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Antiviral activity of ST081006 against the dengue virus

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ABSTRACT

Dengue virus, the causative agent for the dengue fever, infects approximately 50–100 million people worldwide per year. The high incidence of dengue fever, along with its potential to develop into a severe, life-threatening form, resulted in great interest in the discovery of an antiviral against it. In this study, we constructed a DENV2-EGFP infectious clone, established a fluorescence-based, high-throughput screening platform, and conducted a screen for anti-DENV compounds on a flavonoid-derivative library, Amongst the hits identified, ST081006 was found to be a strong inhibitor of the DENV replication. Time-course studies suggest that the presence of ST081006 is necessary to inhibit successive rounds of virus replication. Further investigations demonstrated that ST081006 affects the synthesis of both viral protein and viral RNA, and one anti-DENV mechanism is the direct inhibition of viral protein synthesis. The replication of all dengue serotypes, along with that of the enterovirus EV-A71, was shown to be affected by ST081006. Attempts to generate ST081006-resistant DENV were unsuccessful, and thus hints at host factors as potential drug target. Together, these results suggest that ST081006 affect DENV replication, likely by acting on a target involved in the viral protein and/or RNA synthesis pathway.

1. Introduction

The dengue virus (DENV) is a small, enveloped, positive-sense RNA virus from the genus *Flavivirus* and the family *Flaviviridae* (Kuno et al., 1998; Kuhn et al., 2002). The DENV exists in four serotypes, DENV1-4, and is classified in accordance to the antigenicity of its structural Envelope (E) protein. Transmission of the DENV is mainly mediated by the mosquito species *Aedes aegypti* and *Aedes albopictus*, where human infection occurs after the infected mosquito takes a blood meal from its human host (WHO, 2012). Infection by the DENV results in the dengue fever (DF), which is characterised by an acute fever that is often accompanied by other subclinical manifestations including headache, myalgia, arthralgia, and maculopapular rash (Gubler, 2010). These symptoms typically last for two to seven days and resolve spontaneous even without intervention. For most patients, the end of this stage marks the start of the recovery phase, which sees the resolution of symptoms and the reduction of infection biomarkers (Yacoub et al., 2014). A small subset of the patients, however, enter the critical phase, characterised by life-threatening thrombocytopenia and plasma leakage. These manifestations of the critical phase may lead to shock (dengue shock syndrome, DSS), or haemorrhage (dengue haemorrhagic fever, DHF).

The dengue virus is now prevalent in more than 100 countries worldwide (WHO, 2018). With more than two-fifth of the world's population living at risk of dengue infection, it occurs at a staggering number of 50–100 million infections per year. Further, infection cases are disproportionately distributed to people native to the earth's tropical and subtropical belt, such as the Southeast Asia and Latin America (The Lancet Editorial, 2013). Yet, despite the social and economic burden the disease bears on global society, there is currently no effective prophylactic or therapeutic measure against it. The attempted development of a dengue vaccine has been complicated by the oft co-circulation of the four different serotypes, as the vaccine would ideally have to provide immunity against all serotypes to justify the use of a tetravalent formulation. The absence of a suitable animal model and biomarkers for immunity further adds to the challenge (Ghosh and Dar, 2015). In addition, while various drugs, such as balapiravir (Nguyen et al., 2012), chloroquine (Tricou et al., 2010), celgosivir (Low et al., 2009), lovastatin (Whitehorn et al., 2015), and prednisolone (Tam et al., 2012) have entered clinical trial for potential therapeutic

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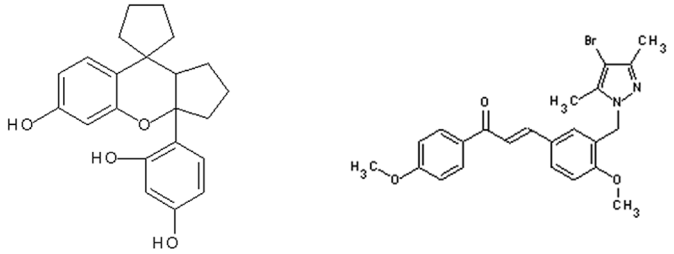

ST081006 **ST070252**

Fig. 1. Chemical structure of ST081006 and ST070252. Following a high-throughput screen and validation of hits, two compounds, ST081006 and ST070252, were selected for further investigation.

measures during dengue infection, none has shown to be sufficiently efficacious to warrant its use as an antiviral against the DENV. Thus, the search for an antiviral that can resolve dengue infection before its development into severe dengue continues.

Our search for a dengue antiviral delved into flavonoids, a group of compounds with varied phenolic structures that are derived from plants, where they serve to regulate plant growth. As a result of their biochemical activity, they are often able to affect other biological systems in numerous ways. Against pathogens, some flavonoids have been reported to kill and inhibit bacteria, fungi, and protozoa, while others have been demonstrated to inhibit viral enzymes (Havsteen, 2002). This, along with their potential for low side effects and ease of accessibility from plants, has raised interest in the scientific community for their potential use in drug development.

In this study, we report the establishment of a phenotypic screening platform for inhibitory compounds against the DENV. A high-throughput screen on the flavonoid-derivative library identified various compounds that were shown to be able to inhibit dengue infection. Amongst them, ST081006 (Fig. 1), a synthetic flavonoid, was subsequently validated and shown to be a strong inhibitor of DENV replication.

Treating DENV-infected cells with ST081006 results in a reduction in virus titer as demonstrated by viral plaque assay. A time-course study established that ST081006 is unlikely to act on the entry or early postentry events of the DENV replication cycle. Further, the continued presence of ST081006 is necessary to prevent titer recovery from successive replication cycles of the virus. Employing qRT-PCR to quantify both positive- and negative-sense viral RNA, reduction in viral RNA copy numbers was observed when infected cells were treated to ST081006. Subsequent Western blot analysis of viral proteins revealed that viral protein synthesis is inhibited in the presence of ST081006. The inhibition of viral protein translation by ST081006 was then further demonstrated through the use of a translation reporter clone. The data suggest that ST081006 inhibits DENV replication via direct inhibition of viral protein synthesis and possibly viral RNA replication as well, and that it may serve as an antiviral against the DENV for dengue management. Subsequent failure to generate a ST081006-resistant mutant hints at host factors as possible drug target. Finally, ST081006 was demonstrated to inhibit the replication of all dengue serotypes and that of the enterovirus EVA-71.

2. Materials and methods

2.1. Cell lines and viruses

Baby hamster kidney (BHK-21; ATCC) cells were cultured in Roswell Park Memorial Institute 1640 media (RPMI-1640; Sigma-Aldrich). HuH-7 human hepatoma cells, human rhabdomyosarcoma cells (RD; ATCC CCL-136™), and human choriocarcinoma cells (JEG-3; ATCC) were cultured in Dulbecco's Modified Eagle media (DMEM; Sigma-Aldrich). Human umbilical vein endothelial cells (HUVEC; ATCC) were cultured in Endothelial Cell Growth Medium 2 (EGM-2; Lonza). *Aedes albopictus* clone cells (C6/36; ATCC) were cultured in L-15 media (Sigma-Aldrich). All cell lines were cultured in their respective media supplemented with 10% heat-inactivated fetal calf serum (HI-FCS; PAA). BHK-21, HuH-7, RD, JEG-3, and HUVEC were grown in vented T-75 flasks at 37 °C with 5% CO_2, whereas C6/36 were grown in angled neck T-75 flasks at 28 °C.

DENV2 (New Guinea C strain; NGC; Accession no.: KM204118) and DENV2-EGFP infectious clone were used in this study. For the DENV2 (NGC), virus propagation was done by infecting confluent C6/36 cells, and thereafter incubating the infected cells in L15 media with 2% HI-FCS for 4–6 days, or until cytopathic effect (CPE) was observed in 50% of the cells. The supernatant was then harvested and stored at −80 °C and quantified using viral plaque assay.

2.2. Viral plaque assay

Viral plaque assays were performed on 24-well plates using BHK-21 cells at a seeding density of $6*10^4$ cells/ml in 1 ml of RPMI-1640 with 10% HI-FCS. After seeding, the plate were incubated in 37 °C with 5% CO_2 overnight. On the following day, the virus samples to be quantified were serially diluted to obtain a 10^1–10^6 fold dilution. The seeded BHK-21 cells were then infected with the diluted virus samples in separate wells. The plate was then incubated in 37 °C with 5% CO_2 for 1 h for viral adsorption. Post incubation, all wells were washed twice with 1× PBS and overlaid with 1 ml of 1% carboxymethyl cellulose (CMC) in RPMI-1640 with 2% heat-inactivated FCS. The plate was then incubated for six days for DENV samples, three days for CHIKV samples, and four days for ZIKV samples. Thereafter, the overlay media was replaced with 1 ml of crystal violet staining solution and the plate was incubated in room temperature overnight. The crystal violet staining solution was then removed, and the number of plaques in each well was counted manually.

