SUPPLEMENTARY DOCUMENT

SUPPLEMENTARY MATERIALS AND METHODS

Time-of-addition assay. A time-of-compound-addition assay was performed to approximate the step of the virus life cycle that was inhibited by compound. BHK21 cells were plated at a density of 5 × 10^4 cells per well in 24-well plates containing 0.5 ml of culture medium, followed by incubation for 16h at 37°C, and synchronously infected with DENV at an MOI of 0.1. At different indicated time points before or after infection, 12 μM of BP13944 or 0.12 % DMSO was treated to the cells. The culture medium were collected at 72 h post infection and subjected to plaque assay as described above. The mean values and S.E.M. from three independent experiments are plotted.

Protein sequence alignment. Sequences of NS3 protease domain (residues 1-184) of DENV-1 (strain Hawaii), DENV-2 (strain PL046), DENV-3 (strain H-87), DENV-4 (strain H-241), and JEV (strain RP9) were collected from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/). The sequences alignment was computed using ClustalW.

NS3 protein and unwinding reaction. The DENV-2 NS3 protein (strain PL046) was expressed as His tagged-SUMO-NS3 fusion protein in *E. coli* BL21 (DE3) cells. The His-SUMO-NS3 protein was purified by Ni-NTA column chromatography as previously described (83). The purified protein was treated with SUMO protease, and then the His tagged-SUMO domain and the full-length NS3 protein were resolved by a second Ni-NTA column chromatography, and the full-length NS3 protein was collected from the unbound fractions.

RNA oligos T16 and B41 and DNA oligo B16 were purchased from commercial source. The B41 RNA oligo was 5'-end labeled by [γ-^{32}P] ATP and T4 polynucleotide kinase. T16/B41 RNA duplex was made by heat-denaturation and renaturation of complementary molecules at a 1:1. The RNA duplex was gel purified for the unwinding assay.

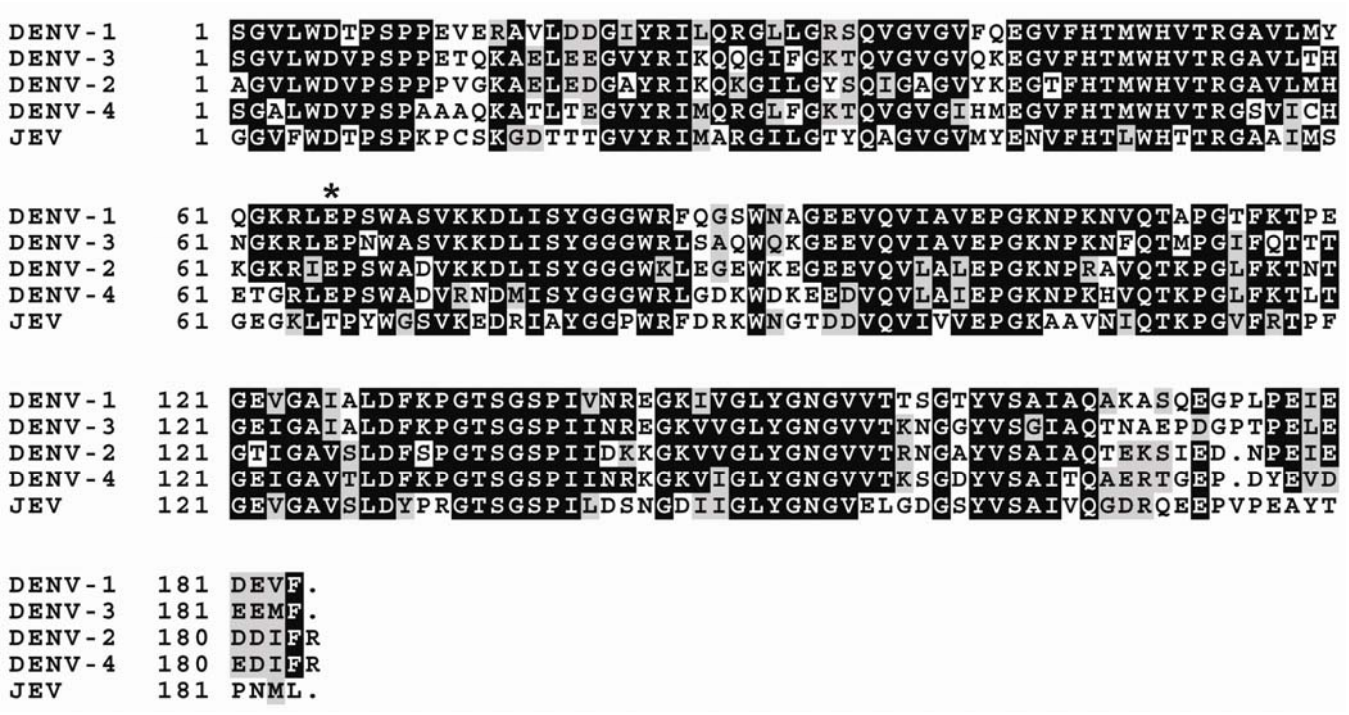
The standard unwinding assay contained 2 nM pre-formed, gel purified T16/B41 RNA duplex,

