Supplemental Information

A Stem Cell-Based High Content Screen to Discover Drug Candidates that Inhibit and Repress Zika Virus Infection in the Fetal and Adult Brain

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Inventory of Supplemental Information

Supplementary Figures:

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Figure S1

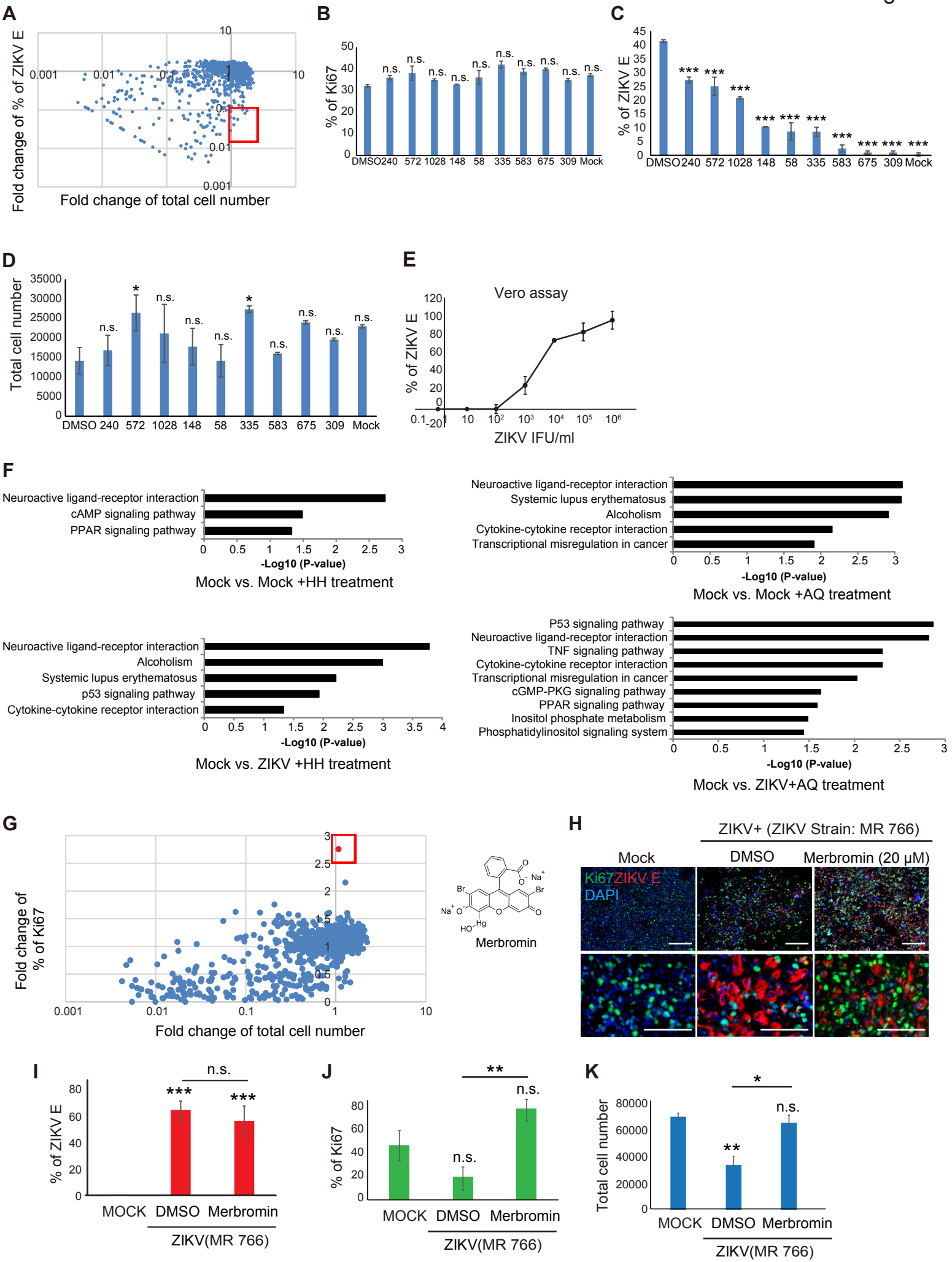

A
Fold change of % of ZIKV E
Fold change of total cell number
B
% of Ki67
DMSO 240 572 1028 148 58 335 583 675 309 Mock
C
% of ZIKV E
D
Total cell number
E
Vero assay
% of ZIKV E
ZIKV IFU/ml
F
Neuroactive ligand-receptor interaction
cAMP signaling pathway
PPAR signaling pathway
-Log10 (P-value)
Mock vs. Mock +HH treatment
Neuroactive ligand-receptor interaction
Systemic lupus erythematosus
Alcoholism
Cytokine-cytokine receptor interaction
Transcriptional misregulation in cancer
-Log10 (P-value)
Mock vs. Mock +AQ treatment
Neuroactive ligand-receptor interaction
Alcoholism
Systemic lupus erythematosus
p53 signaling pathway
Cytokine-cytokine receptor interaction
-Log10 (P-value)
Mock vs. ZIKV +HH treatment
P53 signaling pathway
Neuroactive ligand-receptor interaction
TNF signaling pathway
Cytokine-cytokine receptor interaction
Transcriptional misregulation in cancer
cGMP-PKG signaling pathway
PPAR signaling pathway
Inositol phosphate metabolism
Phosphatidylinositol signaling system
-Log10 (P-value)
Mock vs. ZIKV+AQ treatment
G
Fold change of % of Ki67
Fold change of total cell number
Merbromin
H
ZIKV+ (ZIKV Strain: MR 766)
Mock
DMSO
Merbromin (20 μM)
Ki67ZIKV E
DAPI
I
% of ZIKV E
MOCK DMSO Merbromin
ZIKV(MR 766)
J
% of Ki67
MOCK DMSO Merbromin
ZIKV(MR 766)
K
Total cell number
MOCK DMSO Merbromin
ZIKV(MR 766)

Figure S1. A high content chemical screen identifies anti-ZIKV drug candidates that promote proliferation in hNPCs. Related to Figure 1.

(A) Two dimensional analysis of anti-ZIKV drug screen. X-axis represents the fold change of total cell number, which was calculated by dividing the total cell number of the chemical treated well by the average total cell number of DMSO treated wells. Y-axis represents the fold change of the percentage of ZIKV infected cells, which was calculated by dividing the percentage of ZIKV E expressing cells of the chemical-treated well by the average percentage of ZIKV E expressing cells in DMSO-treated wells. The compounds, in which the fold change of total cell number >1 and the fold change of the percentage of ZIKV infection <20% were picked for subsequent evaluation.
