Figure S1

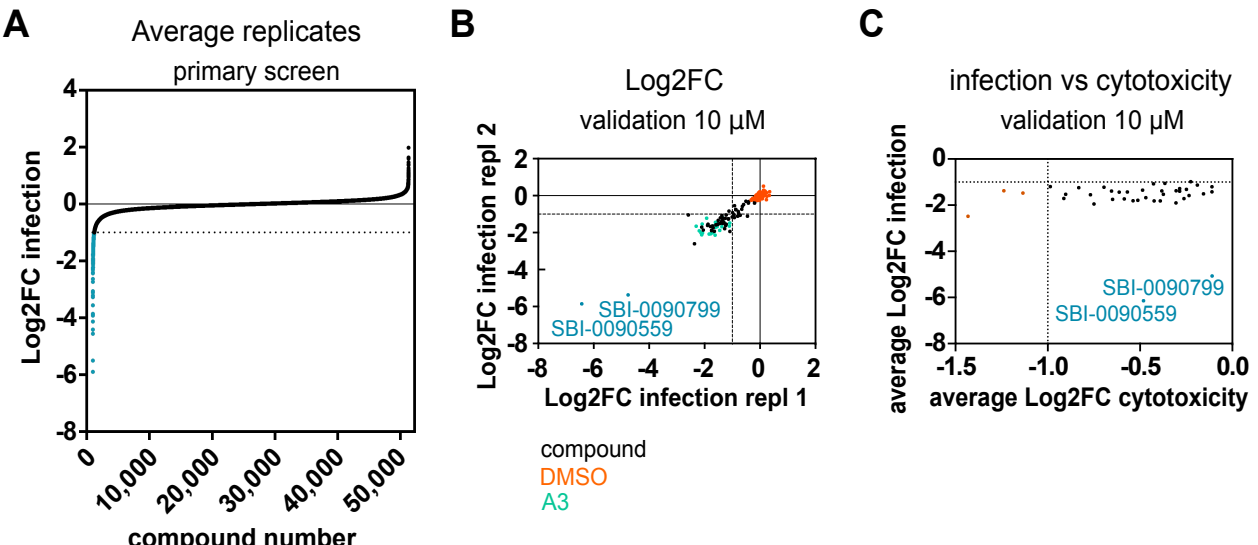
A
Average replicates
primary screen
Log2FC infection
compound number
B
Log2FC
validation 10 µM
Log2FC infection repl 2
Log2FC infection repl 1
SBI-0090799
SBI-0090559
compound
DMSO
A3
C
infection vs cytotoxicity
validation 10 µM
average Log2FC infection
average Log2FC cytotoxicity
SBI-0090799
SBI-0090559

Figure S1: Primary screen and validation. (A) The activity distribution of each compound based on the average of the Log_2 fold change (Log2FC) of each replicate for ZIKV % of infection is shown. Each dot indicates the average Log2FC of the screening replicates for each drug (black dots). The dotted line indicates the selection criteria corresponding to 50% inhibition of the infection after normalization. Compounds reaching that cut-off are indicated in cyan. (B) Correlation plot indicating the Log_2 fold change (Log2FC) of each compound (black dots) in the 2 replicates of the validation screen, after normalization to the median of each plate for ZIKV % of infection, across all screening plates. Values corresponding to DMSO (orange dots), and A3 (cyan dots) treated wells are also represented. (C) Dot plot indicating the average Log_2 fold change (Log2FC) of each compound in the 2 replicates of the validation screen, after normalization to the mean of the DMSO wells, for cell count (cytotoxicity, x axis) versus ZIKV % of infection (infection, y axis), for compounds reaching the criteria of selection of at least 50% inhibition of infection and less than 50% decrease in cell count (black dots). Compounds not meeting the cytotoxicity criteria are indicated by orange dots. The top2 compounds selected for follow-up studies are indicated in cyan.

