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A novel flavivirus entry inhibitor, BP34610, discovered through high-throughput screening with dengue reporter viruses

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ABSTRACT

Dengue virus (DENV) is a global health problem that affects approximately 3.9 billion people worldwide. Since safety concerns were raised for the only licensed vaccine, Dengvaxia, and since the present treatment is only supportive care, the development of more effective therapeutic anti-DENV agents is urgently needed. In this report, we identified a potential small-molecule inhibitor, BP34610, via cell-based high-throughput screening (HTS) of 12,000 compounds using DENV-2 reporter viruses. BP34610 reduced the virus yields of type 2 DENV-infected cells with a 50% effective concentration (EC_{50}) and selectivity index value of 0.48 ± 0.06 μM and 197, respectively. Without detectable cytotoxicity, the compound inhibited not only all four serotypes of DENV but also Japanese encephalitis virus (JEV). Time-of-addition experiments suggested that BP34610 may act at an early stage of DENV virus infection. Sequencing analyses of several individual clones derived from BP34610-resistant viruses revealed a consensus amino acid substitution (S397P) in the N-terminal stem region of the E protein. Introduction of S397P into the DENV reporter viruses conferred an over 14.8-fold EC_{90} shift for BP34610. Importantly, the combination of BP34610 with a viral replication inhibitor, ribavirin, displayed synergistic enhancement of anti-DENV-2 activity. Our results identify an effective small-molecule inhibitor, BP34610, which likely targets the DENV E protein. BP34610 could be developed as an anti-flavivirus agent in the future.

1. Introduction

Dengue virus (DENV) (serotypes 1–4), a mosquito-borne virus, belongs to the family *Flaviviridae*, a group of enveloped RNA viruses. More than 70 members of the *Flavivirus* genus are important human pathogens that cause significant morbidity and mortality (Ye et al., 2013). DENV is a public health threat to an estimated 3.9 billion people living in dengue epidemic areas and leads to 390 million human infections annually (Bhatt et al., 2013; Murray et al., 2013). Approximately 500,000 cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) have been reported among more than 128 countries, causing over 20,000 dengue-related deaths per year (Organization., 2012; Shepard et al., 2011; World Health Organization., 2012).

DENV consists of a 10.7-kb single-stranded, positive-polarity RNA genome associated with multiple copies of capsid proteins. DENV RNA is translated as a single polyprotein upon entering the host cell and is cleaved by host proteases and the virus-encoded two-component protease (NS2B/NS3pro) into three structural proteins (capsid [C], premembrane [prM], and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) to initiate viral replication (Chambers et al., 1990; Falgout et al., 1991; Rice et al., 1985). Three structural proteins and host cell membranes form the viral particles.

The fusion protein of flaviviruses, known as E protein, is the external protein of the virion. The E protein contains three soluble domains (residues 1–394: central I domain, extended fingerlike domain II, and immunoglobulin-like domain III) and a membrane-proximal so-called “stem” region (residues 395–450), which mainly mediates viral

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entry in a pH-dependent manner (Stiasny and Heinz, 2006; Zhang et al., 2003). The configuration of the stem region changes throughout the viral entry/fusion/maturation process (Allison et al., 1999; Zhang et al., 2004). The E protein is highly flexible and undergoes significant conformational changes during the maturation and fusogenic stages of the viral life cycle (Bressanelli et al., 2004; Modis et al., 2004; Zhang et al., 2004).

In silico docking and compound library screening approaches have been applied to the drug discovery of entry inhibitors targeting various regions of the envelope protein. For example, one attractive target of entry inhibitors is located at the hydrophobic pocket between domains I and II of the E protein (Clark et al., 2016; De Burghgraeve et al., 2012; de Wispelaere et al., 2018; Kampmann et al., 2009; Kaptein et al., 2010; Lian et al., 2018; Modis et al., 2003; Pitts et al., 2019; Poh et al., 2009; Wang et al., 2009; Yang et al., 2007; Zhou et al., 2008). Since the stem region of the envelope protein needs to interact with the E trimer during DENV endosomal membrane fusion, the stem region has been regarded as a good target to identify molecules able to block the viral entry/fusion stage (Costin et al., 2010; Hrobowski et al., 2005; Lok et al., 2012; Nicholson et al., 2011; Schmidt et al., 2010a, b). Several entry inhibitors have been reported as heparin sulfate mimetics targeting the receptor binding site of the E protein (Alen et al., 2009, 2011; Hidari et al., 2008; Ichiyama et al., 2013; Pujol et al., 2012; Rees et al., 2008; Vervaeke et al., 2013). Despite the tremendous efforts to discover anti-DENV inhibitors, no clinically approved antiviral therapeutic agents are available for humans, and disease treatment is limited to supportive care (Lo and Perng, 2016; Noble et al., 2010; Rather et al., 2017; Simmons et al., 2012; World Health Organization. et al., 2009).

In this study, we performed a cell-based high-throughput screening (HTS) using DENV-2 reporter viruses to identify a small-molecule inhibitor of DENV, BP34610. In addition to all four serotypes of DENV, BP34610 significantly inhibited JEV viral yields. Time-of-addition experiments and drug resistance studies of BP34610 suggest that BP34610 may affect the early stage of viral entry and target the DENV E protein. Furthermore, the combination of BP34610 with ribavirin displayed a synergistic anti-DENV effect. The data presented here may provide a new combination therapeutic strategy for the treatment of flavivirus infection.

2. Materials and methods

2.1. Cell lines

Baby hamster kidney (BHK21) (ATCC CCL-10) and human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose and 5% fetal bovine serum (FBS). *Aedes albopictus* C6/36 cells (ATCC CRL-1660) were cultured in RPMI 1640 supplemented with 5% FBS. Virus-infected cells were grown in their respective media supplemented with 2% FBS. Huh7.5 cells were grown in DMEM supplemented with 4.5 g/L glucose, 10% FBS, and nonessential amino acids (NEAA, Gibco). All mammalian cells were cultured at 37 °C in 5% CO_2, and C6/36 cells were maintained at 28 °C in 5% CO_2.

2.2. Virus strains

Dengue virus type 1 (DENV-1) (strain Hawaii), dengue virus type 3 (DENV-3) (strain H87), and dengue virus type 4 (DENV-4) (strain H241) were provided by the Centers for Disease Control, Taiwan. DENV-2 (strain PL046) and Japanese encephalitis virus (JEV) (strain RP-9) (Chen et al., 1996) were both provided by C. L. Liao (Institute of Microbiology and Immunology, National Defense Medical Center, Taiwan). Infectious cDNA clone of DENV-2 strain 16681 was kindly provided by Dr. Nopporn Sittisombut (Sriburi et al., 2001; Yang et al., 2013). DEN viruses and JEV stock virus were prepared in C6/36 cells by infecting at a multiplicity of infection (MOI) of 0.1 and 0.01 PFU/cell, respectively. Then, the supernatant was harvested and stored in 20% FBS at −80 °C. Virus titers were determined by a plaque-forming assay on BHK21 cells. Normally, titers of DENV-2, JEV, and adenovirus type 5 (Ad5) virus stock are approximately 1×10^8, 1×10^9, and 1×10^{10} PFU/ml, respectively.

