured using qRT-PCR and normalized to that with the DMSO treatment. Values represent mean ± s.d. (n = 3 cultures; $^{**}P < 0.01$; $^{***}P < 0.001$; One-way ANOVA for comparison with the DMSO treatment).



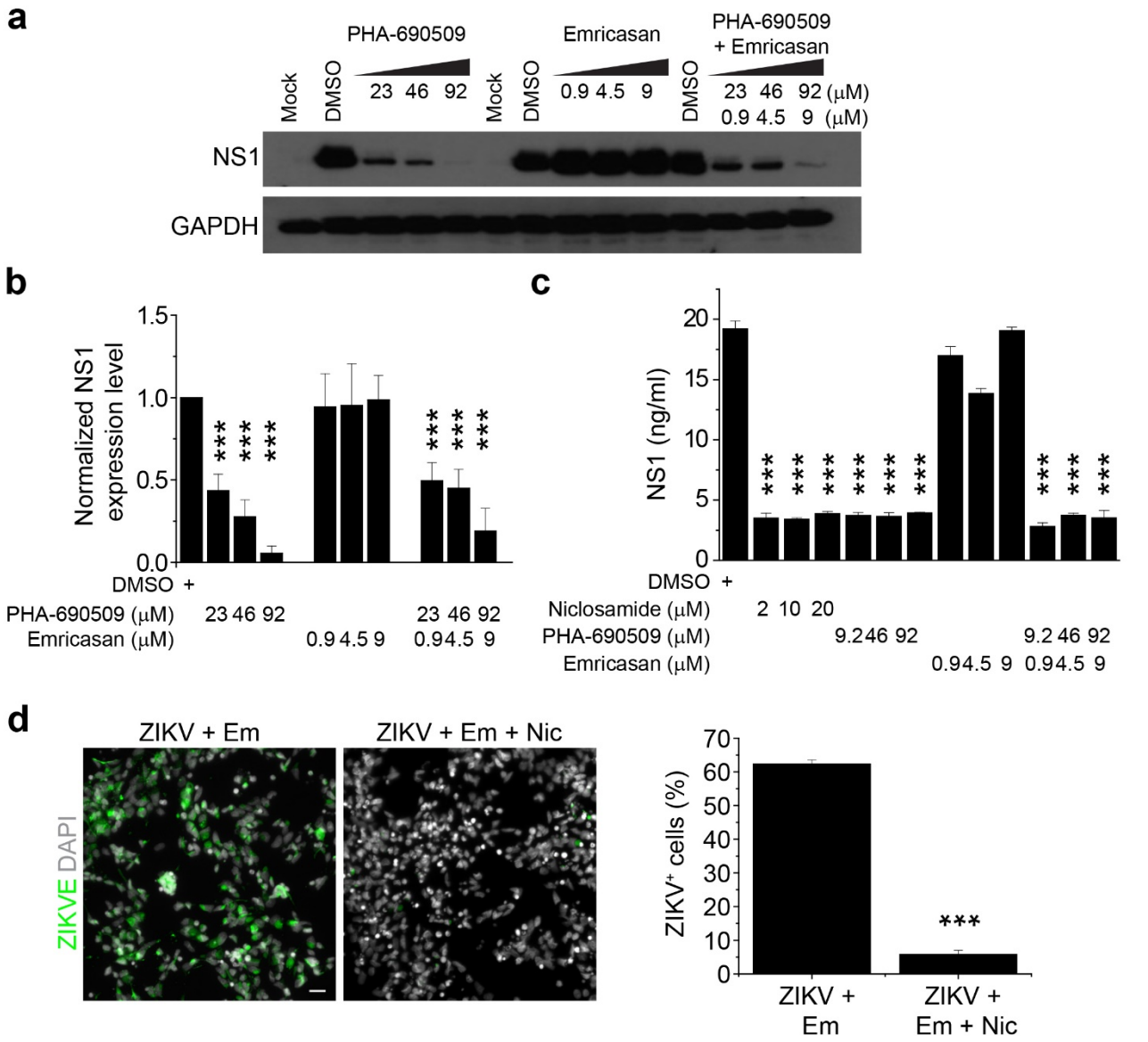
Supplementary Figure 3. Niclosamide inhibits a post-entry step in the ZIKV infection lifecycle. SNB-19 cells were treated with 2 μM Niclosamide or DMSO for 1 hour prior to infection. Cells were incubated with viral inoculum at MOI = 1 for 2 hours on ice, then at 37°C (denoted as 0 hr time point). Cells were washed with PBS and total RNA was collected and purified at each time point for qRT-PCR analysis of ZIKV RNA. Relative ZIKV RNA levels after normalizing to GAPDH RNA in the same sample are plotted. Values represent mean $\pm$ s.d. (n = 3 cultures; $^{**}P < 0.01$; $^{***}P < 0.001$; One-way ANOVA for comparison with the DMSO treatment).