(B-D) The quantification of proliferation rate (B), the percentage of ZIKV E^+ cells (C) and total cell number (D) of hNPCs at 72 h after ZIKV infection with primary hit compounds or DMSO treatment (n=3). *p* values were calculated by one-way repeated measures ANOVA with a Bonferroni test for multiple comparisons. **p*<0.05, ***p*<0.01 and ****p*<0.001.
(E) Vero re-infection assay using different ZIKV (MR766 strain) titers to determine the sensitivity.
(F) Gene ontology pathway analysis of genes that are more than two fold differentially expressed in Mock vs. HH, Mock vs. HH+ZIKV, Mock vs. AQ and Mock vs. AQ+ZIKV among the genes selected in Figure 1L.
(G) Two dimensional analysis of compound screen and the chemical structure of the hit compound Merbromin. The analysis sought compounds that increase cell proliferation rates in ZIKV-infected hNPCs. The X-axis represents the fold change of total cell number, which was calculated by dividing the total cell number of the chemical treated wells by the average of total cell number of DMSO treated wells. The Y-axis represents the fold change of cell proliferation rate, which was calculated by dividing the percentage of $Ki67^+$ cells of the chemical treated well by the average of the percentage of $Ki67^+$ cells of DMSO treated wells. One hit compound, for which the fold change of total cell number >1 and the fold change of the percentage of $Ki67^+$ cells > 2.5 was picked for follow-up evaluation.
(H-K) Immunocytochemical analysis (H), the quantification of the percentage of ZIKV E^+ cells (I), the quantification of the proliferation rate (J) and the quantification of the total cell number (K) of hNPCs at 72 h after ZIKV infection with 20 μM Merbromin or DMSO treatment (n=3). Cells were fixed and stained for Ki67 (green), ZIKV E (red) and DAPI (blue). Scale bars, 100 μm. *p* values were calculated by unpaired two-tailed Student's t-test. **p*<0.05, ***p*<0.01 and ****p*<0.001, unless otherwise stated.