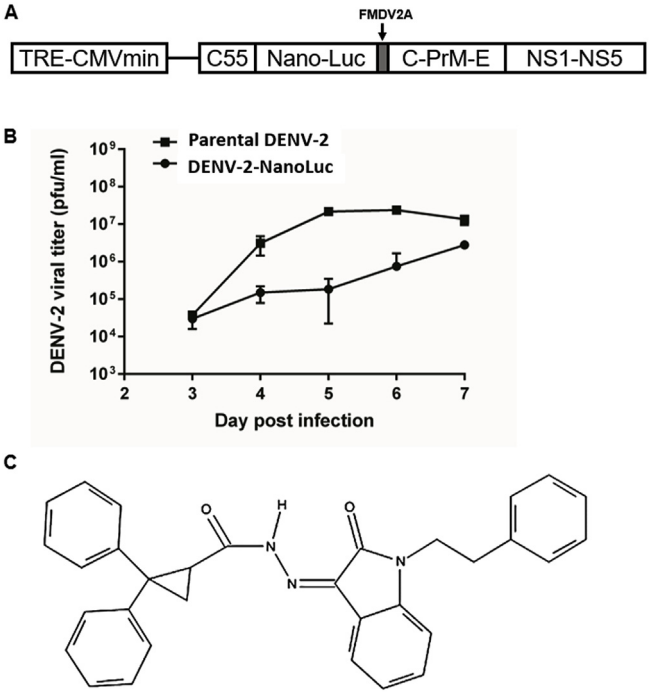


Fig. 1. (A) Schematic of the DNA-launched DENV-2 infectious cDNA clone, pDENV-2-NanoLuc, containing Nano luciferase reporter gene. The TRE-CMVmin promoter, Nano-Luc (inserted at the amino acid residue 55 of core gene), FMDV2A, DENV2 full genome, inverted HDV ribozyme, and SV40 poly-A signal are indicated. (B) Viral titer of DENV-2-Parental and DENV-2-NanoLuc. The mean values and S.E.M. from three independent experiments are plotted. (C) Chemical structure of BP34610.

2.3. Construction of DENV-2 reporter infectious cDNA clone

The Dengue virus serotype 2 (DENV-2) infectious cDNA clone, pTight-DENV2 (Pu et al., 2014), was used as a parental construct for the reporter virus. We inserted a 2422 b.p. Nano luciferase gene (Promega) in the 5′ end of the viral genome. Nano luciferase DNA fragment was inserted at the 261 b.p. of the viral genome to preserve the first 55 amino acids of core protein followed by FMDV2A autoprotease and full-length core protein (Fig. 1A). To add homologous recombination sites, primers pTight/F (5′-CGAGGTAGGCGTGTACGG-3′) and 1434/R (5′-TGGTGTTATTTTGATTTC-3′) were used to amplify the synthesized fragment. The construct was later cotransformed into yeast cells with pTight-DENV2 linearized by SacI and SphI to make pDENV-2-NanoLuc.

2.4. High-throughput screening

The high-throughput screening was done at the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Taiwan. The compound libraries used in this primary screening are purchased from Chemical Diversity Lab (San Diego, CA) and had > 95% purity. Compounds from a compound library with diverse structures were provided as DMSO stock solutions at 10 mM. Nano-Glo® Luciferase Assay (Promega) was applied to measure the luciferase activity of DENV-2 reporter infectious clone by incubating with various compounds from compound libraries at the concentration of 10 μM.

2.5. Cytotoxicity assay

Sensitivity of cell lines to BP34610 were examined using a MTS-based tetrazolium reduction assay CellTiter 96 AQueous Non-Radioactive (Promega G5430). Briefly, BHK21 cells were plated at a density of 1.8×10^4 cells per well in 96-well plates containing 120 µl of culture medium for 6 h. Serial diluted compounds or DMSO (positive control) were added and incubated for an additional 72 h. MTS reagent was then added into each well and incubated for 1 h at 37 °C in a humidified 5% CO_2 atmosphere before reading at a wavelength of 490 nm using an ELISA plate reader. All data are presented as means ± S.E.M. from three independent experiments.

2.6. Plaque-forming assay

BHK21 cells were plated at a density of 2.25×10^5 cells per well in 6-well plates containing 1 ml of culture medium overnight, and then 0.1 ml of serially diluted virus solution was added to ~70–80% confluent BHK21 cells. After adsorption for 2 h, the virus solutions were replaced either with 0.75% methyl cellulose (Sigma, M-0512) containing DMEM and 2% FBS for DENV-2-infected cells or 1.2% methyl cellulose containing DMEM and 2% FBS for JEV-infected cells. On the sixth day postinfection, the methyl cellulose solution was removed from the wells, and the cells were fixed and stained with crystal violet solution (1% crystal violet, 0.64% NaCl and 2% formaldehyde) (Lin et al., 2000). The same protocol was applied for the plaque assay of adenovirus except HEK293 cells were used for adenovirus infection and 0.5% agarose for overlay.

2.7. Viral yield reduction assay in cultured cells

BHK21 cells were plated at a density of 1.5×10^5 cells per well in 12-well plates containing 0.5 ml of culture medium and incubated for 4 h at 37 °C. Compounds (500 µl) were added into wells for 16 h before the addition of DENV-2, JEV, or adenovirus. The plates were incubated for another 72 h (DENV-2 and JEV) or 24 h (adenovirus) at 37 °C in a humidified 5% CO_2 atmosphere. To quantify the viral yield from infected cells in the presence of BP34610, the supernatant of cells treated with the compound was harvested and subjected to viral titer determination by a plaque-forming assay in BHK21 (DENV or JEV) or HEK293 (adenovirus) cells. The mean values and S.E.M. were calculated from three independent experiments. The detection limit was set at 10 PFU/ml.

2.8. RNA-launched DENV-2 reporter replicon assay

RNA transcription, transfection and transient replicon assay. DENV-2 *Renilla* luciferase replicon plasmid, DV2Rep (Pu et al., 2011), was linearized with XbaI. DNA was phenol-chloroform extracted, precipitated, and used as a template for in vitro transcription with an SP6 Message mMachine kit (Ambion). The RNA was quantified with a spectrophotometer and stored at −80 °C. RNAs were transfected into BHK21 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transient replicon assay was performed for quantification of compound-mediated inhibition of viral translation and reduction of viral RNA synthesis. BHK21 cells were seeded in 24-well plates (1×10^4 cells per well) and incubated overnight. Lipofectamine 2000 (Invitrogen) was mixed with 0.5 µg of wild-type or mutant dengue virus replicon RNA for one-well transfection, and cells were transfected according to the manufacturer's instructions. BP34610 at 5 µM control medium was added to each well and assayed for luciferase activities at the times indicated. Duplicate wells were lysed for luminometry. To perform the luciferase assays, 10 µl of lysate was mixed with 50 µl of Renilla Luciferase Assay Reagent (Promega).