Figure S2

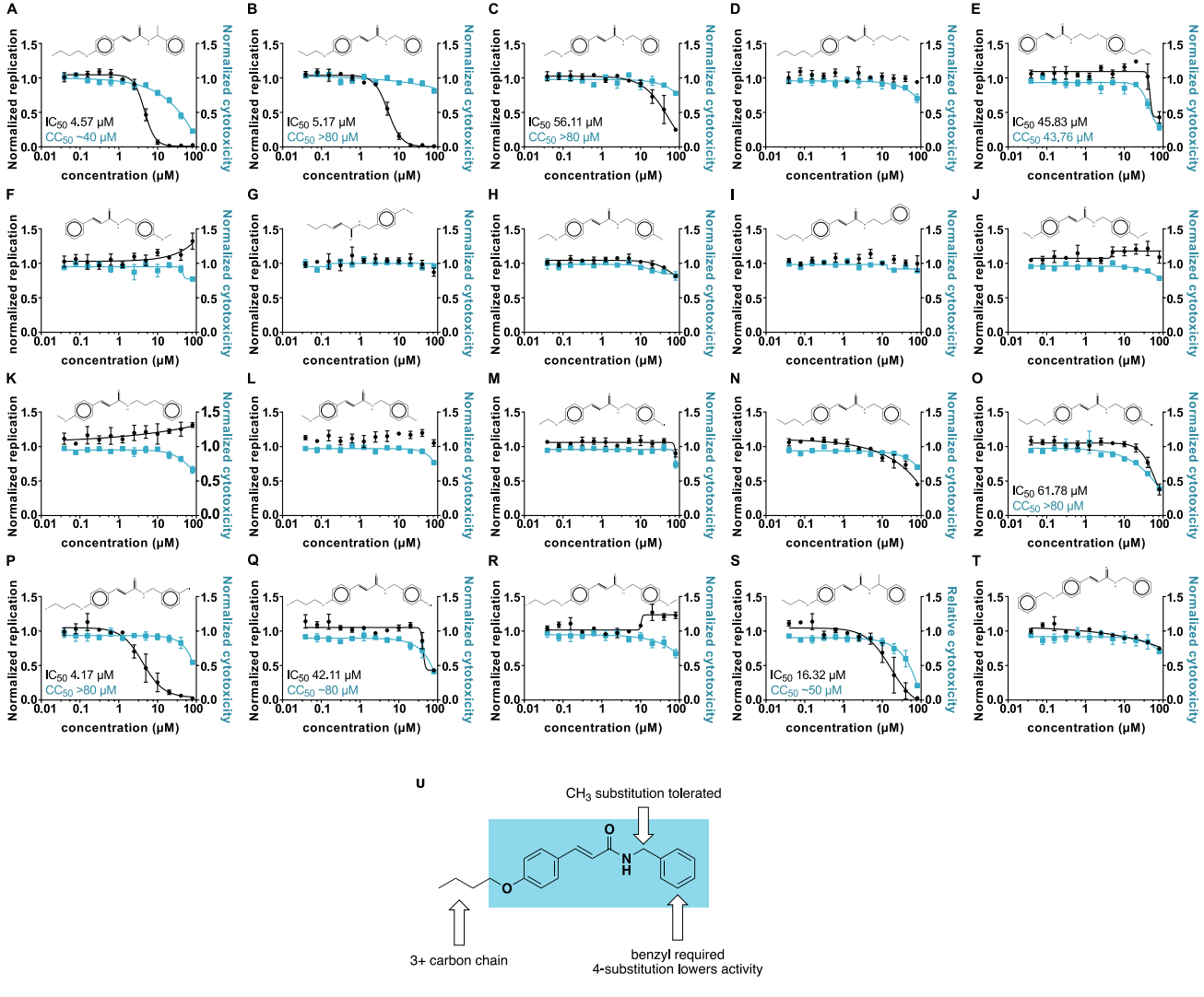
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
Normalized replication
Normalized cytotoxicity
Relative cytotoxicity
concentration (μM)
IC50 4.57 μM
CC50 ~40 μM
IC50 5.17 μM
CC50 >80 μM
IC50 56.11 μM
CC50 >80 μM
IC50 45.83 μM
CC50 43,76 μM
IC50 61.78 μM
CC50 >80 μM
IC50 4.17 μM
CC50 >80 μM
IC50 42.11 μM
CC50 ~80 μM
IC50 16.32 μM
CC50 ~50 μM
U
CH3 substitution tolerated
3+ carbon chain
benzyl required
4-substitution lowers activity