Figure S2

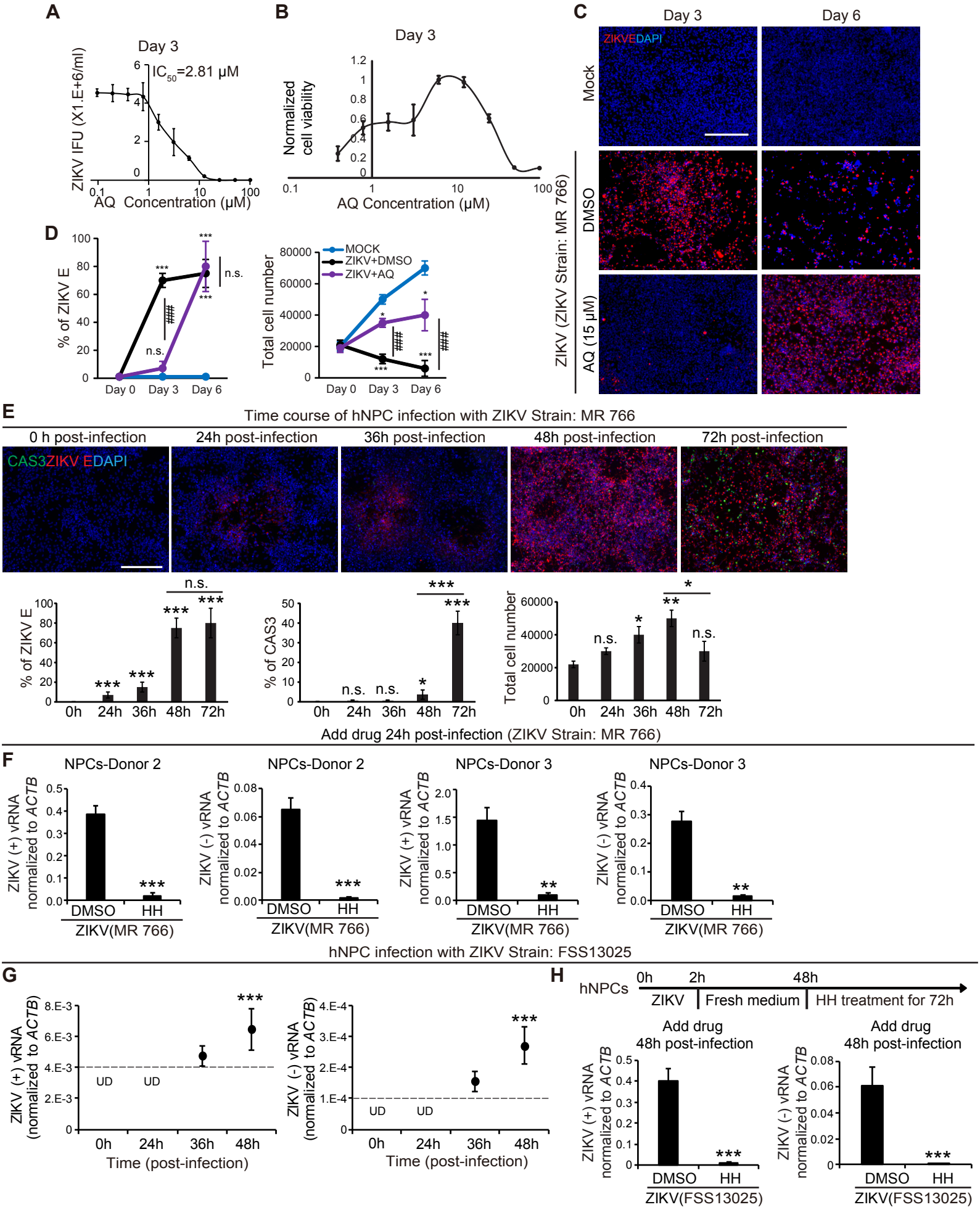

A
Day 3
IC50=2.81 μM
ZIKV IFU (X1.E+6/ml)
AQ Concentration (μM)
B
Day 3
Normalized cell viability
AQ Concentration (μM)
C
Day 3
Day 6
ZIKVEDAPI
Mock
DMSO
AQ (15 μM)
ZIKV (ZIKV Strain: MR 766)
D
% of ZIKV E
n.s.
Day 0
Day 3
Day 6
MOCK
ZIKV+DMSO
ZIKV+AQ
Total cell number
E
Time course of hNPC infection with ZIKV Strain: MR 766
0 h post-infection
24h post-infection
36h post-infection
48h post-infection
72h post-infection
CAS3ZIKV EDAPI
% of ZIKV E
% of CAS3
Total cell number
0h
24h
36h
48h
72h
Add drug 24h post-infection (ZIKV Strain: MR 766)
F
NPCs-Donor 2
NPCs-Donor 2
NPCs-Donor 3
NPCs-Donor 3
ZIKV (+) vRNA normalized to ACTB
ZIKV (-) vRNA normalized to ACTB
DMSO
HH
ZIKV(MR 766)
hNPC infection with ZIKV Strain: FSS13025
G
ZIKV (+) vRNA (normalized to ACTB)
ZIKV (-) vRNA (normalized to ACTB)
UD
Time (post-infection)
H
hNPCs
0h
2h
48h
ZIKV
Fresh medium
HH treatment for 72h
Add drug 48h post-infection
Add drug 48h post-infection
ZIKV(FSS13025)
ZIKV(FSS13025)

Figure S2. HH, but not AQ, efficiently eliminates virus in ZIKV infected hNPCs. Related to Figure 2.
(A) Inhibitory curve of AQ calculated based on infectious particles in supernatant as determined by the Vero reinfection assay (n=3).
(B) Effective curve of AQ calculated based on normalized cell viability (n=3).