2.9. Isolation and characterization of BP34610-resistant virus

BHK21 cells (6×10^4 cells/well) were seeded in 24-well plates for 4 h and then treated with 5 µM BP34610 for 16 h. The cells were infected with parental DENV-2 or viruses from passages 1–8 at an MOI of 0.1. At 72 h postinfection, the supernatants were harvested from BHK21 cells for the next passage. After eight virus passages, total viral RNA from a pool of BP34610-resistant DENV-2 was collected, and virion RNAs were extracted using QIAamp Viral RNA kits (QIAGEN). Viral RNAs were amplified by RT-PCR using SuperScript III one-step RT-PCR kits (Invitrogen). The PCR products of Core to NS1 and NS1 to NS5B were gel-purified and subcloned in pTight-DENV2 (Pu et al., 2014) to replace the parental sequence through homologous recombination in yeast. Finally, BP34610-resistant infectious clones were purified from yeast cells and reamplified in *E. coli* strain C41. After enzyme digestion, only infectious clones harboring the right inserts were subjected to DNA sequencing analyses.

2.10. Construction of molecular clones containing specific mutations

To create point mutations derived from resistant clones, the amino acid substitution S397P was introduced into the pDENV-2-NanoLuc plasmid. The PCR products were gel-purified and joined by overlapping PCR to form fragments containing a single mutation for homologous recombination with linearized pDENV-2-NanoLuc plasmids. The mutant DENV-2 reporter infectious cDNA plasmids were purified from yeast cells and then reamplified and maintained in *E. coli* strain C41. All constructs were sequenced to confirm the presence of the desired mutation and ensure there were no additional changes.

2.11. Statistical analysis

The data were analyzed using GraphPad Prism software (San Diego, CA, USA). The statistical significance of the differences between the means of the experimental groups was analyzed using Student's *t*-test with Welch's correction to measure the two-tailed P values. P values less than 0.05 were considered statistically significant. Analysis of synergism was performed using the CalsuSyn software program (Biosoft, Ferguson, MO, USA). A combination index (CI) < 0.9 indicates a synergistic effect.

3. Results

The availability of a cell-based high-throughput DENV luciferase virus provides an ideal system to screen new antiviral compounds. In this study, we utilized an infectious luciferase virus of DENV-2 (strain 16681) propagated in BHK21 cells for high-throughput screening (HTS). The infectious luciferase viruses cDNA, pDENV-2-NanoLuc, was constructed by inserting Nano luciferase gene into the core gene within DENV-2 genome (Fig. 1A). The infectious luciferase viruses for HTS were harvested at 72 h post-transfection from transiently transfected HEK293 cells with pDENV-2-NanoLuc plasmids to avoid the possible instability of DENV-Luc virus for long-term passage. Characterization of harvested luciferase viruses by RT-PCR revealed the existence of Nanoluciferase gene. Compared to WT DENV-2, DENV-2-NLuc viruses displayed mild defect in the kinetics curve of virus titers derived from HEK293 cells transfected with infectious cDNA clone (Fig. 1B). HTS was performed in a 96-well format with a Z' value of 0.85. We started with a compound library containing 12,000 diverse compounds, of which 85 hit compounds were discovered to effectively suppress DENV-2 luciferase virus replication activity to less than 10% at 10 µM. Approximately 6 of the 85 hit compounds were obtained after second screening of their intrinsic cytotoxicity (a 50% cytotoxic concentration, CC_{50} > 15 µM) and false positive compounds. One of the inhibitor, BP34610 (Fig. 1C), inhibited virus replication with an EC_{50} of 0.48 ± 0.06 µM and inhibited 99% of DENV-2 virus yield at 5 µM

Table 1
The efficacy and cytotoxicity of BP34610 in cell-based assays.

BP34610 potency				
DENV-2 viral yield reduction (μM)[a]		Cytotoxicity MTS assay (μM)[b]		Selective index (CC_{50}/EC_{50})[c]
EC_{50}	EC_{90}	CC_{50}	CC_{90}	
0.48 ± 0.06	5.67 ± 2.17	94.55 ± 1.77	> 100	197

[a] EC_{50} and EC_{90}(effective concentration) values were derived from the 50% and 90% reduction in virus yield.
[b] CC_{50} and CC_{90}values were determined as a 50% and 90% drop in MTS signals. The values represent the means and STDEV from triplicate experiments.
[c] Selectivity index calculated from CC_{50} divided by EC_{50}.

Table 2
BP34610 (5 μM) selectively inhibits viral yield of flaviviruses.

	Virus titer (% of control)
Absence of compound	100
DENV-2	0.7 ± 0.05
JEV	0.7 ± 0.06
Adenovirus	84.6 ± 6.07

A plaque reduction assay of DENV-2, JEV, and adenovirus were employed. The numbers represent the percentages of values relative to the DMSO-treated controls (100%).

(Tables 1 and 2). To verify that the primary observed antiviral activity was not due to compound-mediated cytotoxicity, we performed a cytotoxicity assay based on the cellular metabolism of an MTS-based tetrazolium salt. No suppression of cell viability was observed when the cells were incubated with 50 μM BP34610. The CC_{50} of the compound was found to be 94.55 ± 1.77 μM, and the selectivity index (CC_{50}/EC_{50}) value was 197 (Table 1).

To examine the antiviral activity of BP34610, a viral yield reduction assay (Fig. 2) was performed against DENV-2. The DMSO- and BP34610-treated BHK21 cells were infected with DENV-2 virus (MOI of 0.1 or 1), and the viral titers in the culture medium were assayed at 72 h postinfection by plaque forming assay. The compound BP34610 reduced viral yields in a dose-dependent manner at MOIs of 0.1 and 1. At a concentration of 2 μM, BP34610 suppressed the DENV-2 virus by 100-fold at an MOI of 0.1. When compound concentration was increased to