Supplementary Figure 4. Additional CDKis and non-CDK kinase inhibitors that were tested for antiviral activity. (**a**) Top, chemical structures of nine additional CDKis that inhibited ZIKV infection. Bottom, representative western blot images of SNB-19 cells treated with 1 μM of each indicated compound for 1 hour prior to infection with ZIKV-FSS13025 and harvested 24 hours post infection. (**b**) Left, chemical structures of four non-CDK kinase inhibitors tested on ZIKV infection. Right, representative western blot images of SNB-19 cells treated with 1 μM of each indicated non-CDK kinase inhibitor 1 hour prior to infection with PRVABC59 and harvested 24 hours post infection.



Supplementary Figure 5. Analysis of potential toxicity of CDKis on human astrocyte viability and hNPC proliferation. (**a**) Human iPSC-derived astrocytes (BJ line) were treated with each indicated compound (cpd) for 25 hours prior to cell viability analysis as measured in relative luciferase units for ATP production. Values represent mean ± s.d. (n = 3 cultures; ***P < 0.001; **P < 0.01; One-way ANOVA for comparison with the DMSO treatment). RLU: relative luminescence units. (**b**) hNPCs were treated with saline, PHA-690509 (PHA, 1 μM) or Seliciclib (5 μM),1 hour prior to ZIKV PRVABC59 infection (MOI = 0.08). At 72 hours post infection, EdU (10 μM) was added to hNPCs and cells were cultured for additional 4 hours prior to fixation and staining for EdU and DAPI. Values represent mean ± s.e.m. (n = 3 cultures; ***P < 0.001; One-way ANOVA for comparison with the mock treatment). (**c**) Day 20 forebrain-specific brain organoids were treated with PHA-690509 (1 μM) or Seliciclib (5 μM) for 3 days and hNPC proliferation was evaluated by phospho-Histone 3 (PH3) expression within the ventricular zone. Values represent mean ± s.e.m. (n = 8 organoids; P > 0.05; One-way ANOVA for comparison with the mock treatment).



Supplementary Figure 6. Additional benefit of combinatorial treatment with two classes of compounds. (**a-b**) Human astrocytes were treated with DMSO, PHA-690509, Emricasan, or PHA-690509 and Emricasan at indicated concentrations for 1 hour prior to infection with ZIKV-FSS13025. Cell lysates were harvested 24 hours post infection and analyzed for ZIKV-NS1 and GAPDH protein levels by western blot. Shown are representative western blot images (**a**) and quantification (**b**). Data were normalized to that of the DMSO treatment. Values represent mean ± s.d. (n = 3 cultures; *P < 0.001; One-way ANOVA for comparison with the DMSO treatment). (**c**) Secreted NS1 levels from DMSO-, Niclosamide-, PHA-690509-, or Emricasan-treated astrocytes. Astrocytes were infected as in (**a**) and supernatants were collected 24 hours post infection for analysis by NS1 ELISA. Values represent mean ± s.d. (n = 3 cultures; *P < 0.001; One-way ANOVA for comparison with the DMSO treatment). (**d**) Combined treatment of hNPCs with Emricasan (Em) and Niclosamide (Nic). hNPCs were treated with 15 μM Emricasan 1 hour prior to addition of PRVABC59 (MOI = 0.08). At 72 hours post infection, 1 μM Niclosamide or DMSO was added to hNPCs and cells were cultured for an additional 48 hours prior to staining with anti-ZIKVE. Shown are representative images of immunostaining for ZIKVE (green) and DAPI (gray, left; Scale bar: 20 μm) and quantification (right). Values represent mean ± s.e.m. (n = 3 cultures; ***P < 0.001; One-way ANOVA).