(C and D) Immunocytochemistry analysis (C) and quantification of the percentage of ZIKV E^+ cells and total cell number (D) of hNPCs after ZIKV infection treated with 15 μM AQ or DMSO or mock infection (n=3). Cells were fixed and stained for ZIKV E (red) and with DAPI (blue). Scale bars, 100 μm. *p* values were calculated by one-way repeated measures ANOVA with a Bonferroni test for multiple comparisons. **p*<0.05, ***p*<0.01 and ****p*<0.001.
(E) Time course staining and quantification of the percentage of ZIKV E^+ cells, the percentage of $CAS3^+$ cells, and the total cell number at 24 h, 36 h, 48 h and 72 h post-infection.(n=3)..
(F) qRT-PCR analysis of (+) strand and replicating (-) strand of ZIKV (MR766 strain) vRNA level in hNPCs from two additional donors. hNPCs from different donors were treated with 25 μM HH at 24 h post-infection.
(G) The expression dynamics of ZIKV vRNA (FSS13025 strain) in hNPCs. qRT-PCR analysis to quantify the (+) strand and replicating (-) strand ZIKV vRNA levels in hNPCs at different time points post-infection. The dash line shows qRT-PCR detection limit. *p* values were calculated by one-way repeated measures ANOVA with a Bonferroni test for multiple comparisons. **p*<0.05, ***p*<0.01 and ****p*<0.001. UD, undetectable.
(H) qRT-PCR analysis of hNPCs that were treated with 25 μM HH or DMSO at 48 h post-ZIKV (FSS13025 strain) infection and maintained for additional 3 days.
p values were calculated by unpaired two-tailed Student's t-test. **p*<0.05, ***p*<0.01 and ****p*<0.001, unless otherwise stated.