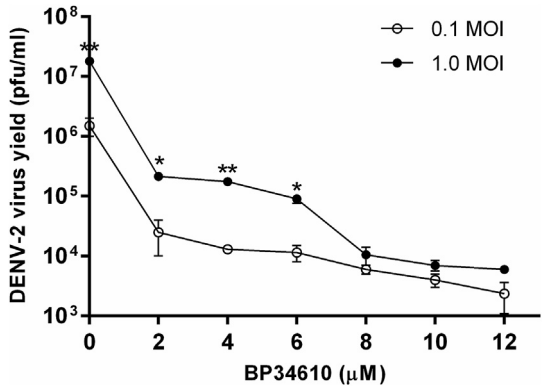
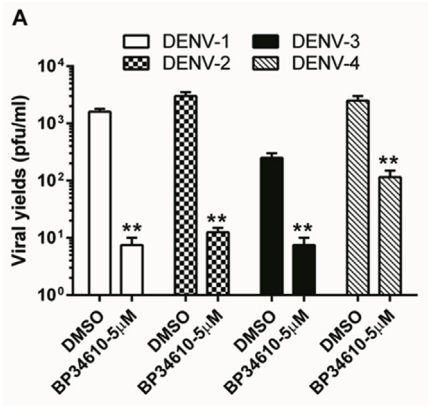


Fig. 2. Dose-dependent inhibition of DENV-2 by BP34610. The DENV-2 viral yield was calculated after treating BHK21 cells with increasing concentrations of BP34610 and then infecting them with DENV-2 at an MOI of 0.1 (open circles) and 1 (filled circles) as described in the Materials and Methods. The viral yields in culture medium at 72 h postinfection were determined by plaque-forming assays. The mean values and S.E.M. from two independent experiments are plotted. **, $P < 0.01$, and *, $P < 0.05$, compared with the o.1 MOI group. Statistical significance was calculated by using an unpaired Student *t*-test as described in Materials and Methods.



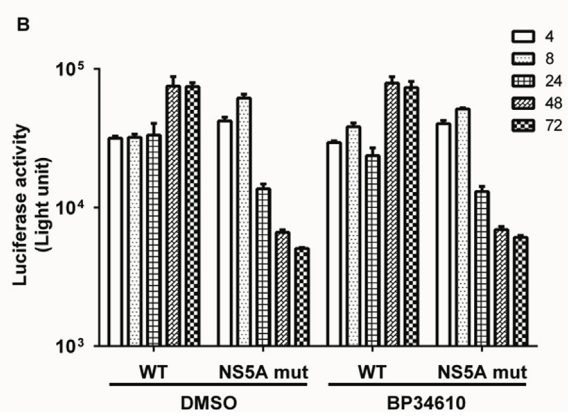


Fig. 3. Inhibition of all four serotypes of DENV by BP34610 but not at the stage of viral translation and replication. (A) BHK21 cells were incubated with 5 μM BP34610, and then infected with four serotypes of DENV at a MOI of 0.01. The viral yield in culture medium was determined by plaque-forming assays at 72 h postinfection. The mean values and S.E.M. from three independent experiments are plotted. **, $P < 0.001$. (B) Kinetics of transient expression of wild-type and replication-defective NS5A mutant dengue replicons in BHK21 cells. The luciferase activity in cytoplasmic extracts prepared from BHK21 cells transfected with either wild-type or NS5A mutant replicon RNAs was measured at different time points (4, 8, 24, 48, and 72 h).

12 μM, a 1000-fold reduction in the viral titer was observed at both MOIs of 0.1 and 1. BP34610 displayed better inhibitory effect on DENV-2 at MOI = 0.1 than MOI = 1 when the compound concentration is between 2 and 6 μM. These results indicate that BP34610 is a potential inhibitor of DENV-2. Furthermore, we performed a viral yield reduction assay in parallel with all four serotypes of DENV. As illustrated in Figs. 3 and 5 μM BP34610 decreased the viral titers of all four DENV serotypes by 10- to 100-fold at an MOI of 0.01. BP34610 dramatically decreased the viral loads of DENV serotypes 1 to 3 compared with that of serotype 4. These results indicate that BP34610 is a potentially therapeutic compound against all four serotypes of DENV.

To examine the antiviral spectrum of BP34610, a viral yield reduction assay was also performed against JEV, and adenovirus. BHK21 cells were infected with JEV or DENV (MOI of 0.1) in the presence or absence of 5 μM BP34610. HEK293 cells were infected with adenovirus (MOI of 0.1), respectively, in the presence or absence of 5 μM BP34610. Comparison of the effects of BP34610 on these four viruses demonstrated that the viral yields of JEV and DENV were dramatically reduced by 99% in the presence of BP34610 (Table 2). In contrast, BP34610 failed to suppress adenovirus, a DNA virus, replication (Table 2). These results indicate that BP34610 is a potential inhibitor of flaviviruses.

To further validate the mechanistic function of BP34610, either WT or defective NS5A mutant DENV-2 *Renilla* luciferase replicon plasmid, DV2Rep, was transcribed in vitro and transfected into BHK21 cells to

determine if BP34610 affects the viral translation or viral RNA synthesis. Because the luciferase activity peaked both within the first 8 h post-transfection and after 24 h post-transfection (representing viral translation and RNA replication, respectively), we measured luciferase activity at 4, 8, 24, 48, and 72 h post-transfection. Luciferase activity was monitored at 4, 8, 24, 48, and 72 h post-transfection (Fig. 3B). The luciferase activity of WT replicon reached a peak level at approximately 48 h post-transfection and was maintained at that level until 72 h post-transfection in the absence or presence of BP34610. In contrast, NS5A mutant replicon luciferase activity gradually decreased from 8 to 72 h post-transfection. BP34610 had a minimal effect on the Rluc signal detected from 4 to 72 h post-transfection of WT or NS5A mutant replicon in the presence or absence of BP34610 (Fig. 3B). The results indicated BP34610 does not apparently affect either viral translation or viral RNA replication.

To define the effect of BP34610 on viral entry, a time-of addition experiment was performed. DENV2 was incubated with cells in the presence of 5 μM BP34610 or DMSO (control). The cells were pre-incubated 24 h (−24 h), 18 h (−18 h), or 0 h (0 h) with BP34610, then infected with DENV-2 (MOI = 0.1) simply by adding viruses onto cells without changing medium. The medium was harvested for titers from infected cells at 72 h postinfection. For cells treated with BP34610 after virus infection, cells were infected with first DENV-2 (MOI = 0.1), treated with BP34610 at 2 h (+2 h) or 6 h (+6 h) postinfection by adding BP34610 on infected cells without changing medium. Similarly, the medium was harvested for titers from infected cells at 72 h postinfection. Viral titers were monitored at −24, −18, 0, +2, and +6 h postinfection. The viral titer was significantly reduced by 94% and 95% at −24 and −18 h incubation with BP34610, respectively (Fig. 4). Less reduction in viral titers was found when cells were first infected with DENV-2 and treated with BP34610 at 2 h or 6 h postinfection. The results implied BP34610 may inhibit DENV-2 at the early stage of virus infection.