Figure S2: SAR studies on SBI-0090799. (A-T) Huh-7.5 cells were pre-treated for 16 h with increasing concentrations of the indicated compounds and then infected with ZIKV MR766 at MOI 0.3. Twenty-four h after infection, cells were fixed and analysed by immunofluorescence imaging. For each condition, the percentage of infection was calculated as the ratio of the number of infected cells stained for ZIKV envelope protein to number of cells stained with DAPI (left axis, black line). In parallel, Huh-7.5 cells were treated with increasing concentrations of compound, and 30 h later cell viability was assessed by measurement of the ATP content (right axis, cyan line). Data represent the mean ± SEM of n=3, normalized to mean values for DMSO-treated wells. Whenever possible, the anti-ZIKV IC_{50} values and the CC_{50} values were determined by non-linear regression analysis and are indicated. (U) Chemical structure of SBI-0090799 highlighting in blue the active part of the molecule and indicating the chemical requirements and tolerated substitutions for its antiviral activity.

Figure S3

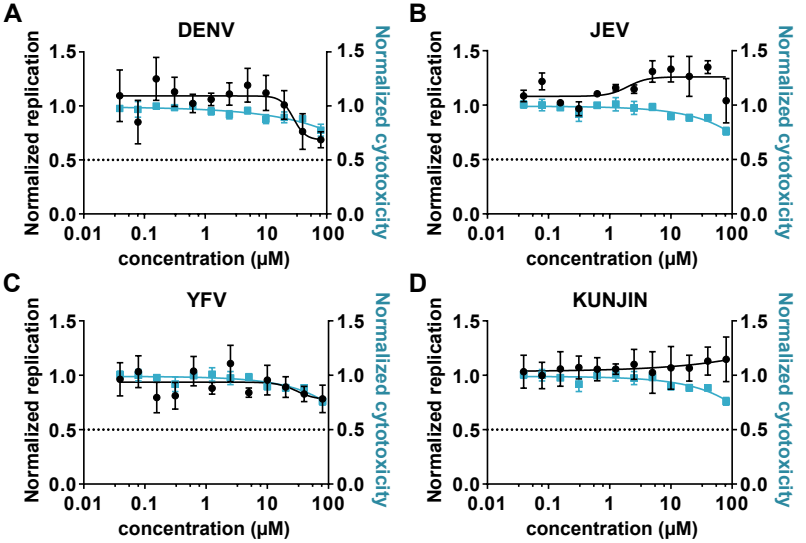
A
DENV
B
JEV
C
YFV
D
KUNJIN
Normalized replication
Normalized cytotoxicity
concentration (μM)
0.0
0.5
1.0
1.5
0.01
0.1
1
10
100

Figure S3: SBI-0090799 does not inhibit other mosquito borne flaviviruses. Huh-7.5 cells were pre-treated for 16 h with increasing concentrations of SBI-0090799 and then infected with DENV-2 16681 (A), JEV SA14-14-2 (B), YFV-17D (C), or KUNV (D) at an MOI 0.5, 2.5, 0.5 and 1.6, respectively. Twenty-four h after infection, cells were fixed and analysed by immunofluorescence imaging. For each condition, the percentage of infection was calculated as the ratio of the number of infected cells stained for the flaviviral envelope protein to the number of cells stained with DAPI (left axis, black line). In parallel, Huh-7.5 cells were treated with increasing concentrations of compound, and 30 h later cell viability was assessed by measurement of the ATP content (right axis, cyan line). Data represent the mean ± SEM of n=2 (A) or n=3 (B-D), normalized to mean values for DMSO-treated wells.