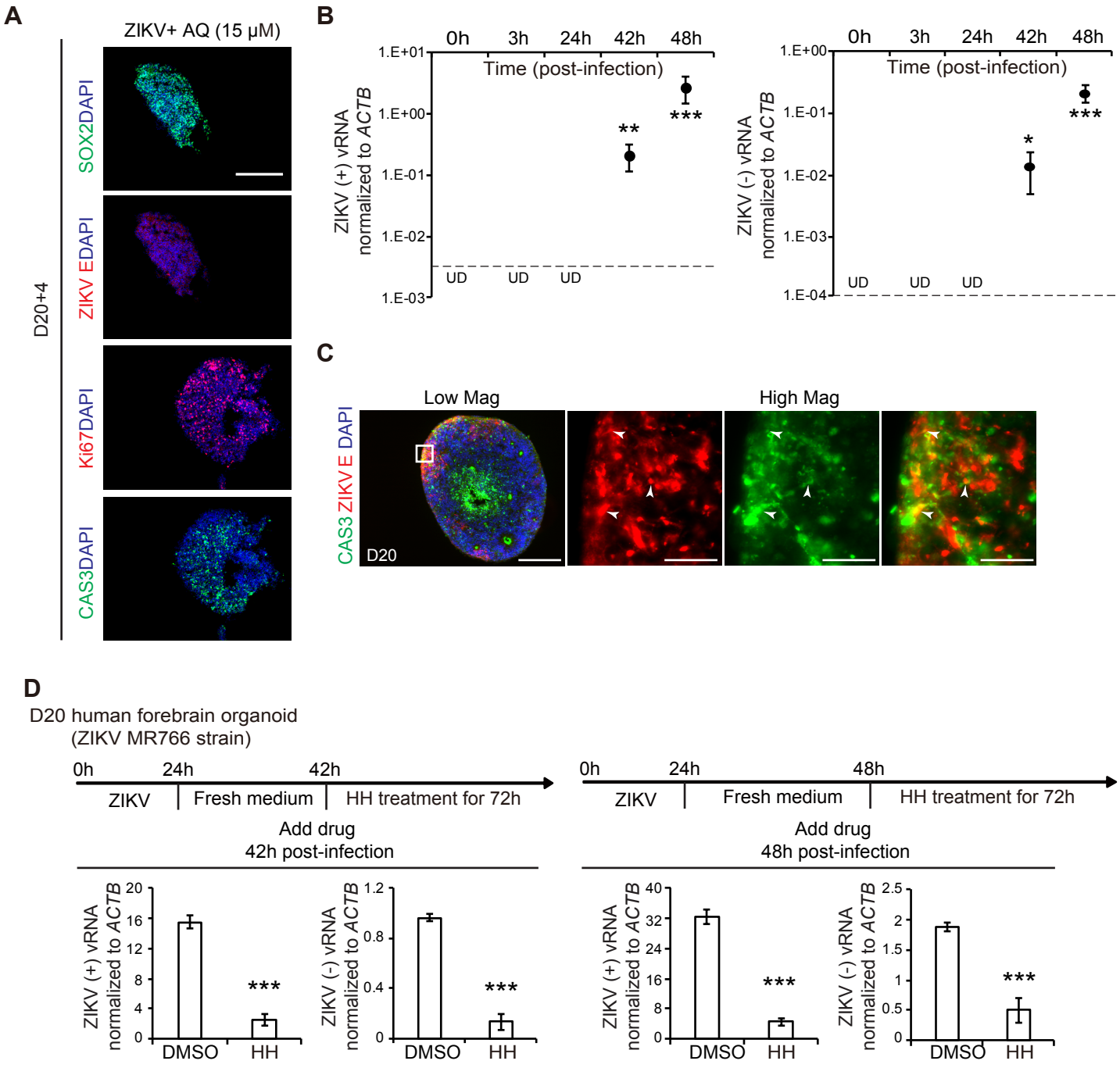

A
ZIKV+ AQ (15 μM)
D20+4
SOX2DAPI
ZIKV EDAPI
Ki67DAPI
CAS3DAPI
B
0h 3h 24h 42h 48h
Time (post-infection)
ZIKV (+) vRNA normalized to ACTB
1.E+01
1.E+00
1.E-01
1.E-02
1.E-03
UD UD UD
**
***
ZIKV (-) vRNA normalized to ACTB
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
*
C
Low Mag
High Mag
CAS3 ZIKV E DAPI
D20
D
D20 human forebrain organoid
(ZIKV MR766 strain)
0h 24h 42h
ZIKV
Fresh medium
HH treatment for 72h
Add drug
42h post-infection
0h 24h 48h
48h post-infection
ZIKV (+) vRNA normalized to ACTB
ZIKV (-) vRNA normalized to ACTB
DMSO
HH

Figure S3. HH, but not AQ, suppresses virus propagation in ZIKV-infected human forebrain organoids. Related to Figure 3.