Virus variants that are resistant to the inhibitory effects of a compound provide evidence that the compound is acting on a virus-specific target and not on a cellular target. To identify the antiviral mechanism and the molecular target of BP34610, we generated BP34610-resistant DENV-2 by eight serial passages of DENV-2 in BHK21 cells in the presence of 5 μM BP34610. Genomic RNA from viruses capable of growing in the presence of BP34610 was isolated and amplified by reverse transcription PCR to generate cDNA. The obtained sequencing data (Core to NS5) revealed that three of the four clones had accumulated a

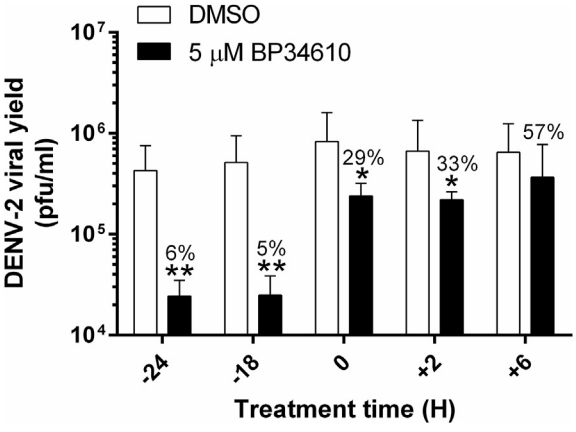


Fig. 4. Inhibition of DENV-2 at the early stage of infection by BP34610. BHK21 cells were treated with either 5 μM BP34610 or control DMSO at various time points of pre- or postinfection with DENV-2 at MOI = 0.1. Viral yield was measured at 72 h postinfection. The numbers above the BP34610-treated times represent the percentages of the viral titers relative to the DMSO-treated controls (100%). The error bars represent the S.E.M. from three independent experiments. *, $P < 0.05$; **, $P < 0.005$.

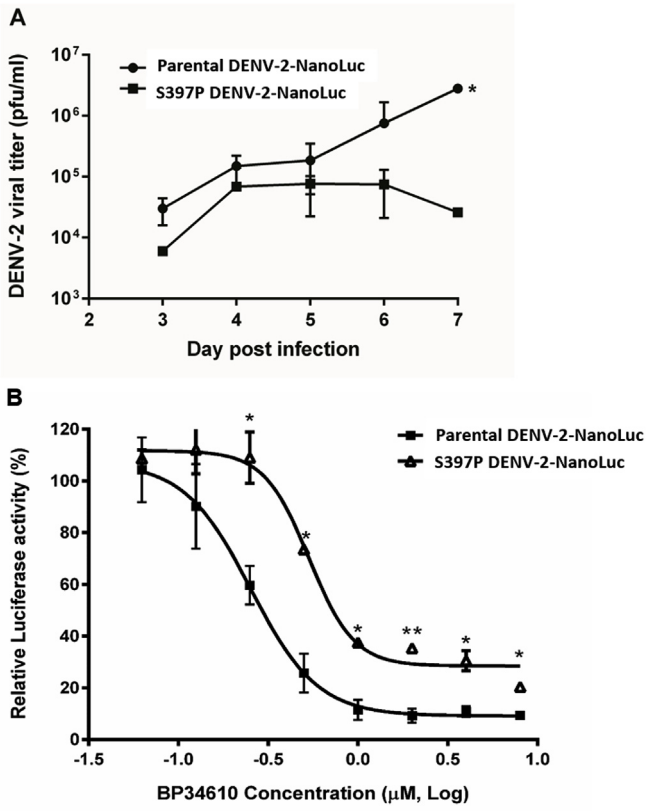


Fig. 5. S397P substitution within the DENV envelope domain in the DENV-2 luciferase virus conferred drug resistance to BP34610. (A) The growth kinetic curves of wild-type and S397P mutant DENV-2 luciferase viruses. Either WT or S397P pDENV-2-NanoLuc infectious cDNA plasmid was transfected into BHK21 cells, and the virus titers of each clone were monitored at the indicated time points postinfection. The mean values and S.E.M. from three independent experiments are plotted. *, $P < 0.05$, compared with the S397P group. (B) Drug resistance analyses of recombinant parental and S397P substitution DENV. Parental and S397P substituted DENV-2 luciferase virus were pre-incubated with BP34610 at the indicated concentrations for 1 h. BHK21 cells were infected with the compound-treated DENV-2 luciferase virus at an MOI of 0.05. The EC_{50}s were determined by calculating the luciferase activity at 24 h postinfection. The mean values and S.E.M. from two independent experiments are plotted. **, $P < 0.01$, and *, $P < 0.05$, compared with the parental group.

serine (S)-to-proline (P) substitution at amino acid position 397 of the Envelope gene (Table 3). The other mutations were found to be sporadic mutations within the Core to NS5 region. The DNA sequencing results implied that the consensus S397P substitution may be related to drug resistance to BP34610.

To determine if the S397P mutation affects DENV-2 replication, the S397P substitution was introduced into the envelope domain of the parental full-length DENV-2 infectious cDNA clone, pDENV-2-NanoLuc. Virus spreading assay was performed to compare the virus titers of transfected BHK21 cells with either WT or S397P pDENV-2-NanoLuc infectious cDNA plasmid (Fig. 5A). Apparently, the virus titers derived from BHK21 cells transfected with S397P infectious cDNA plasmid were slightly lower than those transfected with WT infectious cDNA plasmid three to seven days post-transfection. The results suggested the successful isolation of S397P-resistant mutation is not due to the advantageous growth curve of S397P virus.

To confirm that the S397P mutation was correlated with reduced drug susceptibility, the S397P substitution was introduced into the envelope domain of the parental full-length DENV-2 infectious cDNA clone with a Nano-Luc reporter, pDENV-2-NanoLuc. The grow kinetics curve of S397P reporter viruses displayed slower spreading activity compared to parental DENV-2 reporter viruses (Fig. 5A). The S397P-

Table 3
Sequence analysis of the mutant gene from clones derived from BP34610-resistant DENV-2.

Gene	Encoded amino acid at position:													
	Envelope						NS2B		NS3					NS4A
a.a. position	129	139	284	332	397	459	60	114	47	147	300	327	402	17
Parental	V	I	K	P	S	I	K	I	H	V	I	Q	W	M
Clone														
1					P						T		R	
2		L			P		I							
3	A			L	P			M		A		R		I
4			R			T			Y					

substituted DENV-2 reporter virus showed reduced susceptibility to BP34610 (EC_{50} of 0.72 ± 0.13 μM), with an EC_{50} value 4-fold higher than that of the wild-type DENV-2 reporter virus (0.18 ± 0.02 μM). Interestingly, the EC_{90}s for the parental and substitution reporter viruses were calculated as 0.54 ± 0.04 μM and > 8 μM, respectively (Fig. 5B). The S397P-substituted reporter virus exhibited dramatically higher resistance than the parental DENV-2 reporter virus. These findings indicate that the S397P substitution is sufficient to reduce BP34610 susceptibility.