Figure S4

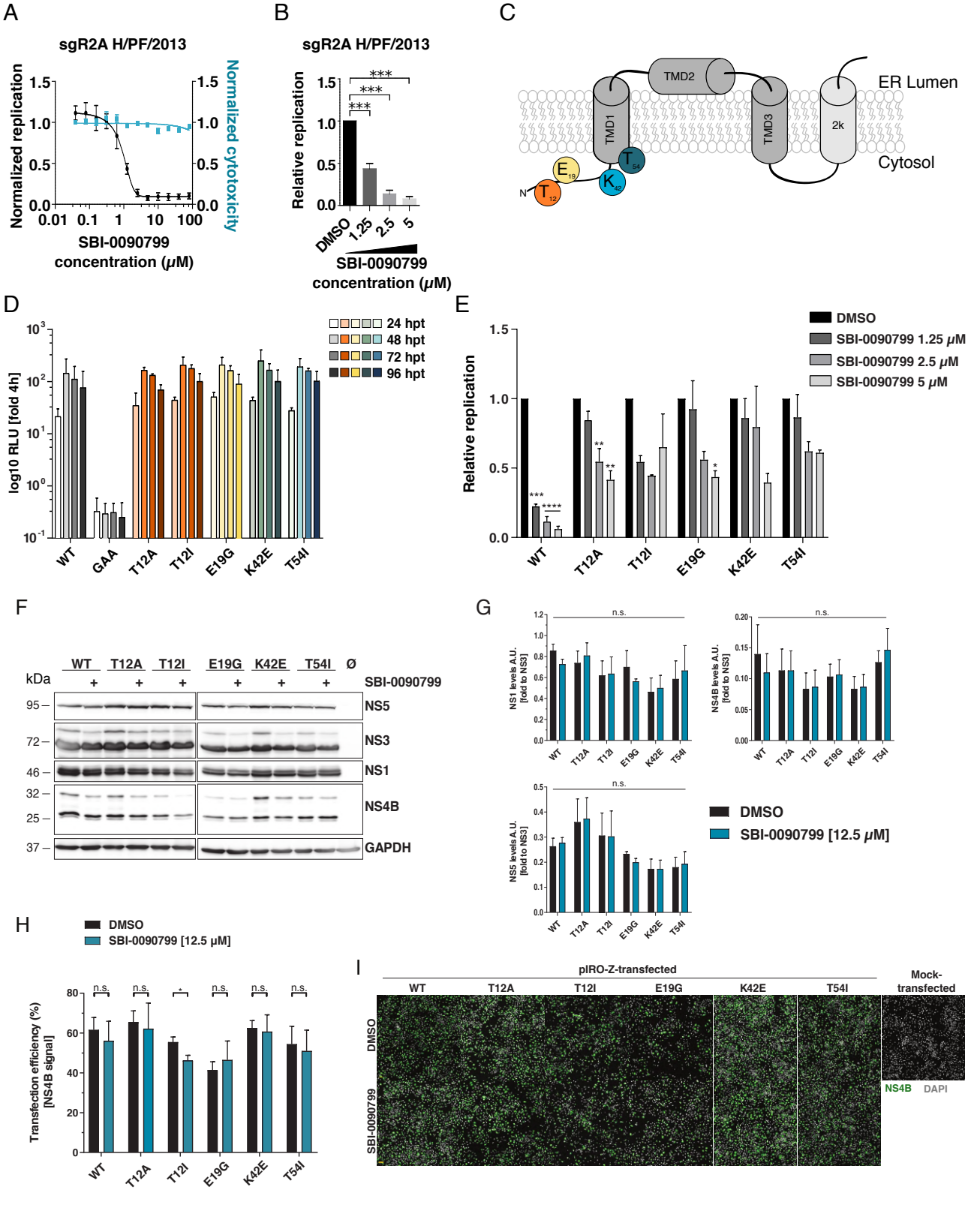
A
sgR2A H/PF/2013
Normalized replication
Normalized cytotoxicity
SBI-0090799 concentration (μM)
B
sgR2A H/PF/2013
Relative replication
DMSO
1.25
2.5
5
SBI-0090799 concentration (μM)
C
TMD2
TMD1
TMD3
2k
ER Lumen
Cytosol
D
log10 RLU [fold 4h]
24 hpt
48 hpt
72 hpt
96 hpt
WT
GAA
T12A
T12I
E19G
K42E
T54I
E
Relative replication
DMSO
SBI-0090799 1.25 μM
SBI-0090799 2.5 μM
SBI-0090799 5 μM
F
kDa
SBI-0090799
NS5
NS3
NS1
NS4B
GAPDH
G
NS1 levels A.U. [fold to NS3]
NS4B levels A.U. [fold to NS3]
NS5 levels A.U. [fold to NS3]
n.s.
DMSO
SBI-0090799 [12.5 μM]
H
DMSO
SBI-0090799 [12.5 μM]
Transfection efficiency (%) [NS4B signal]
I
pIRO-Z-transfected
Mock-transfected
DMSO
SBI-0090799
NS4B
DAPI