Plaque assay for EV-A71 samples followed similar procedures. Briefly, RD cells, instead of BHK-21, were seeded at a density of $2*10^5$ per well. The cells were then incubated overnight, infected with diluted samples, and washed accordingly. Thereafter, 1 ml of DMEM with 2% heat-inactivated FCS and 1% agarose was added into all wells. The plate was then incubated at 37°C with 5% CO_2 for four days. 1 ml of crystal violet staining solution was then added directly onto of the solidified agarose. The plate was then incubated in room temperature for another day before having all wells flushed with water. The plaques in each well were then counted manually.

2.3. Construction of DNA-launched dengue virus 2 infectious clones

Viral RNA from the DENV2-16681 strain was extracted and purified using the Qiagen viral RNA extraction kit. The viral RNA was then used as a template for cDNA synthesis using the Superscript III reverse transcriptase (Life Technologies, Thermo Fisher). The cDNA was then used as a template for infectious clone construction.

The Ptight promoter, consisting of a tetracycline regulatory element and a minimal CMV promoter (TRE-minCMV), was cloned from the pTRE-Tight vector (Clontech). The hepatitis D virus ribozyme (HDVr) sequence and the porcine teschovirus 2A ribosomal skipping sequence (P2A) were each synthesised as a gBlock (Integrated DNA Technologies). The SV40-poly(A) signal sequence was cloned from the pcDNA3.1 vector (Life Technologies, Thermo Fisher). The ubiquitin cDNA sequence was cloned from mRNA from a human cell line. The EGFP reporter gene was cloned from the pEGFP-N1 vector (Clontech).

We then constructed several PCR-amplicon fragments using conventional PCR and fusion-PCR using the Q5 High-Fidelity DNA Polymerase (New England Biolabs). Where necessary, primers with overhangs were used to add restriction sites to the termini of the PCR-

amplicons.

Fragment AB consist of the TRE-minCMV cassette fused to the cDNA sequence corresponding to DENV2-5′UTR-Cap-prM. Fragments BC, DEF and EFGHIJ consists of the cDNA sequences corresponding to the DENV2-5′UTR-Cap-prM-Env, Env-NS1-NS2A-NS2B, and NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 respectively. Fragment KL consists of the cDNA sequence corresponding to the DENV2-NS5-3′UTR fused to the HDVr sequence and the SV40-Poly(A) sequence.

The DENV2 infectious clones were constructed by conventional molecular cloning methods. The NotI restriction site was introduced at the 5′ terminus of Fragment AB. The MluI restriction site was introduced at each of the 3′termini of fragments AB, BC, DEF, EFGHIJ and KL. We also utilised the following naturally occurring restriction sites in the DENV2 cDNA sequence: SalI in Capsid, SphI in Envelope, KpnI in NS2B, and BsrGI in NS5. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs.

For bacterial transformation we used the following *E. coli* strains: MDS42, STBL2, and STBL3. The bacterial were cultured on LB-agar plates or in LB at 25 °C–28 °C. Plasmid extraction and purification was performed using Qiagen midi or maxi prep kits. Plasmid sequences were verified by Sanger sequencing.

The DENV2 infectious clones were cloned into the low-copy number plasmid pSMART-LC-Kan (Lucigen). The NotI and MluI restriction sites were first cloned in place of the plasmid's multiple-cloning-site. Fragment AB was then cloned into the pSMART vector via the NotI and MluI restriction sites to construct plasmid pDV2-AB. Fragment BC was cloned into pDV2-AB via the SalI and MluI restriction sites to construct plasmid pDV2-ABC. Fragment DEF was cloned into pDV2-ABC via the SphI and MluI restriction sites to construct plasmid pDV2-A-F. Plasmid pDV2-A-F was found to have an unstable poly(A) region in nucleotides 556–568 of NS2A (CAGCAAAAAACAG). This was stabilised by introducing silent mutations that changed the sequence to (CAACAGAA AACGG); this did not alter the NS2A peptide sequence.

Fragment EFGHIJ was then cloned into the stabilised pDV2-A-F via the KpnI and MluI restriction sites to construct plasmid pDV2-A-J. Fragment KL was cloned into pDV2-A-J via the BsrGI and MluI restriction sites to construct the infectious clone plasmid pDV2-16681, where the DENV2 sequence is flanked by the TRE-minCMV promoter at the 5′terminus and by the HDVr-SV40-Poly(A) sequence at the 3′terminus.

To construct the reporter virus, a transgenic recombinant cassette was cloned between nucleotides 75 and 76 of the capsid coding region. The recombinant cassette (EGFP-P2A-UBB-smC75) consists of the EGFP reporter gene, the P2A sequence, the ubiquitin gene, and finally smC75 sequence. The smC75 sequence corresponds to the first 75 nucleotides of the capsid coding region which has been modified with silent mutations: the encoded peptide sequence remains the same.

To construct the DENV-2 translation reporter-WT clone (Fig. 1), the EGFP reporter gene was replaced with the Firefly luciferase (F. luc) reporter gene. The GDD catalytic triad in the NS5 coding region was also deleted by conventional fusion PCR and cloning methods. The F. luc reporter gene was chosen for the detection of viral protein synthesis as it is more sensitive compared to EGFP or Western blot assays. The GDD catalytic triad forms the essential catalytic core of the NS5 RDRP. Deletion of the GDD triad means the translation reporter is stuck at first round of protein synthesis of the viral replication cycle as it cannot progress to the RNA replication stage.

2.4. Rescue and titration of DNA-launched virus

Virus rescue took place by co-transfection of BHK-21 cells with the DENV2 infectious clone plasmid with an accessory plasmid pTofa (pTet-Off-Advanced, Clontech) that expresses the tetracycline trans-activator. *In vitro* RNA transcription was not required. Transfection was performed with the Jetprime reagent (Polyplus), according to the manufacturers instructions. At 24 h post-transfection, the cell culture media was changed to RPMI with 2% FCS and 2 g/L of $NaHCO_3$. To harvest the virus, the supernatant was removed and filtered through a 0.45 PES syringe filter (Sartorius), it was then stored as aliquots at −80 °C. For titration of the reporter virus, BHK-21 cells were first infected with reporter virus stock. At 2-days post-infection, the cells were re-suspended with PBS-EDTA, fixed with PFA, and then analysed by flow-cytometry. Reporter virus titres were calculated from the percentage of GFP-positive cells as FFU/ml (fluorescence forming units). Reporter virus titre typically peaked at day 5 post-transfection at 1.2×10^6 to 1.5×10^6 FFU/ml.

2.5. Immunofluorescent detection of DENV2-Env expression in cells

BHK-21 cells were infected with either the wildtype DENV2 or DENV2-EGFP at approximately MOI 10. Cells were fixed and stained 2 days p.i. Cells were fixed for 10 min with 4% PFA, 0.1% Triton. Primary staining was with mouse anti-DENV-Env, clone DE2 from abcam at 1:20 dilution in PBS with 3% BSA. Secondary staining was with goat anti-mouse, alexa-fluor 532, at 1:500 dilution in PBS with 3% BSA.

2.6. Flavonoid-derivatives library

The primary screen for anti-DENV compounds were performed on the flavonoid-derivatives library (TimTec). The list of the 500 flavonoids in this library can be found at https://www.timtec.net/flavonoid-derivatives.html. The compounds were reconstituted in DMSO to a final concentration of 200 µg/ml in 96-well plates and stored in −20 °C.

2.7. Primary screen

The primary screen was done on HuH-7 cells on 96-well plates, with a seeding density of $1.6*10^4$ cells in 100 µL of DMEM with 10% HI-FCS. Seeded cells were incubated overnight at 37 °C with 5% CO_2 before being infected with infectious DENV2-EGFP at a MOI of 3 for 2 h. The wells were washed and the drugs from the library were added to their respective wells for a final concentration of 20 µg/ml 0.1% DMSO and 0.5 µM emetine were used as negative and positive controls respectively. The plates were then incubated for 48 h before fixing with 4% PFA and 0.1% TX and stained with DAPI. The wells were imaged with Operatta High Content Imaging System with Harmony High Content Imaging and Analysis Software (PerkinElmer).

2.8. Data acquisition

Five non-overlapping images were taken for each well at 10× magnification using fluorescein and DAPI channels. The images were analysed using CellProfiler (Carpenter et al., 2006). The virus inhibition for each compound was calculated as follows: $\frac{(IR_N - IR_T)}{IR_N} \times 100\%$. IR_N and IR_T refer to the average infection rate of negative control and flavonoid-treated well respectively.