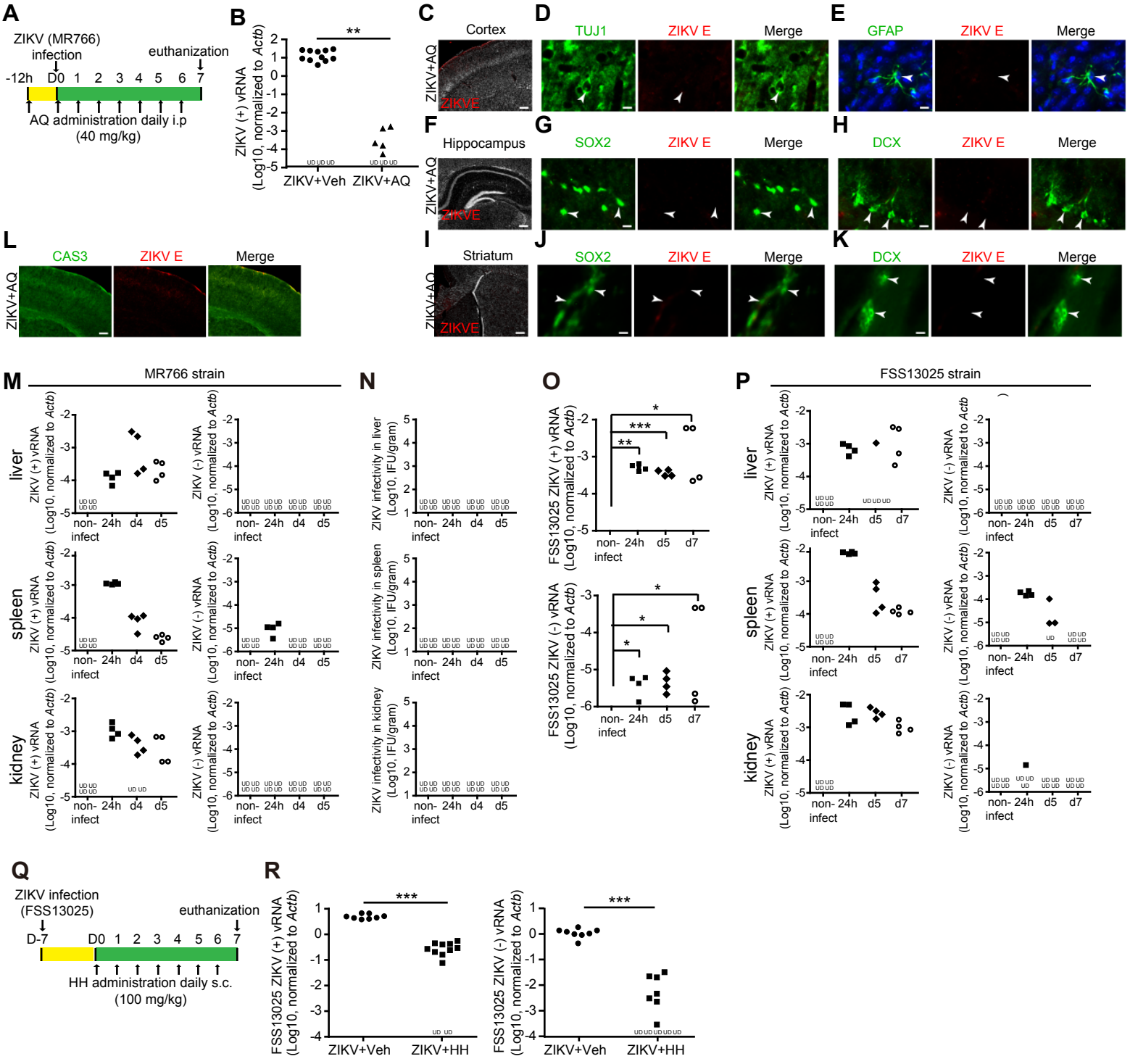
(A) AQ reduced ZIKV infection, but also induced toxicity in short-term organoid cultures. Immunocytochemical analysis of D20+4 ZIKV-infected organoids treated with 15 μM AQ or DMSO or with mock infection (n=3). D20+4 organoids were stained for SOX2 (green), ZIKV E (red) and with DAPI to monitor the percentage of ZIKV E^+ cells. D20+4 organoids were also stained for Ki67 (green), CAS3 (red) and with DAPI to monitor the cell proliferation and apoptosis rate. Treatment with AQ reduces ZIKV infection, but increases cell apoptosis rate. Scale bars, 100 μm.

(B) The expression dynamics of ZIKV vRNA (MR766 strain) in human forebrain organoids. qRT-PCR analysis to quantify the (+) strand and replicating (-) strand vRNA levels in the human forebrain organoids at different time points post-infection. The dash line shows qRT-PCR detection limit. *p* values were calculated by one-way repeated measures ANOVA with a Bonferroni test for multiple comparisons. **p*<0.05, ***p*<0.01 and ****p*<0.001. UD, undetectable.