Figure S4: NS4A mutations conferring resistance against SBI-0090799 do not affect ZIKV replication fitness. (A-B) Effect of SBI-0090799 on the replication of the sgR2A H/PF/2013 (GeneBank ID KJ776791) replicon and viability of Huh7/Lunet-T7 cells treated with various concentrations of the compound. Viral replication was assessed by measuring the *Renilla* luciferase activity 24 h post-transfection. (A) The graph shows the percentage of replication (mean ± SEM) relative to the DMSO control. *Renilla* counts were normalized to 4 h post-transfection values reflecting transfection efficiency. Cell viability was determined by quantifying ATP levels 24 h post-treatment. The graph shows the percentage of ATP levels of compound treated cells (mean ± SEM) relative to DMSO treated control cells (triplicates per condition). (B) The graph shows the percentage of replication (mean ± SEM) relative to the DMSO control. (***, $p<0.0001$). (C) Schematic representation of the ZIKV NS4A membrane topology along with sites of resistance mutations. (D) Effects of NS4A mutations on ZIKV replication fitness was assessed by measuring the *Renilla* luciferase activity at 24 h, 48 h, 72 h, and 96 h post transfection. Relative light units (RLU) were normalized to the 4-hour value, reflecting transfection efficiency. The graph shows the mean of 2 independent experiments using 2 independent RNA preparations. (E) Viral replication fitness of NS4A wild-type and NS4A mutants was analyzed in the presence of 3 different concentrations of SBI-0090799. The graph shows the percentage of viral replication relative to the DMSO control from 2 independent experiments (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). (F) Huh7/Lunet-T7 cells were transfected with the respective pIRO-Z constructs in the presence of either SBI-0090799 [12.5 µM] or DMSO [0.125% v/v] and 18 h later cells were lysed and lysates were subjected to western blot analysis. Polyprotein processing and abundance of viral proteins was determined by probing for NS5, NS3, NS1 and NS4B. GAPDH was used as loading control. (G) Relative polyprotein processing efficiency and abundance of cleavage products was calculated by densitometry of the western blots and normalizing the signals of NS1, NS4B or NS5 to NS3 expression levels in each sample to the respective DMSO control. Values represent mean ± SEM of 3 independent experiments. n.s., not significant. (H) Transfection efficiencies were determined by quantifying the number of NS4B-positive cells using a custom-made macro for the FIJI software package. Error bars represent the SEM of three independent experiments (n.s., not significant; *, $p<0.05$). (I) Representative

immunofluorescence images of pIRO-Z transfected cells in the presence of either SBI-0090799 [12.5 μM] or DMSO [0.125% v/v], or with mock-transfected cells (Control). Nuclear DNA was stained with DAPI. Scale bar: 100 μm.