100 nM full-length NS3 protein, 20 mM HEPES (pH7.5), 2 mM DTT, 10 mM NaCl, 1 mM $MgCl_2$/ATP, 500 nM of trap DNA B16, and 10% DMSO or BP13944 of the indicated concentration.

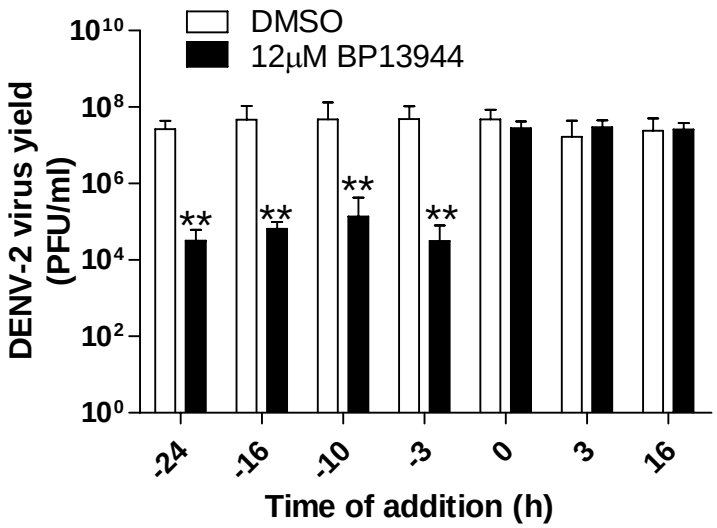
The RNA duplex, NS3 protein, and 10% DMSO or BP13944 were pre-incubated in the reaction buffer for 30 min at 37°C. The unwinding reaction was initiated by the addition of $MgCl_2$/ATP and B16 trap DNA. After a 30 min reaction at 37°C, the unwinding reaction was terminated by the treatment of protease K (in 1% SDS and 0.1 M EDTA) for 30 min. Different nucleic acid species were resolved on a 20% polyacrylamide-3 M urea-0.5x TBE gel at 4^oC. The gel was dried, and the radiolabeled RNA species were quantified using Phosphoimager and Image Quant software.

SUPPLEMENTARY TABLE 1. Oligonucleotide primers, hydrolysis probe, RNA and DNA oligos employed for cDNA synthesis, PCR amplification, and unwinding reation