Standard care or single-agent therapies for viral infections often lead to the production of quasispecies, which increases the possibility of clinical drug resistance. Therefore, more effective and better-tolerated combination therapies to decrease the emergence of viral resistance are greatly needed. To evaluate the effect of BP34610 when used in combination with other DENV inhibitors, the inhibitory activity of pairwise combinations of ribavirin with BP34610 was analyzed in cells infected with DENV-2 luciferase virus. BP34610 was mixed with ribavirin, and serial dilutions of each mixture were generated thereafter. The results revealed that the combination of BP34610 with ribavirin produced synergistic effects on anti-DENV activity at the 50%, 75%, and 90% effective doses (Table 4). No cytotoxicity was observed for BP34610 or ribavirin at the concentrations used in these experiments.

4. Discussion

In this study, we used DENV-2 reporter viruses to identify BP34610 as a DENV-2 inhibitor that shows broad inhibitory activity against DENV-1, 2, 3, 4, and JEV in vitro. Sequencing analyses of isolated BP34610-resistant DENV-2 viruses revealed that an S397P mutation was responsible for the drug resistance to BP34610. Time addition experiments and BP34610-resistance studies suggested that BP34610 may affect the early stage of viral entry and target the DENV E protein. Moreover, the result of the combination study using BP34610 with ribavirin supports the continued exploration of BP34610 as a component of a new combination therapeutic strategy for the treatment of flavivirus infection.

BP34610 dramatically inhibited all four serotypes of DENV and JEV and significantly reduced viral yields. However, BP34610 failed to inhibit adenovirus, a DNA virus (Table 2). This result indicated that BP34610 is an effective, broad-spectrum flavivirus inhibitor that may target the flavivirus E protein. Since the inhibitory effect of BP34610 on DENV-2 and JEV is greater than that on adenovirus, it is anticipated that the different susceptibilities of DENV-2, JEV, and adenovirus to BP34610 may result from different amino acid sequences of the E protein. E protein sequences from DENV-2 and JEV were aligned and compared to correlate with the highly similar susceptibilities of DENV-2 and JEV to BP34610. The degree of conservation of the envelope amino acid sequence of DENV-2 compared to those of JEV was shown to correspond to 47.6% identity (74.8% similarity) (data not shown). The high similarity in the amino acid sequence identity between virus E proteins may explain the susceptibilities of DENV-2 and JEV to BP34610. Interestingly, our observation showed that DENV-2 after incubation with BP34610 and gel filtration still maintained infectivity (unpublished results), suggesting BP34610 may not bind non-specifically to the viral lipid bilayer and irreversibly inactivates/denatures the virus. However, BP34610 lost the inhibitory effect to DENV-2 after the incubation of BP34610 with DENV-2 and removal of unbonded BP34610 by gel filtration. The phenomenon may be explained by either the interaction between BP34610 and E protein may not be strong or the gel filtration matrix may non-specifically bind BP34610 and reduced the amount of BP34610 associated with E protein. Further biochemical studies are needed to show the interaction between BP34610 and E protein.

Time-of-addition experiments implied that BP34610 interferes with an early event in the dengue virus life cycle (Fig. 4). BP34610 did not apparently affect either at the stage of viral translation or viral RNA replication in the transient DENV-2 reporter replicon assays (Fig. 3B). The S397P substitution in the envelope region moderately reduced virus spreading activity (Fig. 5A), implying the resistance to BP34610 conferred by S397P (Fig. 5B) is not resulting from the advantageous replication characteristics of the recombinant S397P virus. It suggested that BP34610 inhibits DENV by targeting the E protein. Since the S397 residue is located within the hydrophobic stem region of the DENV E protein, BP34610 likely reduces virus yields by targeting the DENV stem region and affecting viral entry, fusion, or maturation stage. Previous studies showed that the stem region has been used as a target to identify molecules able to block the viral entry/fusion stage (Hrobowski et al., 2005; Schmidt et al., 2012; Schmidt et al., 2010a, b). Several stem-specific inhibitors have been discovered that show anti-DENV activity, although no drug resistance studies have been reported. Interestingly, S397P is in proximity to the junction region between the soluble domain (residues 1–394) and the hydrophobic domain (395–450) of the E protein. Therefore, the possibility of the soluble domain being targeted by BP34610 cannot be excluded. Taken together, our data strongly suggest that BP34610 might target the E protein by affecting the translocation and rearrangement of domains or interfering with the membrane-fusion process, thereby blocking the virus entry process. The precise mode of action remains to be characterized.

The combination study using cells infected with DENV-2 luciferase virus demonstrated the synergistic antiviral actions of BP34610 and ribavirin (Table 4). More importantly, none of these combinations enhanced host cell toxicity, and no antagonistic antiviral effects were

Table 4
Anti-DENV activity of BP34610 in combination with ribavirin. BP34610 in combination with ribavirin yielded synergistic effects at the 50%, 75%, and 90% effective doses.

BP34610/ribavirin ratio	CI value for:			Influence
	ED_{50}	ED_{75}	ED_{90}	
1:5	0.82	0.60	0.45	Synergy
1:5	0.80	0.68	0.65	Synergy

observed. Resistance to clinical antiviral chemotherapy has become a major issue in the management of patients with chronic viral infections. To achieve sustained viral responses, it is necessary to develop effective combination therapies, especially those targeting distinct DENV viral targets. For instance, ribavirin, which has known in vitro antiviral activities, is currently being used synergistically in combination therapy for DENV infections (Chang et al., 2011; Rattanaburee et al., 2015; Takhampunya et al., 2006). Given that BP34610 can yield a high level of synergy with "clinical standard care" inhibitors and targets the DENV envelope, BP34610 may potentially be developed for use in future anti-DENV cocktails. Such combinational therapy can provide major advantages over single-drug therapy and represents an attractive paradigm for improving current virologic response rates.

In conclusion, the data presented in this study demonstrate the anti-flavivirus activity of BP34610. The in vitro combination of BP34610 with ribavirin, a viral replication inhibitor, led to a synergistic anti-DENV response in cell culture. Importantly, the S397P substitution in the DENV E protein conferred a high level of resistance to BP34610. BP34610 may target the E protein to block the early stage of viral infection. Further work is required to determine the detailed molecular mechanism by which BP34610 inhibits DENV entry and/or fusion. Molecular understanding of small-molecule inhibitors targeting the intermediates in the fusion-inducing arrangements of E proteins will facilitate anti-flavivirus drug discovery.

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References

Alen, M.M., De Burghgraeve, T., Kaptein, S.J., Balzarini, J., Neyts, J., Schols, D., 2011. Broad antiviral activity of carbohydrate-binding agents against the four serotypes of dengue virus in monocyte-derived dendritic cells. PLoS One 6, e21658.

Alen, M.M., Kaptein, S.J., De Burghgraeve, T., Balzarini, J., Neyts, J., Schols, D., 2009. Antiviral activity of carbohydrate-binding agents and the role of DC-SIGN in dengue virus infection. Virology 387, 67–75.