Figure S5

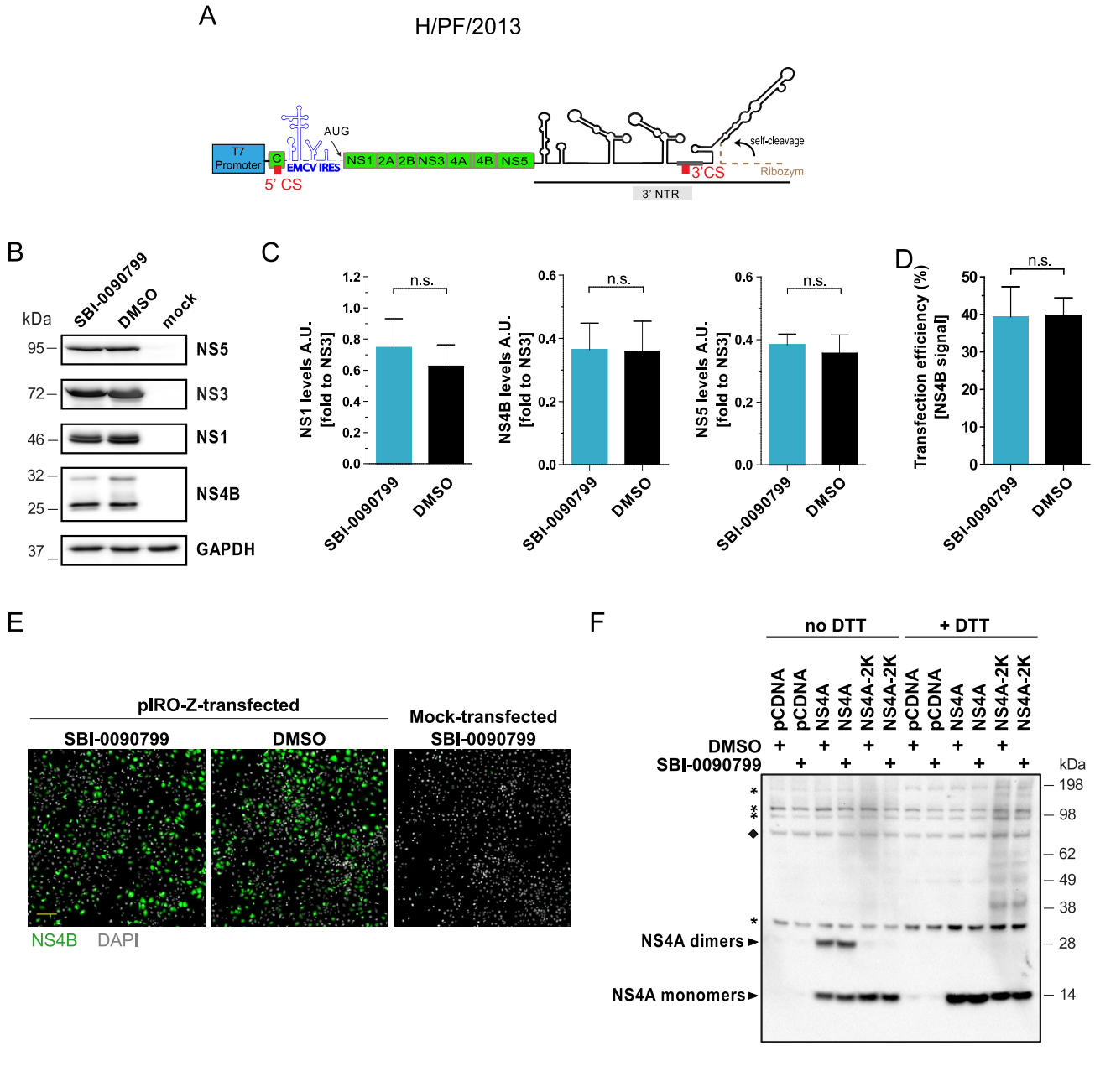
A
H/PF/2013
T7 Promoter
C
EMCV IRES
AUG
NS1 2A 2B NS3 4A 4B NS5
5' CS
3' CS
self-cleavage
Ribozym
3' NTR
B
kDa
SBI-0090799
DMSO
mock
95
72
46
32
25
37
NS5
NS3
NS1
NS4B
GAPDH
C
NS1 levels A.U. [fold to NS3]
NS4B levels A.U. [fold to NS3]
NS5 levels A.U. [fold to NS3]
n.s.
SBI-0090799
DMSO
D
Transfection efficiency (%) [NS4B signal]
n.s.
SBI-0090799
DMSO
E
pIRO-Z-transfected
Mock-transfected
SBI-0090799
DMSO
SBI-0090799
NS4B DAPI
F
no DTT
+ DTT
pCDNA
pCDNA
NS4A
NS4A
NS4A-2K
NS4A-2K
DMSO
SBI-0090799
kDa
198
98
62
49
38
28
14
NS4A dimers
NS4A monomers