(C) The immunostaining of ZIKV E and CAS3 in human forebrain organoids at 42 h post-infection. Scale bars, 100 μm in low mag figure panel; Scale bars, 20 μm in high mag figure panels.

(D) qRT-PCR analysis of human forebrain organoids that were treated with 25 μM HH or DMSO at 42 h or 48 h post-ZIKV (MR766 strain) infection and maintained for additional 3 days. *p* values were calculated by unpaired two-tailed Student's t-test. **p*<0.05, ***p*<0.01 and ****p*<0.001, if not mentioned specifically.

Figure S4


A
ZIKV (MR766) infection
euthanization
-12h D0 1 2 3 4 5 6 7
AQ administration daily i.p (40 mg/kg)
B
ZIKV (+) vRNA (Log10, normalized to Actb)
ZIKV+Veh ZIKV+AQ
C
Cortex
ZIKV+AQ
ZIKVE
D
TUJ1
ZIKV E
Merge
E
GFAP
ZIKV E
Merge
F
Hippocampus
G
SOX2
H
DCX
I
Striatum
J
K
L
CAS3

M
MR766 strain
liver
spleen
kidney
ZIKV (+) vRNA (Log10, normalized to Actb)
ZIKV (-) vRNA (Log10, normalized to Actb)
non-infect
24h
d4
d5
N
ZIKV infectivity in liver (Log10, IFU/gram)
ZIKV infectivity in spleen (Log10, IFU/gram)
ZIKV infectivity in kidney (Log10, IFU/gram)
O
FSS13025 ZIKV (+) vRNA (Log10, normalized to Actb)
FSS13025 ZIKV (-) vRNA (Log10, normalized to Actb)
d7
P
FSS13025 strain

Q
ZIKV infection (FSS13025)
euthanization
D-7 D0 1 2 3 4 5 6 7
HH administration daily s.c. (100 mg/kg)
R
FSS13025 ZIKV (+) vRNA (Log10, normalized to Actb)
FSS13025 ZIKV (-) vRNA (Log10, normalized to Actb)
ZIKV+Veh ZIKV+HH

Figure S4. AQ and HH suppresses ZIKV infection *in vivo*. Related with Figure 4.

(A) Scheme of prophylactic treatment of AQ *in vivo*. AQ was administrated to SCID-beige mice 12 h before infection with MR766 virus strain. AQ was injected at a dose of 40 mg/kg/day subcutaneously and euthanized at day 7.

(B) qRT-PCR analysis of ZIKV vRNA (+) strand in brains of ZIKV-infected mice treated with vehicle (n=14) or AQ (n=8).

(C-E) Immunohistochemical analysis of ZIKV in adult cortex of AQ-treated ZIKV infected mice (C), and co-staining with neuron marker TUJ1 (D) and astrocyte marker GFAP (E). AQ suppresses infection in adult cortex. Scale bar=100 μm in (C) and 10 μm in (D, E).

(F-H) Immunohistochemical analysis of ZIKV in adult hippocampus of AQ-treated ZIKV infected mice (F), and co-staining with neural progenitor marker SOX2 (G) and immature neuron marker DCX (H). AQ suppresses infection in adult hippocampus. Scale bar, 100 μm in (F) and 10 μm in (G, H).

(I-K) Immunohistochemical analysis of ZIKV in adult striatum of AQ-treated ZIKV infected mice (I), and co-staining with neural progenitor marker SOX2 (J) and immature neuron marker DCX (K). AQ suppresses infection in adult striatum. Scale bar, 100 μm in (H) and 10 μm in (J, K).

(L) Immunohistochemical analysis of cell apoptosis in brain of ZIKV infected mice treated with AQ. No apoptosis is detected in the presence of AQ. Scale bar, 100 μm.

(M, N) Expression kinetics of (+) strand (S4M, left) and (-) strand (S4M, right) of ZIKV vRNA and infectious viral particles (N) of MR766 strain in liver, spleen, and kidney. n is larger or equal to 4 mice at each time points. The mice were inoculated with 1 $x10^6$ PFU of virus through intraperitoneal injection and then euthanized at 24 h, 4 days and 5 days post-infection.