The robustness of the assay was determined using the Z-factor (Zhang, 1999). 64 wells of a 96-well plate were seeded with HuH-7 and infected with DENV2-EGFP as per the primary screen. Half the wells were then treated with 0.1% DMSO as negative control while the other half were treated with 0.5 µM emetine as positive control. The Z-factor was then calculated as follows: $Z - factor = \frac{3(\sigma_A - \sigma_B)}{|\mu_A - \mu_B|}$, where σ refers to standard deviation, µ refers to mean of the percentage infection, A refers to positive control, and B refers to negative control.

2.9. Hits validation

Selected hits from the primary screen, ST081006 and ST070252, were validated using cell viability assay and titer reduction assay. Post screening, assays were conducted using DENV2 instead of its cognate infectious clone.

Cell viability assay involves evaluating the viability of the compound-treated cells with the alamarBlue™ Cell Viability Reagent (ThermoFisher). Briefly, HuH-7 cells were seeded on a 96-well plate with a seeding density of $7.5*10^3$ cells per well and incubated overnight at 37 °C with 5% CO_2. The cells were then treated to the selected hit at a range of concentrations and incubated for 48 h. Post incubation, the media was replaced with maintenance media with 1× alamarBlue™ Cell Viability Reagent (ThermoFisher), and the plate was further incubated at 37 °C, 5% CO_2 for 2.5 h. Fluorescence readings of all wells were taken using the Infinite™ 200 series microplate reader (Tecan) with emission and excitation wavelengths of 585 and 570 nm respectively. The cell viability of each well was calculated using the following formula:

$$Cell\ viability\ (\%) = \frac{fluorescence\ of\ compound\ treated\ well}{fluorescence\ of\ mock\ treated\ well} * 100$$

Titer reduction assay was performed to validate the anti-DENV activity of the hits. HuH-7 cells were seeded on a 96-well plate with a seeding density of $7.5*10^3$ cells per well and incubated overnight at 37 °C with 5% CO_2. The cells were then infected with DENV2 at a MOI of 1 for 1 h at 37 °C. Post infection, the cells were washed twice with 1× PBS, and were thereafter treated with the selected hit at a range of concentrations in DMEM with 2% HI-FCS. The plate was then incubated at 37 °C with 5% CO_2 for 48 h. After which, the supernatant was harvested, and the virus titer was determined using viral plaque assay.

The entire validation procedure was repeated for one of the selected hit, ST081006, using the primary cell line, HUVEC. The seeding density for HUVEC cells was $7*10^3$ cells per well on a 96-well plate. The same procedures for cell viability assay and titer reduction assay were performed as described.

2.10. Time-course assays

Time-course assays include the pretreatment, time-of-addition, and time-of-removal assays.

For all time-course assays, HuH-7 cells were seeded on a 96-well plate, infected with DENV2, and washed with 1× PBS as per the titer reduction assay under hits validation. For pretreatment assay, the cells were pretreated with 10 μM of ST081006 or 0.1% DMSO for 2 h at 37 °C with 5% CO_2. The supernatant was then removed and the cells were washed twice with 1× PBS, before being infected with DENV2 at a MOI of 1 for 1 h. Post infection, the cells were was twice with 1× PBS, and incubated in DMEM with 2% HI-FCS for 48 h. The supernatant were then harvested and quantified using viral plaque assay.

For the time-of-addition assay, the cells were incubated in 100 μL of DMEM with 2% HI-FCS at 0 h post infection (hpi). After which, at 0, 12, 24, 36, and 48 hpi, ST081006 were added into the wells for a final concentration of 10 μM. For the time-of-removal assay, cells were incubated in DMEM with 2% HI-FCS and 10 μM ST081006 at 0 hpi. At the same timepoints as with the time-of-addition assays, the media was then aspirated and replaced with DMEM containing 2% HI-FCS only. For both assays, 0.1% DMSO in place of 10 μM ST081006 served as vehicle control. At 48 hpi, supernatant of all wells were harvested and quantified using viral plaque assay.

2.11. qRT-PCR quantification of viral RNA

HuH-7 cells were seeded on a 24-well plate at a density of $5*10^4$ cells per well and incubated overnight at 37 °C with 5% CO_2. The cells were then infected with DENV2 at an MOI of 1 for 1 h, before being post-treated with either 10 μM ST081006, 0.1% DMSO (as vehicle control), or 5 μM emetine (as positive control). At 12, 24, and 48 hpi, the supernatant of all wells were aspirated and total RNA extraction was performed on the cells using QIAGEN's RNeasy Mini Kit in accordance to the manufacturer's protocol.

Two sets of reverse transcription, one using the forward primer complementary to the negative sense DENV2 viral RNA and the other using the reverse primer complementary to the positive-sense DENV2 viral RNA, were performed on all samples separately. The sequences for the forward and reverse primer are 5′–tagagacctgggaagagtgatg–3′ and 5′–gctgctagtagggcaagataag–3' (Sigma) respectively. For each reaction, 19 μL of reaction mix was set up comprising of 4 μL M-MLV 5× buffer (Promega), 1 μL of forward or reverse primer, 1 μL of 10 mM dNTP (Promega), 4.5 μL of nuclease free H_2O, and 8.5 μL of the total RNA sample. The tubes were then heated for 70 °C for 10 min and placed on ice thereafter. 1 μL of M-MLV reverse transcriptase diluted to a concentration of 100 u/μL, was added into all tubes. The tubes were then heated at 42 °C for 1 h, followed by 95 °C for 5 min. The resulting cDNA samples were stored at −80 °C.

The reaction mix for qPCR of the cDNA samples was prepared as such: 10 μL of 2× PrimeTime Gene Expression mastermix (Integrated DNA Technologies), 0.5 μL of DENV2 reverse and forward primer each, 0.5 μL of DENV2 probe, 6.5 μL of H_2O, and 2 μL of cDNA sample. The sequence for the DENV2 probe is 5′–FAM/TATGTCATC/ZEN/CGTCAT GGT–3'. qPCR was then performed using the StepOnePlus Real-Time PCR system (Applied Biosystems) with the following cycling conditions: one round of 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 1 min. The threshold values obtained were normalised against the values obtained from β-actin gene as endogenous control. The absolute viral RNA copy numbers were then derived using a standard curve.

The standard curve was obtained by first extracting the total RNA of a virus stock of known titer using the QIAamp Viral RNA Mini Kit (Qiagen) using its provided protocol. The extracted RNA sample was 10-fold serially diluted using H_2O for a final dilution factor of 10^1 to 10^5. qRT-PCR was then performed as described above to obtain the threshold values for the various dilution factors.

2.12. Western blot analysis of viral protein

Cells were seeded and infected in a similar fashion as with the sample preparation for qRT-PCR. For Western blot, cells were with post-treated with 0.1% DMSO as vehicle control or 10 μM of ST081006. At 24 and 30 hpi, the supernatant was discarded and 150 μL of 1× Laemmli SDS-PAGE buffer was added into all wells, before scraping the well with a pipette tip. The cell lysate samples were harvested and stored at −80 °C.

Prior to SDS-PAGE, all samples were heated at 100 °C for 5 min. SDS-PAGE was performed using a 12% SDS-polyacrylamide gel using 20 μL of the sample per well. 5 μL of PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific) was used as protein ladder. The electrophoresis was run at 80 V for stacking and 120 V for resolving, and was terminated when the dye front was visually noted to have reached the end of the gel. Post electrophoresis, the proteins were transferred onto a PVDF membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad) on its default high molecular weight program.

For Western blot, all washing procedure was performed by rocking the membrane in Tris-buffered saline with Tween-20 (TBS-T) for 5 min. The blot was first blocked with 2% bovine serum albumin (BSA; MP Biomedicals; w/v) in TBS-T at room temperature with rocking for 1 h and washed thrice. The blot was then cut into three pieces according to the expected band size of the protein of interest.

For actin, the respective blot was incubated in mouse anti-β-actin monoclonal antibody diluted 1:10000 in TBS-T with 2% BSA for 30 min at room temperature. Washing was then performed thrice, before incubation in secondary anti-mouse goat polyclonal IgG antibody conjugated with horseradish peroxidase (Millipore) diluted 1:10000 in TBS-T containing 2% BSA for 30 min at room temperature. The blot was then washed thrice again, then rocked in SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific) for 2 min, before being visualized using the C-Digit® Blot Scanner.

The two other blots were probed with rabbit anti-dengue NS4B antibody (GeneTex), and mouse anti-envelope antibody (GeneTex) respectively. Both blots were incubated in their respective antibodies diluted 1:5000 in TBS-T with 2% BSA overnight at 4 °C with rocking. The blots were then wash thrice, before being incubated in secondary anti-mouse, or anti-rabbit goat polyclonal IgG antibody conjugated with horseradish peroxidase, diluted 1:10000 in TBS-T with 2% BSA for 1 h at room temperature with rocking. The blots were developed and visualized as per the actin blot.