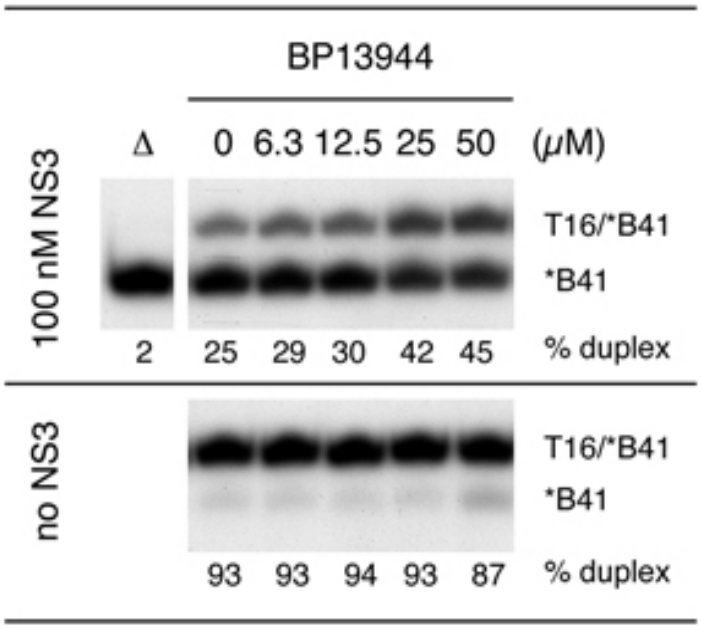
Oligonucleotides	Sequence
SacI-Sp6-5UTR-F	5′-AGTGAATTGTAATACGACTCACTATAGGGCGAATTGGAGCTCATTTAGGTGACACTATAGAGTTG-3′
C102-R	5′-GGGGTCGTACACCTTGGAAGCCAT TGCAGTTCTGCGCCTCCTGTTCAA-3′
Neo-R	5′-GAAGAACTCGTCAAGAAGGCGATA-3′
DV2.PS-R	5′-AGAACCTGTTGATTCACAAGC-3′
DV2.L1-R	5′-CATTCCATTTTCTGGCGTTCT-3′
DV2.U2-F	5’-AAGGTGAGATGAAGCTGTAGTCTC-3’
DV2.P1	5’-CTGTCTCCTCAGCATCATTCCAGGCA-3’
IRES-F	5’-CTCAAGCGTATTCAACAAGGG-3’
pCMV-F	5′- TGTCGAGTTTACTCCCTATCAGTGATAGAGAACGTATGTCGAGGTAGGCGTGTACGGTGG -3′
CMV-5′UTR-R	5′-GTCGGTCCACGTAGACTAACAACTGACGGTTCACTAAACGAGCTCTGC-3′
CMV-5′UTR-F	5′-GCAGAGCTCGTTTAGTGAACCGTCAGTTGTTAGTCTACGTGGACCGAC-3′
3UTR-F	5′-GAAGCAGGAGTTCTGTGGTAGAAAGC-3′
HDVr-R	5′-GGGCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGTCCCGCCTCCCTTAGCCATCCGAGTGGACG TGCGTCCTCCTTCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCAGAACC TGTTGATTCAACAGCACCATTCCATTTTCTG-3′
D2/3817-F	5′-AGCACCATACCAGAGACC-3′
E66G-F	5′-AAAGAGGATTGGACCATCATGGG-3′
E66G-R	5′-CCCATGATGGTCCAATCCTCTTT-3′
D2/5800-R	5’-TAGTATAACTGGTTTCATGCAGCG-3’
T16	5'-AGCACCGUAAAGACGC-3'
B41	5’-GCGUCUUUACGGUGCUUAAAACAAAACAAAACAAAACAAAA-3’
B16	5’-GCGTCTTTACGGTGCT-3’



Supplementary Figure 1. Alignment of the amino acid sequences of NS3 protease domain from DENV-1, 2, 3, and 4, and JEV. The amino acid sequence alignments of the NS3 protease domain (residues 1-184) of DENV-1 (strain Hawaii), DENV-2 (strain PL046), DENV-3 (strain H-87), DENV-4 (strain H-241), and JEV (strain RP9) are presented. Black boxes indicate identical and conserved amino acids. * indicates the BP13944 drug resistance mutation.



Supplementary Figure 2. Time-of-addition analysis of BP13944. BHK-21 cells were synchronously infected with DENV-2 at M.O.I of 0.1, and treated with 12 μM of BP13944 or 0.12 % DMSO at the indicated times before or after infection. Time point 0 h indicated DENV-2 addition time. The culture medium at 72 h post-infection were collected and quantified for viral yields. (** $P < 0.0001$) (n=3) The mean values and S.E.M. from three independent experiments are plotted.



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48 **Supplementary Figure 3. The effect of BP13944 on DENV NS3 unwinding activity.** Representative
49 gels of the unwinding of 3'-tailed RNA duplex T16/B41 by 100 nM NS3 protein (top panel) or no NS3
50 protein (bottom panel) in the presence of different concentration of BP13944. Reaction mixtures were
51 treated with proteinase K and analyzed on a 20% polyacrylamide-3 M urea gel. Substrate duplex
52 T16/B41 and the unwound B41 are shown at the left of the autoradiograph. The remaining RNA duplex
53 after a 30 min reaction at 37^{o}C is indicated at the bottom of the autoradiograph. "Δ" represents the
54 heat-denatured T16/B41 RNA duplex.

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