(O) Expression kinetics of (+) strand vRNA (left) and (-) strand vRNA (right) of ZIKV (FSS13025 strain) in mouse brain. N is larger or equal to 4 mice at each time points. The mice were inoculated with (1 $x10^7$ PFU) through intraperitoneal injection. The mice were euthanized at 24 h, 5 days and 7 days post-infection.

(P) Expression kinetics of (+) strand (left) and (-) strand (right) of FSS13025 strain ZIKV vRNA in liver, spleen, and kidney. N is larger or equal to 4 mice at each time points. The mice were inoculated with 1 $x10^7$ PFU of virus through intraperitoneal injection and then euthanized at 24 h, 5 days and 7 days post-infection.

(Q) Scheme of therapeutic treatment of HH on ZIKV (FSS13025 strain) infected mice. The SCID-beige mice were infected with ZIKV (FSS13025 strain, 1 $x10^7$ PFU). The mice at 7 days post-infection were treated with 100 mg/kg HH or vehicle daily for 7 days. The mice were euthanized after 7 days treatment and RNA from brains was analyzed using qRT-PCR. The data were presented in Figure S4R.

(R) qRT-PCR analysis of (+) strand vRNA (left) and (-) strand vRNA (right) in the brains of mice treated with vehicle (n=8) or HH (n=12) for 7 days starting at day 7 post-infection.

p values were calculated by one-way repeated measures ANOVA or two-way repeated measures ANOVA with a Bonferroni test for multiple comparisons. **p*<0.05, ***p*<0.01, ****p*<0.001. UD, undetectable.

Related with Figure 4.

Table S1. Information of confirmed hit compounds. Related to Figure 1.

Chemical name	CAS number	Therapeutic group	Number in library
Proglumide	6620-60-6	Anti-ulcerative	240
Mometasone furoate	83919-23-7	Anti-inflammatory	572
Benzathine benzylpenicillin	5928-84-7	Anti-bacterial	1028
Canrenoic acid potassium salt	2181-04-6	Anti-hypercholesterolemic	148
Oxethazaine	126-27-2	Local anesthesic	58
Zaprinast	37762-06-4	Erectogen	335
Papaverine hydrochloride	61-25-6	Vasodilator	583
Hippeastrine hydrobromide	22352-41-6	Hypotensor	675
Amodiaquin dihydrochloride dihydrate	6398-98-7	Antimalarial	309

Table S2. Primer sets used in RT and qPCR, and probes used in qPCR. The strand-specific tag is highlighted by italic font. Related to Figure 1 to 4, and STAR Methods.

ZIKV (+) vRNA-RT	*TACTTGTACAGCTCGTCCATG*CCACTAACGTTCTTTTGCAGACAT
ZIKV (+) vRNA-PCR forward	CCGCTGCCCAACACAAG
ZIKV (+) vRNA-PCR reverse	TACTTGTACAGCTCGTCCATG
ZIKV (+) vRNA-PCR probe	5'-/56-FAM/AGCCTACCT/ZEN/TGACAAGCAATCAGACACTCAA/3IABkFQ/-3'
ZIKV (-) vRNA-RT	*AACAGCCACAACGTCTATATC*CCGCTGCCCAACACAAG
ZIKV (-) vRNA-PCR forward	AACAGCCACAACGTCTATATC
ZIKV (-) vRNA-PCR reverse	CCACTAACGTTCTTTTGCAGACAT
ZIKV (-) vRNA-PCR probe	5'-/56 FAM/TTGAGTGTC/ZEN/TGATTGCTTGTCAAGGTAGGCT/3IABkFQ/-3'
Human ACTB-RT	CCTGGATAGCAACGTACATGG
Human ACTB-PCR forward	CCTTGCACATGCCGGAG
Human ACTB-PCR reverse	ACAGAGCCTCGCCTTTG
Human ACTB-PCR probe	5'-/5HEX/TCATCCATG/ZEN/GTGAGCTGGCGG/3IABkFQ/-3'
Mouse Actb-RT	CTGGATGGCTACGTACATGC
Mouse Actb -PCR forward	ATGCCGGAGCCGTTGTC
Mouse Actb -PCR reverse	GCGAGCACAGCTTCTTTG
Mouse Actb -PCR probe	5'-/5HEX/CCGCCACCA/ZEN/GTTCGCCATG/3IABkFQ/-3'