2.13. Firefly luciferase translation reporter assay

HuH-7 cells were seeded at a density of 1×10^7 cells per well in a 24-well plate in DMEM supplemented with 10% HI-FCS and incubated overnight at 37 °C with 5% CO_2. In this experiment, the DENV-2 translation reporter-WT clone was transfected (Fig. 1). Transfection was performed with according to the jetPRIME® protocol (Polypus transfection®). For each reaction, a mixture of 50 µL of jetPRIME® buffer (Polypus transfection®), 400 ng of plasmid DNA, 100 ng of accessory plasmid (pTofa), and 1 µL of jetPRIME® transfection reagent (Polypus transfection®) were prepared. The mixture was then mixed thoroughly and incubated for 10 min at room temperature. Subsequently, for every one reaction, a 52 µl volume of the mix was added into each well of the seeded 24-well plates. The plates were rocked gently and incubated at 37 °C with 5% CO_2. At 6 h post-transfection (hpt), the media was removed and replaced with DMEM supplemented with 2% HI-FCS, along with 10 µM of ST081006, 0.5 µM of emetine as positive control, or 0.1% DMSO as negative control.

At 48 and 72 hpt, the transfected cells were lysed and the F. luc signals were analysed. All procedures were done in accordance to the manufacturer's protocol using the ONE-Glo™ Luciferase Assay System, Promega. The F. luc signals were read using a luminometer (Promega) following the instrument's ONE-Glo protocol.

2.14. Generation of a drug resistant mutant

Cells were seeded and infected in a similar fashion as with the sample preparation for qRT-PCR. Post infection, cells were with post-treated with media only (wild type strain), 0.1% DMSO (vehicle control strain) or 10 µM of ST081006 (drug-treated strain). The cells were then incubated for 48 h before its supernatant was harvested and split into different tubes and stored at −80 °C. The supernatant in the tubes was either used for viral titer quantification or for passaging the next generation of the virus strain.

A portion of the supernatant was then used to infect newly seeded cells to generate the second passage of the virus. After infection, the infected cells were post-treated to the same conditions: the cells infected with the wild type strain were treated with media only, and so on. For subsequent passages, the infected cells were incubated for 48 h, or until approximately 50% CPE was observed, up to 72 hpi, in order to maintain the virus titer. This procedure was repeated for multiple passages until the drug-treated strain gained resistance against the drug, or became unable to recover its titer.

2.15. Assays on other dengue serotypes and positive-sense RNA viruses

The inhibitory profile of ST081006 was tested against other dengue virus serotypes and other positive-sense RNA virus. For the other dengue serotypes, the same procedures were performed as per the section on titer reduction assay under hits validation, replacing DENV2 with the other dengue serotypes. The other dengue serotype tested were DENV1 (EDEN 2928), DENV3 (EDEN 2930), or DENV4 (TCR310A129 DEN4 #4) at a MOI of 1.

For other positive-sense RNA viruses, the chikungunya virus (CHIKV-122508), Zika virus (ZIKV-PRVABC59), and the enterovirus A71 (EV-A71) were tested. Similar procedures were performed as per the section on titer reduction assay under hits validation, replacing DENV2 with the other positive-sense RNA viruses. For CHIKV, HuH-7 cells were infected with CHIKV at a MOI of 1 for 1.5 h, and were incubated for 48 h post infection. For ZIKV, JEG-3 cells were infected with ZIKV at a MOI of 5 for 1 h, and were incubated for 24 h post infection. For EV-A71, RD cells were infected with EV-A71 at a MOI of 1 for 1 h, and were incubated for 12 h post infection. After incubation, the supernatant were harvested and the virus titer were quantified using viral plaque assay. All infections were carried out at 37 °C with 5% CO_2.

3. Results

3.1. Construction & Design of Dengue Virus 2 Infectious Clones

The designs for the DNA-launched DENV2 infectious clone and EGFP-dengue reporter virus infectious clone are a synthesis of several previously published designs (Shustov and Frolov, 2010; Pu et al., 2014; Schoggins et al., 2012; Lin et al., 2012). *Flavivirus* infectious clones can be unstable, for example a previous attempt to construct a DNA launched dengue reporter virus with a normal CMV promoter and a bacterial artificial chromosome was unsuccessful due to instability in bacteria (Usme-Ciro et al., 2014). To retain the benefits of a DNA-launched infectious clone and to reduce the bacterial instability, we utilised a low copy plasmid and adopted the use of a Ptight promoter: a minimal-CMV promoter linked to a tetracycline regulatory element (Pu et al., 2014). The plasmid was further stabilised in bacterial culture by using *E. coli* strains which are tolerant of viral vectors (MDS42, STBL2, STBL3), and by culturing the bacteria under gentle conditions.

The wildtype DENV2 infectious clone backbone, pDV2-16681, was constructed by conventional molecular cloning methods (Fig. 2A). The viral cDNA sequence was cloned from the DENV2-16681 strain.

To construct the reporter virus, we followed an established design where a transgenic recombinant cassette was cloned between nucleotides 75 and 76 of the capsid coding region (Fig. 2B) (Schoggins et al., 2012; Shustov and Frolov, 2010). This preserved the sequence and location of critical RNA elements in the first 75 nucleotides of the capsid sequence. The recombinant cassette is expressed along with the DENV2 genes as a single continuous polyprotein which is then processed into a reporter protein and the complete set of Flaviviral proteins. The smC75 sequence is immediately upstream and contiguous with nucleotide 76 of the native capsid sequence, allowing expression of the full-length capsid protein and thereafter the rest of the DENV2 polyprotein. The silent mutations in smC75 prevents interference with the RNA elements in nucleotides 1–75 of the native capsid sequence (Lin et al., 2012). The P2A sequence allows separation of EGFP from the polyprotein. UBB allows generation of an authentic N-terminus for the capsid protein: when UBB is expressed as part of the polyprotein, deubiquitinating enzymes cleave the polyprotein at the C-terminus of UBB, between the last amino acid residue of UBB and the first residue of the downstream capsid. This reporter virus design does not require an IRES that is typically derived from a non-Flavivirus, usually EMCV. Therefore, expression is driven entirely by Flaviviral promoters, meaning the reporter phenotype is more authentically linked to *Flavivirus* replication. This is critical in drug screens, where a false positive might arise from a drug that does not inhibit *Flavivirus* replication, but instead inhibits ECMV IRES activity.

We also constructed a DENV translation reporter. It possesses Firefly luciferase instead of EGFP as a reporter gene. The GDD catalytic triad in the NS5 coding region was also deleted, which prevents the translation reporter from initiating viral RNA replication.

3.2. Characterisation of DENV2-EGFP

The dengue reporter virus expressing EGFP, DENV2-EGFP, was DNA-launched by direct transfection into BHK-21 cells to produce the passage 1 working stock. The reporter virus was titrated by flow-