Allison, S.L., Stiasny, K., Stadler, K., Mandl, C.W., Heinz, F.X., 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J. Virol. 73, 5605–5612.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., Myers, M.F., George, D.B., Jaenisch, T., Wint, G.R., Simmons, C.P., Scott, T.W., Farrar, J.J., Hay, S.I., 2013. The global distribution and burden of dengue. Nature 496, 504–507.

Bressanelli, S., Stiasny, K., Allison, S.L., Stura, E.A., Duquerroy, S., Lescar, J., Heinz, F.X., Rey, F.A., 2004. Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. EMBO J. 23, 728–738.

Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. 44, 649–688.

Chang, J., Schul, W., Butters, T.D., Yip, A., Liu, B., Goh, A., Lakshminarayana, S.B., Alonzi, D., Reinkensmeier, G., Pan, X., Qu, X., Weidner, J.M., Wang, L., Yu, W., Borune, N., Kinch, M.A., Rayahin, J.E., Moriarty, R., Xu, X., Shi, P.Y., Guo, J.T., Block, T.M., 2011. Combination of alpha-glucosidase inhibitor and ribavirin for the treatment of dengue virus infection in vitro and in vivo. Antivir. Res. 89, 26–34.

Chen, L.K., Lin, Y.L., Liao, C.L., Lin, C.G., Huang, Y.L., Yeh, C.T., Lai, S.C., Jan, J.T., Chin, C., 1996. Generation and characterization of organ-tropism mutants of Japanese encephalitis virus in vivo and in vitro. Virology 223, 79–88.

Clark, M.J., Miduturu, C., Schmidt, A.G., Zhu, X., Pitts, J.D., Wang, J., Potisopon, S., Zhang, J., Wojciechowski, A., Hann Chu, J.J., Gray, N.S., Yang, P.L., 2016. GNF-2 inhibits dengue virus by targeting Abl kinases and the viral E protein. Cell Chem Biol 23, 443–452.

Costin, J.M., Jenwitheesuk, E., Lok, S.M., Hunsperger, E., Conrads, K.A., Fontaine, K.A., Rees, C.R., Rossmann, M.G., Isern, S., Samudrala, R., Michael, S.F., 2010. Structural optimization and de novo design of dengue virus entry inhibitory peptides. PLoS Neglected Trop. Dis. 4, e721.

De Burghgraeve, T., Kaptein, S.J., Ayala-Nunez, N.V., Mondotte, J.A., Pastorino, B., Printsevskaya, S.S., de Lamballerie, X., Jacobs, M., Preobrazhenskaya, M., Gamarnik, A.V., Smit, J.M., Neyts, J., 2012. An analogue of the antibiotic teicoplanin prevents flavivirus entry in vitro. PLoS One 7, e37244.

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., 2018. Inhibition of flaviviruses by targeting a conserved pocket on the viral envelope protein. Cell Chem Biol 25, 1006–1016 e1008.

Falgout, B., Pethel, M., Zhang, Y.M., Lai, C.J., 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J. Virol. 65, 2467–2475.

Hidari, K.I., Takahashi, N., Arihara, M., Nagaoka, M., Morita, K., Suzuki, T., 2008. Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. Biochem. Biophys. Res. Commun. 376, 91–95.

Hrobowski, Y.M., Garry, R.F., Michael, S.F., 2005. Peptide inhibitors of dengue virus and West Nile virus infectivity. Virol. J. 2, 49.

Ichiyama, K., Gopala Reddy, S.B., Zhang, L.F., Chin, W.X., Muschin, T., Heinig, L., Suzuki, Y., Nanjundappa, H., Yoshinaka, Y., Ryo, A., Nomura, N., Ooi, E.E., Vasudevan, S.G., Yoshida, T., Yamamoto, N., 2013. Sulfated polysaccharide, curdlan sulfate, efficiently prevents entry/fusion and restricts antibody-dependent enhancement of dengue virus infection in vitro: a possible candidate for clinical application. PLoS Neglected Trop. Dis. 7, e2188.

Kampmann, T., Yennamalli, R., Campbell, P., Stoermer, M.J., Fairlie, D.P., Kobe, B., Young, P.R., 2009. In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. Antivir. Res. 84, 234–241.

Kaptein, S.J., De Burghgraeve, T., Froeyen, M., Pastorino, B., Alen, M.M., Mondotte, J.A., Herdewijn, P., Jacobs, M., de Lamballerie, X., Schols, D., Gamarnik, A.V., Sztaricskai, F., Neyts, J., 2010. A derivate of the antibiotic doxorubicin is a selective inhibitor of dengue and yellow fever virus replication in vitro. Antimicrob. Agents Chemother. 54, 5269–5280.

Lian, W., Jang, J., Potisopon, S., Li, P.C., Rahmeh, A., Wang, J., Kwiatkowski, N.P., Gray, N.S., Yang, P.L., 2018. Discovery of immunologically inspired small molecules that target the viral envelope protein. ACS Infect. Dis. 4, 1395–1406.

Lin, Y.L., Liu, C.C., Lei, H.Y., Yeh, T.M., Lin, Y.S., Chen, R.M., Liu, H.S., 2000. Infection of five human liver cell lines by dengue-2 virus. J. Med. Virol. 60, 425–431.

Lo, Y.C., Perng, G.C., 2016. Novel concept on antiviral strategies to dengue. Curr Opin Virol 18, 97–108.

Lok, S.M., Costin, J.M., Hrobowski, Y.M., Hoffmann, A.R., Rowe, D.K., Kukkaro, P., Holdaway, H., Chipman, P., Fontaine, K.A., Holbrook, M.R., Garry, R.F., Kostyuchenko, V., Wimley, W.C., Isern, S., Rossmann, M.G., Michael, S.F., 2012. Release of dengue virus genome induced by a peptide inhibitor. PLoS One 7, e50995.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U. S. A. 100, 6986–6991.

Modis, Y., Ogata, S., Clements, D., Harrison, S.C., 2004. Structure of the dengue virus envelope protein after membrane fusion. Nature 427, 313–319.

Murray, N.E., Quam, M.B., Wilder-Smith, A., 2013. Epidemiology of dengue: past, present and future prospects. Clin. Epidemiol. 5, 299–309.

Nicholson, C.O., Costin, J.M., Rowe, D.K., Lin, L., Jenwitheesuk, E., Samudrala, R., Isern, S., Michael, S.F., 2011. Viral entry inhibitors block dengue antibody-dependent enhancement in vitro. Antivir. Res. 89, 71–74.

Noble, C.G., Chen, Y.L., Dong, H., Gu, F., Lim, S.P., Schul, W., Wang, Q.Y., Shi, P.Y., 2010. Strategies for development of dengue virus inhibitors. Antivir. Res. 85, 450–462.

Organization, W.H., 2012. Dengue and Severe Dengue.