Figure S5: SBI-0090799 does not affect pIRO-Z construct transfection efficiency and viral protein abundance. (A) Schematic representation of the pIRO-Z expression plasmid(24). It is composed of the T7 RNA polymerase promoter (blue box), a short sequence of the capsid coding region containing the 5' cyclization sequence (CS), the internal ribosome entry site (IRES) of the encephalomyocarditis virus (EMCV) to allow high level translation of the viral NS1 to NS5 polyprotein coding region and the 3' non-translated region. The hepatitis D virus ribozyme at the 3' end mediating self-cleavage ensures formation of the proper 3' end of the RNA in the cells. (B) Huh7/Lunet-T7 cells were transfected with the pIRO-Z construct in the presence of either SBI-0090799 [12.5 µM] or DMSO [0.125% v/v] for 18 h before being lysed and subjected to western blot analysis. Polyprotein processing was determined by probing for NS5, NS3, NS1 and NS4B. GAPDH was used as loading control. (C) Relative polyprotein processing efficiency was calculated by densitometry of the western blots and normalizing the signals of NS1, NS4B or NS5 to NS3 in each sample to the respective DMSO control. Values represent mean ± SEM of n=3 independent experiments. (D) Transfection efficiencies were determined by quantifying the number of NS4B containing cells using a custom-made macro for the FIJI software package. Error bars represent the SEM of n=3 independent experiments. (E) Representative NS4B-specific immunofluorescence images of pIRO-Z transfected cells treated with either SBI-0090799 [12.5 µM] or DMSO [0.125% v/v], or with mock-transfected and compound treated cells. Nuclear DNA was stained with DAPI. Scale bar: 100 µm. The differences in polyprotein processing efficiency, and transfection efficiency were analyzed using the two-tailed unpaired t-test with Welch's correction. n.s., not significant. (F) 293T/17 cells were transfected with pCDNA, ZIKV NS4A or NS4A-2K and treated 24h later with DMSO or SBI-0090799 [10 µM]. Forty-eight hours post-transfection, cells were harvested, and a western blot was performed comparing reducing (+DTT) and non-reducing (no DTT) conditions. NS4A multimerization was evaluated by immunoblotting of NS4A. Arrowheads indicate cNS4A monomers and dimers. Asterisks indicates a-specific bands recognized by the anti-NS4A antibody and indicating comparable loading conditions (square). An additional non-specific band recognized by the NS5 antiserum is shown as a loading control.