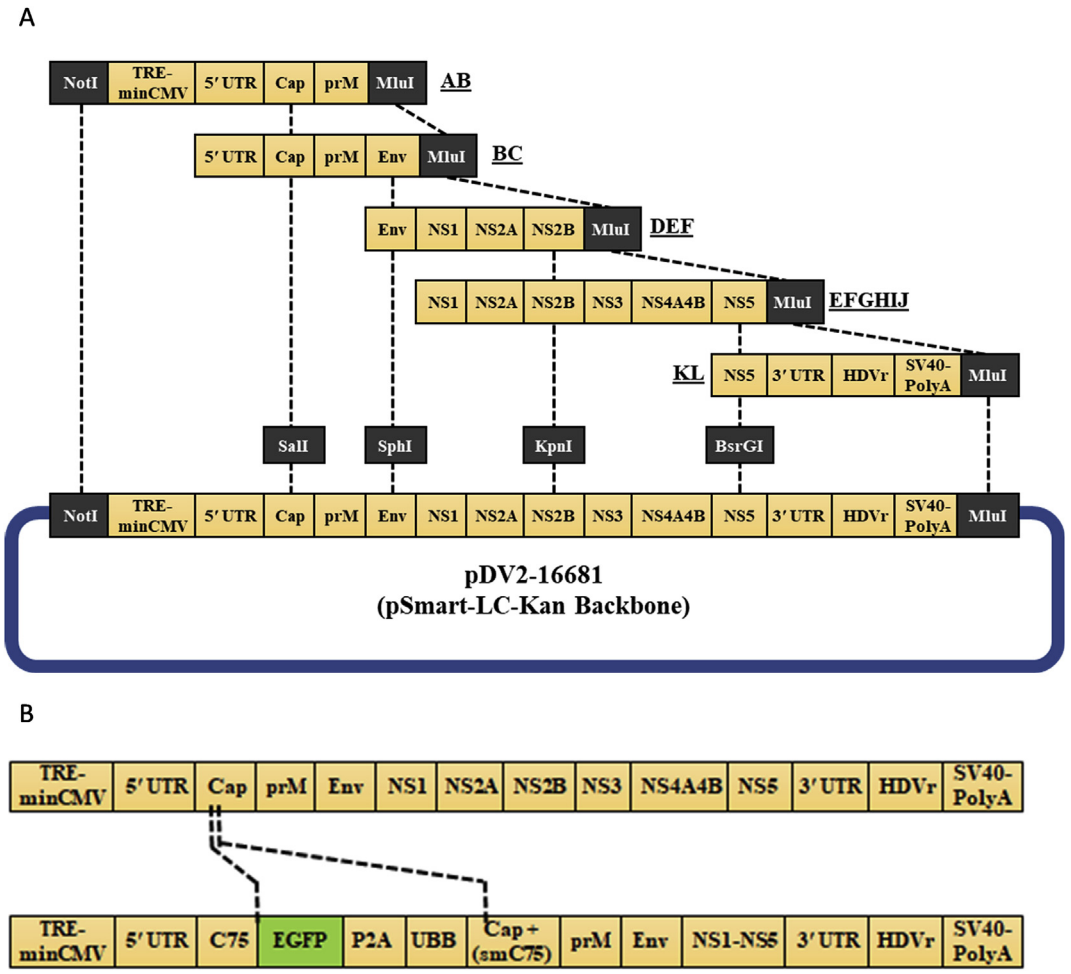

Fig. 2. Construction of a DENV2-EGFP infectious clone. A) Plasmid map and construction details of DNA-launched dengue virus 2 infectious clone. Approximate restriction site locations are shown in dark grey. The PCR-amplicon fragments AB, BC, DEF, EFGHIJ and KL were successively cloned into the pSMART vector in the listed order. B) Genomic map of dengue reporter virus expressing EGFP (DENV2-EGFP). TRE-minCMV: tetracycline regulatory element and a minimal CMV promoter. HDVr: hepatitis D virus ribozyme sequence. P2A: porcine teschovirus 2A ribosomal skipping sequence. SV40-poly(A): polyadenylation signal sequence. UBB: ubiquitin ORF. EGFP: enhanced green fluorescent protein.

cytometry, and passage 1 titers could be as high as 1.5×10^6 FFU/ml, which was sufficient for drug screening. Further virus culture of DENV2-EGFP stocks was not expected to further amplify the reporter virus titers as an increasing proportion of revertant viruses would take over the virus pool with successive passages.

To confirm that DENV2-EGFP undergoes viral replication, we infected BHK-21 cells with either wildtype DENV2 or DENV2-EGFP and analysed DENV2-Env expression by immunofluorescence assay (Fig. 3A). Cells infected with either DENV2 or DENV2-EGFP were positive for DENV2-Env expression, whereas mock infected cells did not. Furthermore, only DENV2-EGFP infected cells showed co-expression of EGFP alongside DENV2-Env. We note that for DENV2-EGFP, EGFP is freed during polyprotein processing, and is not tagged to any specific viral protein, and therefore adopted the typical cytoplasmic and nuclear localisation of free EGFP (Seibel et al., 2007).

Plaque-forming capability of DENV2 and DENV2-EGFP were also tested by plaque assay in BHK-21 cells. Both DENV2 and DENV2-EGFP formed plaques, confirming the viral nature of DENV2-EGFP (Fig. 3B). We note that plaque-formation required a longer period of incubation in DENV2-EGFP and resulted in smaller plaques as compared to DENV2. This was expected as the reporter gene places a burden on viral replication.

3.3. Establishment of a direct fluorescence screening platform and primary screen

Prior to the actual screen, we validated the screening platform to ensure that it is sufficiently specific and sensitive for its intended purpose. EGFP signal was taken as an indicator of infection as the infectious agent used in the screen is a DENV2-EGFP construct. A high amount of EGFP signal was observed when infected cells were treated to 0.1% DMSO as vehicle control. In contrast, minimal EGFP signal was observed for those treated with 0.5 μM emetine, previously shown to be an inhibitor of DENV2 replication (Fig. 4A). Translating of the EGFP signal to percentage infection, a clear separation was observed between infected cells treated with 0.1% DMSO from those treated with 0.5 μM emetine (Fig. 4B), with the Z-factor of this screening platform calculated to be 0.536. This indicates that this is an excellent platform for the separation of compounds that are able to decrease percentage infection from those that are not (Zhang, 1999). Using this platform, a primary screen of the flavonoid-derivatives library for anti-DENV compounds were conducted. From the library, nine compounds exhibited virus inhibition at 45% or greater (Fig. 4C); amongst, ST081006 and ST070252 were selected for further investigations.

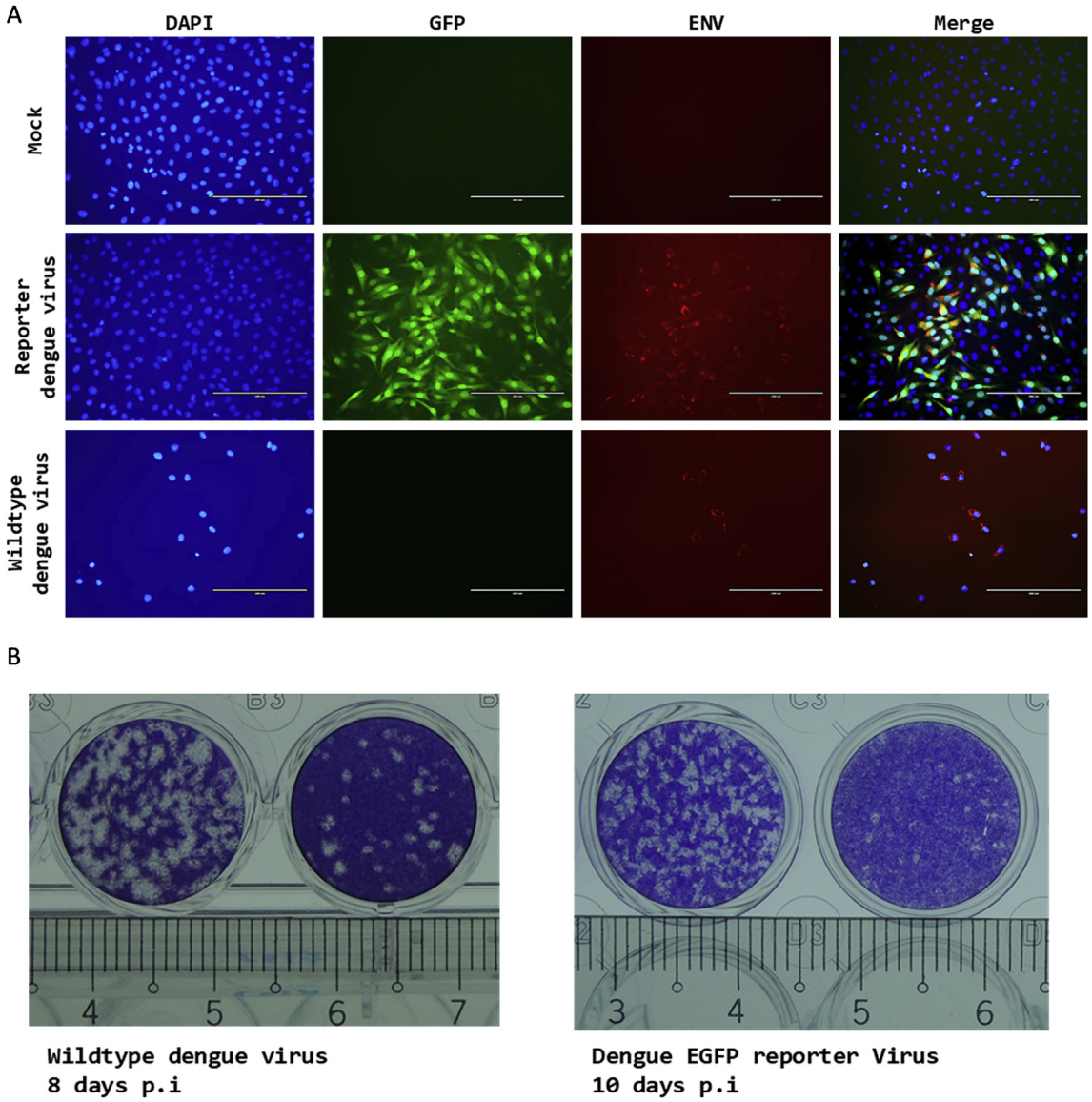

Fig. 3. Characterisation of DENV2-EGFP. A) Immunofluorescence assay for DENV2-Env expression in BHK-21 cells infected with either wildtype DENV2, or DENV2-EGFP, or in mock infected cells. B) Plaque assay showing plaque formation by DENV2 or DENV2-EGFP in BHK-21 cells.