Pitts, J., Hsia, C.Y., Lian, W., Wang, J., Pfeil, M.P., Kwiatkowski, N., Li, Z., Jang, J., Gray, N.S., Yang, P.L., 2019. Identification of small molecule inhibitors targeting the Zika virus envelope protein. Antivir. Res. 164, 147–153.

Poh, M.K., Yip, A., Zhang, S., Priestle, J.P., Ma, N.L., Smit, J.M., Wilschut, J., Shi, P.Y., Wenk, M.R., Schul, W., 2009. A small molecule fusion inhibitor of dengue virus. Antivir. Res. 84, 260–266.

Pu, S.Y., Wu, R.H., Tsai, M.H., Yang, C.C., Chang, C.M., Yueh, A., 2014. A novel approach to propagate flavivirus infectious cDNA clones in bacteria by introducing tandem repeat sequences upstream of virus genome. J. Gen. Virol. 95, 1493–1503.

Pu, S.Y., Wu, R.H., Yang, C.C., Jao, T.M., Tsai, M.H., Wang, J.C., Lin, H.M., Chao, Y.S., Yueh, A., 2011. Successful propagation of flavivirus infectious cDNAs by a novel method to reduce the cryptic bacterial promoter activity of virus genomes. J. Virol. 85, 2927–2941.

Pujol, C.A., Ray, S., Ray, B., Damonte, E.B., 2012. Antiviral activity against dengue virus of diverse classes of algal sulfated polysaccharides. Int. J. Biol. Macromol. 51, 412–416.

Rather, I.A., Parray, H.A., Lone, J.B., Paek, W.K., Lim, J., Bajpai, V.K., Park, Y.H., 2017. Prevention and control strategies to counter dengue virus infection. Front Cell Infect Microbiol 7, 336.

Rattanaburee, T., Junking, M., Panya, A., Sawasdee, N., Songprakhon, P., Suttitheptumrong, A., Limjindaporn, T., Haegeman, G., Yenchitsomanus, P.T., 2015. Inhibition of dengue virus production and cytokine/chemokine expression by ribavirin and compound A. Antivir. Res. 124, 83–92.

Rees, C.R., Costin, J.M., Fink, R.C., McMichael, M., Fontaine, K.A., Isern, S., Michael, S.F., 2008. In vitro inhibition of dengue virus entry by p-sulfoxy-cinnamic acid and structurally related combinatorial chemistries. Antivir. Res. 80, 135–142.

Rice, C.M., Lenches, E.M., Eddy, S.R., Shin, S.J., Sheets, R.L., Strauss, J.H., 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 229, 726–733.

Schmidt, A.G., Lee, K., Yang, P.L., Harrison, S.C., 2012. Small-molecule inhibitors of dengue-virus entry. PLoS Pathog. 8, e1002627.

Schmidt, A.G., Yang, P.L., Harrison, S.C., 2010a. Peptide inhibitors of dengue-virus entry target a late-stage fusion intermediate. PLoS Pathog. 6, e1000851.

Schmidt, A.G., Yang, P.L., Harrison, S.C., 2010b. Peptide inhibitors of flavivirus entry derived from the E protein stem. J. Virol. 84, 12549–12554.

Shepard, D.S., Coudeville, L., Halasa, Y.A., Zambrano, B., Dayan, G.H., 2011. Economic impact of dengue illness in the Americas. Am. J. Trop. Med. Hyg. 84, 200–207.

Simmons, C.P., Farrar, J.J., Nguyen v, V., Wills, B., 2012. Dengue. N. Engl. J. Med. 366, 1423–1432.

Sriburi, R., Keelapang, P., Duangchinda, T., Pruksakorn, S., Maneekarn, N., Malasit, P., Sittisombut, N., 2001. Construction of infectious dengue 2 virus cDNA clones using high copy number plasmid. J. Virol. Methods 92, 71–82.
Stiasny, K., Heinz, F.X., 2006. Flavivirus membrane fusion. J. Gen. Virol. 87, 2755–2766.
Takhampunya, R., Ubol, S., Houng, H.S., Cameron, C.E., Padmanabhan, R., 2006. Inhibition of dengue virus replication by mycophenolic acid and ribavirin. J. Gen. Virol. 87, 1947–1952.
Vervaeke, P., Alen, M., Noppen, S., Schols, D., Oreste, P., Liekens, S., 2013. Sulfated Escherichia coli K5 polysaccharide derivatives inhibit dengue virus infection of human microvascular endothelial cells by interacting with the viral envelope protein E domain III. PLoS One 8, e74035.
Wang, Q.Y., Patel, S.J., Vangrevelinghe, E., Xu, H.Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N.L., Poh, M.K., Phong, W.Y., Keller, T.H., Jacoby, E., Vasudevan, S.G., 2009. A small-molecule dengue virus entry inhibitor. Antimicrob. Agents Chemother. 53, 1823–1831.
World Health Organization, 2012. Global Strategy for Dengue Prevention and Control 2012-2020 (In IRIS). World Health Organization, Geneva.
World Health Organization, 2009. Special programme for Research and training in tropical diseases., World health organization. Dept. Of control of neglected tropical diseases., World Health Organization. Epidemic and Pandemic Alert and Response, pp. 147 Dengue : Guidelines for Diagnosis, Treatment, Prevention and Control, New ed. World Health Organization, Geneva.
Yang, C.C., Tsai, M.H., Hu, H.S., Pu, S.Y., Wu, R.H., Wu, S.H., Lin, H.M., Song, J.S., Chao, Y.S., Yueh, A., 2013. Characterization of an efficient dengue virus replicon for development of assays of discovery of small molecules against dengue virus. Antivir. Res. 98, 228–241.
Yang, J.M., Chen, Y.F., Tu, Y.Y., Yen, K.R., Yang, Y.L., 2007. Combinatorial computational approaches to identify tetracycline derivatives as flavivirus inhibitors. PLoS One 2, e428.
Ye, J., Zhu, B., Fu, Z.F., Chen, H., Cao, S., 2013. Immune evasion strategies of flaviviruses. Vaccine 31, 461–471 31.
Zhang, W., Chipman, P.R., Corver, J., Johnson, P.R., Zhang, Y., Mukhopadhyay, S., Baker, T.S., Strauss, J.H., Rossmann, M.G., Kuhn, R.J., 2003. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat. Struct. Biol. 10, 907–912.
Zhang, Y., Zhang, W., Ogata, S., Clements, D., Strauss, J.H., Baker, T.S., Kuhn, R.J., Rossmann, M.G., 2004. Conformational changes of the flavivirus E glycoprotein. Structure 12, 1607–1618.
Zhou, Z., Khaliq, M., Suk, J.E., Patkar, C., Li, L., Kuhn, R.J., Post, C.B., 2008. Antiviral compounds discovered by virtual screening of small-molecule libraries against dengue virus E protein. ACS Chem. Biol. 3, 765–775.