3.4. ST081006 is a strong inhibitor of the DENV2 replication

When the compounds selected for further validation were incubated with the host cell HuH-7 for 48 h, cell viability remains at above 80% for ST081006 at concentrations up to 15 μM, while all tested concentrations of ST070252 were non-toxic (Fig. 5A and B). When DENV2-infected cells were treated to ST081006, a dose-dependent decrease in virus titer was observed at 48 hpi starting from 5 μM. Meanwhile, a significant decrease in virus titer was observed for ST070252 at 10 μM, and subsequent increase in concentration did not further decrease virus titer (Fig. 5A and B). As ST081006 exhibited a more promising inhibition on DENV2 replication as compared to ST070252, we decided to further our investigation solely on ST081006. Thereafter, using the data obtained, we fitted a nonlinear regression for ST081006, and its CC_{50} and IC_{50} values were calculated to be 25.17 μM and 3.14 μM respectively (Fig. 5C and D). Together, the selective index, taken as the ratio of CC_{50} to IC_{50}, was calculated to be 8.03. Prior to conducting further assays on ST081006, the validation procedure was repeated on the primary cell line HUVEC to ensure that its effect on DENV2 is not cell type specific. ST081006 was demonstrated to be non-toxic to HUVEC at all tested concentrations and a dose-dependent reduction in virus titer was observed starting from 7.5 μM (Fig. 5E).

3.5. The presence of ST081006 is necessary for the continued inhibition of DENV2 replication cycle

To narrow down on the search for drug target of ST081006, time-course assays which manipulate the presence of ST081006 at various timepoints during the infection were used to study its effect on virus titer. Fig. 6A illustrates the methodologies employed for the pretreatment, time-of-addition, and time-of-removal assays. Pretreating the cells to ST081006 for 2 h prior to infection did not lead to any significant difference in virus titer at 48 hpi (Fig. 6B). The time-of-addition assay demonstrated that addition of ST081006 prior to 36 hpi was necessary to decrease the overall virus titer at 48 hpi; this observation is supported by the time-of-removal assay, which showed that the removal of ST081006 only at 36 hpi or later was required to keep virus titer low (Fig. 6C). Together, these data suggest that the continued presence of ST081006 at beyond the initial 24 hpi was necessary to maintain an overall decrease in virus titer at 48 hpi.

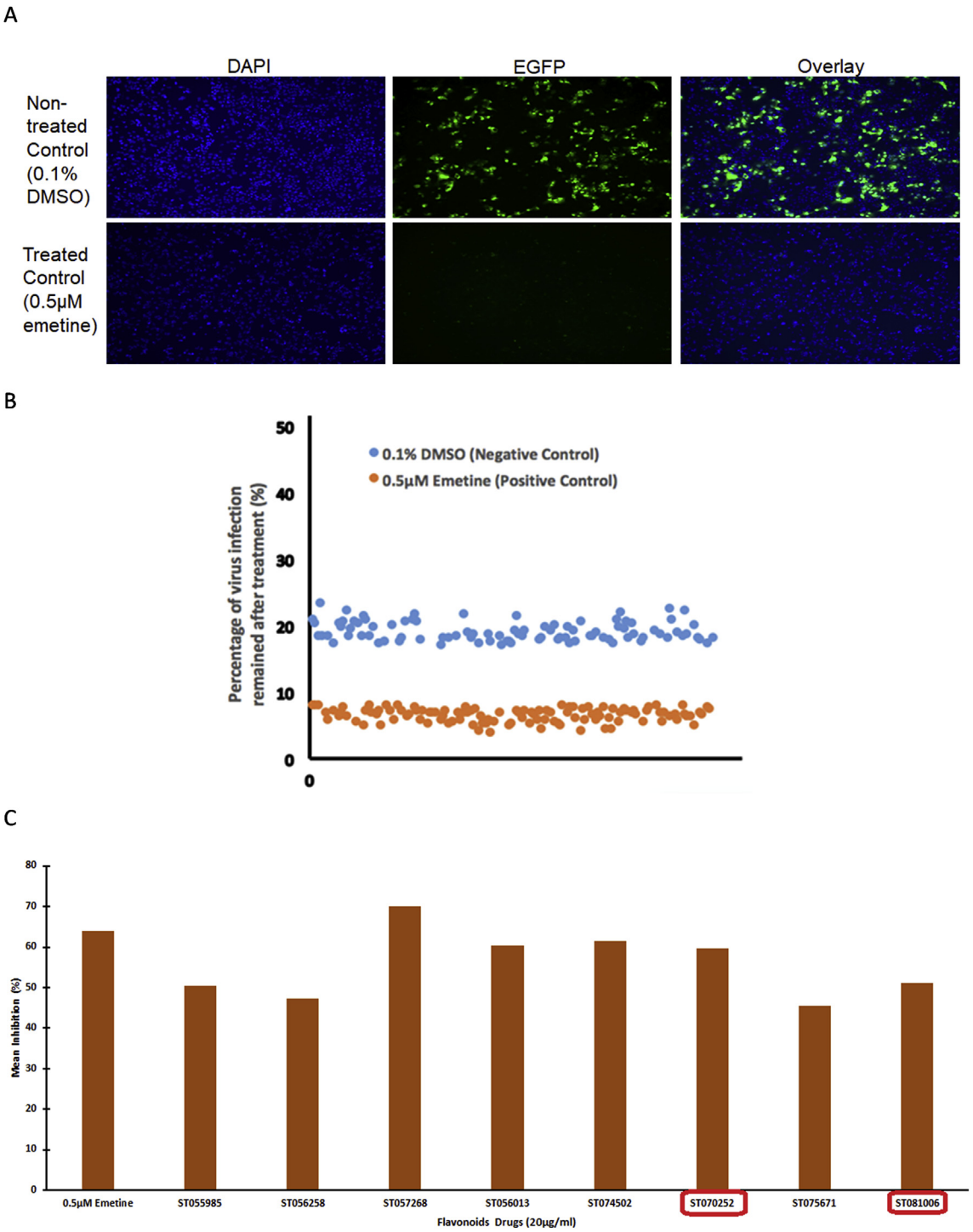

Fig. 4. Establishment of a screening platform for the primary screen. A) Representative direct fluorescence images of DENV-EGFP infected cells treated to 0.1% DMSO as vehicle control or 0.5 μM emetine as positive control. A substantial amount of EGFP signal was detected in the 0.1% DMSO-treated cells while almost no signal could be detected for the 0.5 μM emetine-treated cells. This demonstrates that EGFP signal serves as a good indicator for infection by the DENV-EGFP construct. B) A scatterplot showing the clear separation between infected cells treated with 0.1% DMSO as vehicle control from those treated with 0.5 μM emetine as positive control. The derived Z-factor, at 0.536, indicates that this is an excellent platform for a screening assay. C) Bar graph showing the percentage inhibition of hits with more than 45% percentage inhibition. The two compounds that were further followed up on, ST081006 and ST070252, are shown circled with a red box.

3.6. ST018006 inhibits DENV2 replication by affecting viral protein and/or viral RNA synthesis

Viral RNA replication and viral protein translation are pivotal processes of the virus replication cycle. To determine if the viral RNA replication is affected by the presence of ST081006, qRT-PCR was used to quantify both the positive- and negative-sense viral RNA at 12, 24, and 48 hpi after infection and treatment with either ST081006, 0.1% DMSO (negative control), or 5 μM emetine (positive control). Cell viability assay was first conducted to ensure that the concentration of emetine used was non-toxic (Fig. 7A). From Fig. 7B and C, treatment with ST081006 decreased viral RNA copy number by ~1-log unit for both positive- and negative-sense viral RNA at 24 and 48 hpi. Next, Western blot analysis was performed to determine the level of the structural E

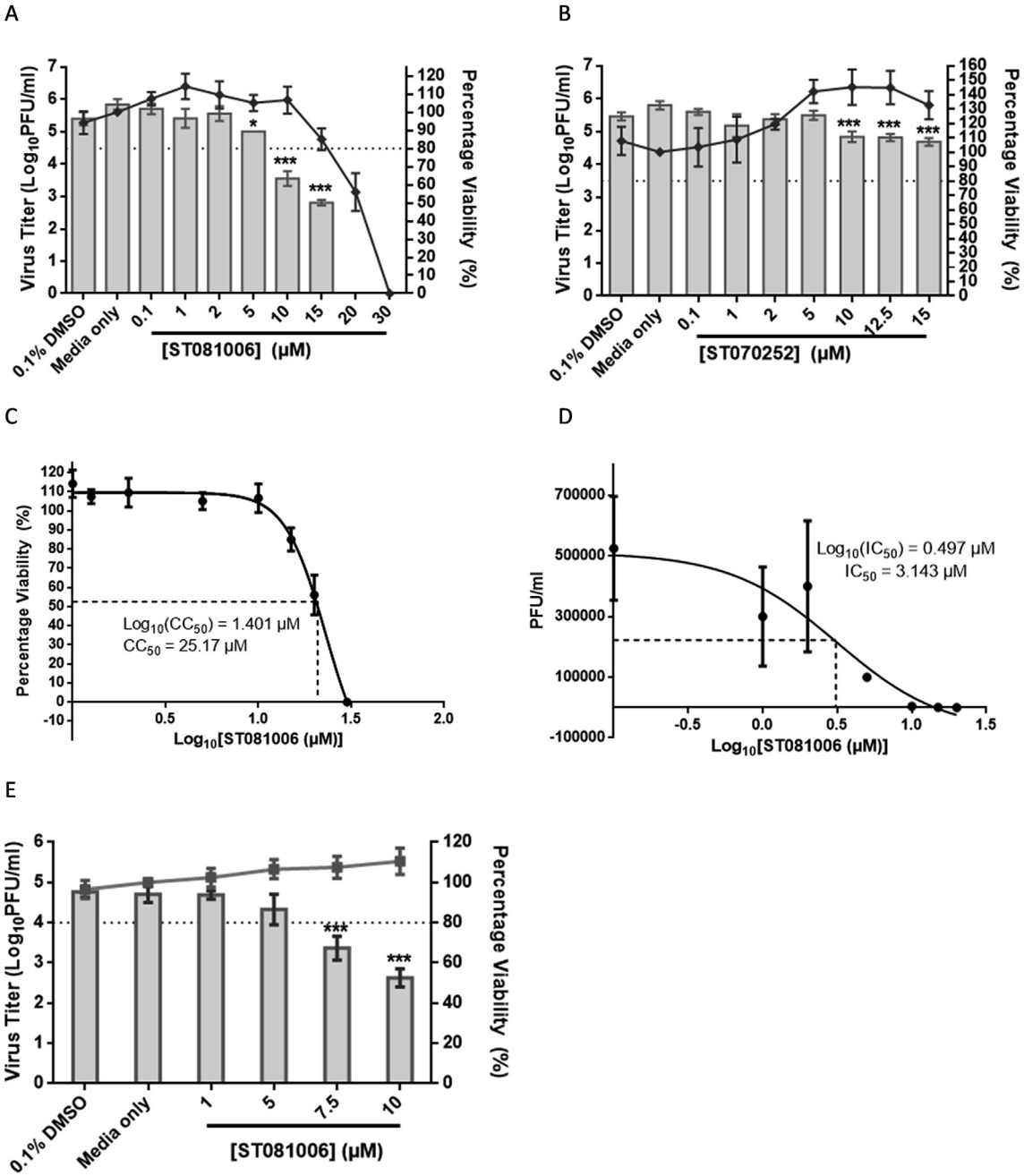

Fig. 5. Cytotoxicity and titer reduction profiling of select hits, ST081006 and ST070252. A) Cytotoxicity and titer reduction property of ST081006. ST081006 is non-toxic to HuH-7 cells at concentrations up to 15 μM, yielding a titer reduction of ~1.9-log unit. B) ST070252 is non-toxic across the range of tested concentrations, but its titer reduction effect plateaus at 0.6-log unit from 10 μM onwards. C, D) CC_{50} and IC_{50} values of ST081006, given at 25.17 μM and 3.14 μM respectively. E) Repeated profiling of ST081006 on the primary cell line, HUVEC. The range of tested concentration was non-toxic in HUVEC, and at 10 μM, resulted in a 2.1-log unit reduction in virus titer. Error bars represent standard error of experimental replicates with n = 3. Statistical analysis was performed using one-way ANOVA with Dunnett's posttest. $^{*}P < 0.05$; $^{***}P < 0.001$.

protein and non-structural NS4B protein at 24 and 32 hpi. The results obtained demonstrated that relative to the lysate harvested from 0.1% DMSO-treated cells, ST081006-treated cells expressed lower amount of viral proteins, and this trend persisted through the two selected time-points (Fig. 7D, E, F). To further resolve the effect of ST081006 on viral protein translation, we constructed a translation reporter clone with a Firefly luciferase (F. luc) reporter gene (Fig. 7G) and conducted a translation assay. F. luc signal of cells transfected with the translation reporter clone and treated with either ST081006, 0.1% DMSO (negative control), or 0.5 μM emetine (positive control) were measured at 48 or 72 hpi. From Fig. 7H, a reduction in F. luc signal was observed from ST081006 treated cells as compared to the untreated cells; at 72 hpi, a further significant reduction in F. luc signal was observed. Taken together, the data obtained from this assay demonstrated that ST081006 has a direct inhibitory effect on the viral protein translation stage of the virus replication cycle.

3.7. DENV2 was unable to generate resistance against ST081006 following multiple passages

We attempted to generate a ST081006-resistant mutant by repeatedly passaging DENV2 over many generations in the presence of

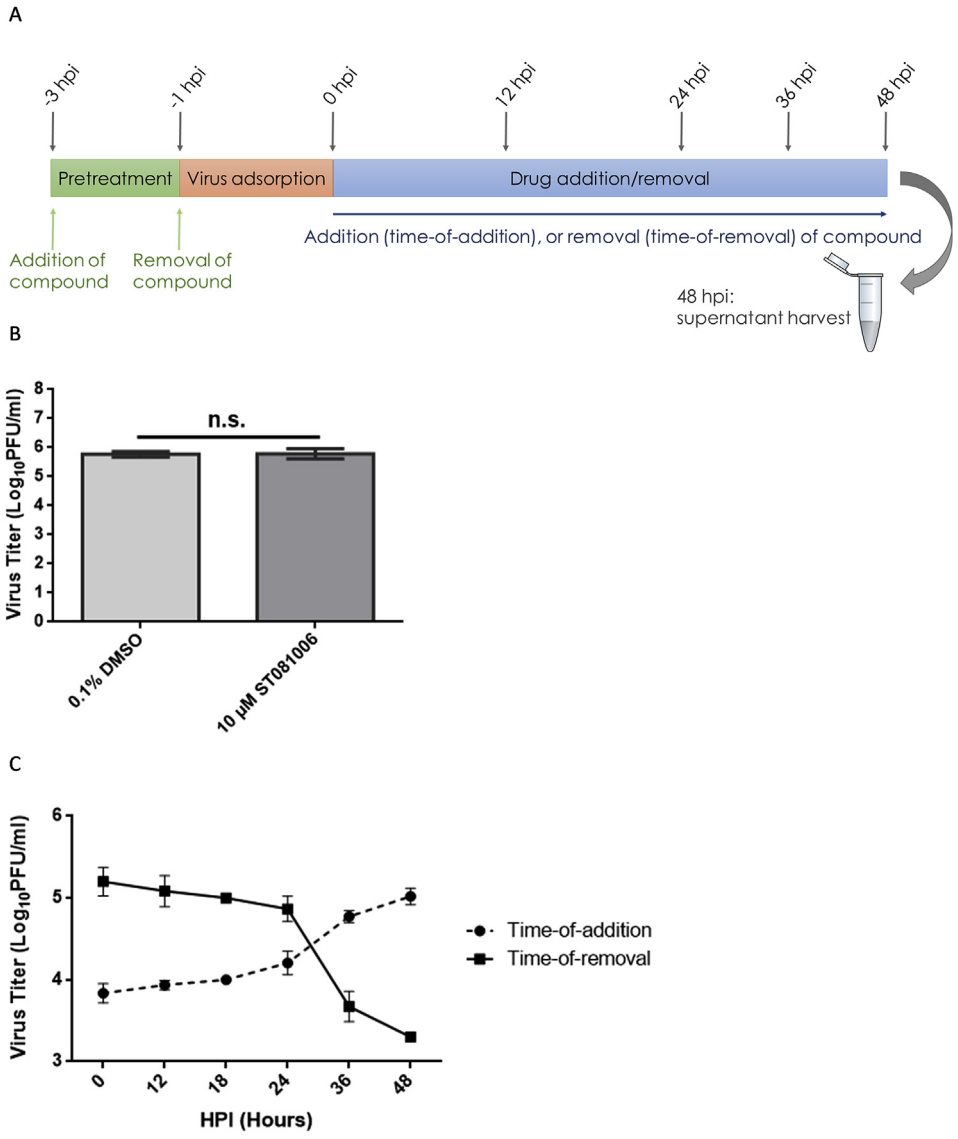

Fig. 6. The presence of ST081006 is necessary for the continued inhibition of virus replication. A) A schematic illustration of the methodologies for the pretreatment, time-of-addition, and time-of-removal assays. B) Pretreating HuH-7 to ST081006 for 2 h prior to infection does not lead to a significant decrease in virus titer as compared to the vehicle control, 0.1% DMSO. C) Virus titer at 48 hpi as 10 μM ST081006 was added, or removed, at various timepoints for the time-of-addition and time-of-removal assays. For time-of-addition assay, virus titer stayed low at 48 hpi when ST081006 was added before 36 hpi, whereas virus titer stayed high when ST081006 was removed before 36 hpi. Error bars represent standard error of experimental replicates with n = 3. Statistical analysis for the pretreatment assay was performed using Student's t-test. n.s. = not significant.

ST081006 (Supplementary Fig. 1). Expectedly, the virus titer of ST081006-treated cells dropped over the passages, and by passage 3, was < 1-log unit. In order to recover the virus titer, infected cells treated with ST081006 were incubated until CPE became prominent, before harvesting the supernatant. This allowed for the recovery of the virus titer (passage 4 to 6). When the supernatant was reverted back to being harvested at 48 hpi, the virus titer for the ST081006-treated cells dipped again (passage 7 to 8). A second period of recovery maintained the virus titer but did not raise it any further (passage 9 to 11). Finally, when the resistance of the virus against ST081006 was tested again for the third time, the virus titer plunged and was beyond recovery by passage 14, demonstrating that DENV2 was unable to generate resistance against ST081006 despite the many rounds of titer recovery provided.

3.8. Inhibitory property of ST081006 extends to other dengue serotypes and EV-A71

We also tested the effect of ST081006 against other dengue serotypes (DENV1, DENV3, and DENV4) on HuH-7, and on other positive-sense RNA viruses (CHIKV, ZIKV, and EV-A71 on HuH-7, JEG-3, and RD cells respectively). ST081006 was able to inhibit the replication of all dengue serotypes indiscriminately, with a dose-dependent reduction in virus titer starting from 5 μM for DENV1 and from 7.5 μM for DENV3 and DENV4 (Fig. 8A, B, and C). Further testings against other positive-sense RNA viruses demonstrated that at 10 μM ST081006 is not effective against CHIKV or ZIKV, but interestingly, is able to inhibit the replication of the enterovirus EVA-71 (Fig. 8D).

4. Discussion

The propensity of DENV in causing outbreaks, along with the lack of any therapeutic measure against it, makes the search for a DENV antiviral an urgent task. In our study we constructed an infectious clone tagged with EGFP for ease in the tracking of virus infection. Characterisation of the constructed clone demonstrated that it is capable of undergoing viral replication and inducing plaque formation in cell culture, validating its use in our subsequent drug screen. Thereafter, we established a phenotypic screening platform with a Z-factor of 0.536, which indicates an excellent platform that can discern between positive and negative hits. An anti-DENV screen conducted on the flavonoid-derivative library yielded nine hits with percentage inhibition above the arbitrarily set threshold of 45%, and these hits were further examined. Amongst these hits, ST055985, ST056258, and ST056013 belong to the flavonoid class of flavone, which contains other members with reported anti-DENV activity, such as luteolin (Peng et al.,

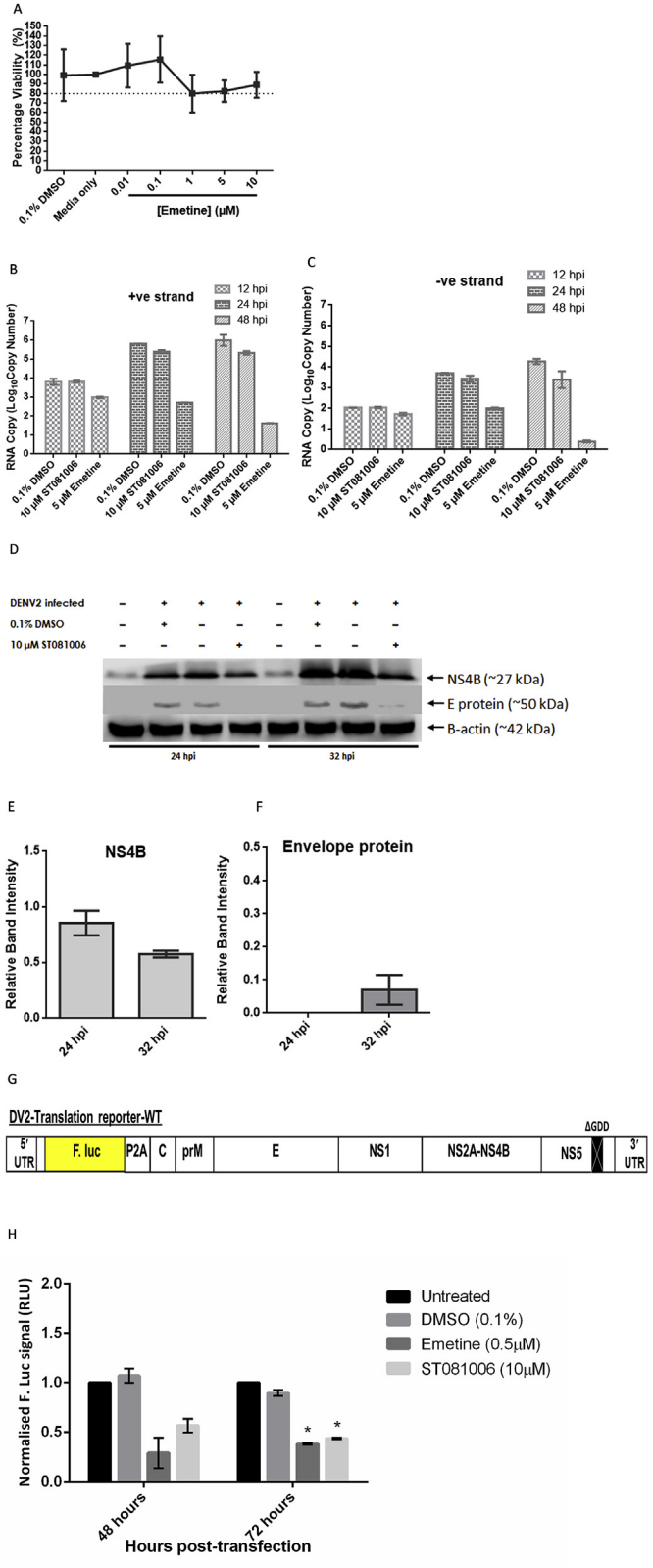

Fig. 7. ST081006 is likely to inhibit DENV2 replication by affecting the viral protein translation and/or viral RNA synthesis pathway. A) Percentage viability of HuH-7 cells treated with varying concentrations of emetine (positive control for qRT-PCR) at 48 h post treatment. Cell viability for all tested concentrations remained above the cytotoxic threshold of 80%. B, C) RNA copy number for the positive and negative sense viral RNA at 12, 24, and 48 hpi, upon treatment with 0.1% DMSO as vehicle control, 5 μM emetine as positive control, or 10 μM ST081006. Treatment with ST081006 leads to a decrease in both positive and negative sense RNA at 24 and 48 hpi. D) Western blot analysis of viral protein at 24 and 32 hpi. Treatment with ST081006 leads to a decrease in both viral E and NS4B proteins. E, F) Densitometry plot of band intensity of ST081006-treated sample relative to the 0.1% DMSO-treated samples. Decrease in relative expression is observed at both timepoints for both viral proteins. G) Genomic map of the DENV2 translation reporter clone. H) F. luc values of lysates from cells infected with DENV2 and treated with ST018006, 0.1% DMSO as negative control, 0.5 μM emetine as positive control, or were untreated. At 48 and 72 hpi, reduction in F. luc signal was observed for ST081006-treated sample; the reduction was significant by 72 hpi. Error bars represent standard error of experimental replicates with n = 2. Statistical analysis for the Firefly luciferase translation reporter assay was performed using one-way ANOVA with Dunnett's posttest.

2017), baicalin (Moghaddam et al., 2014), and chrysin (Du et al., 2016), although none of these hits were previously reported for anti-DENV activity. Based on the criteria of low cytotoxicity, gauged preliminary by the nuclei count from the DAPI stain, and on academic novelty of the compound's effect on DENV, ST070252 was selected for further evaluation. In addition, ST081006, previously reported to inhibit the replication of the hepatitis C virus, a fellow member of the *Flaviviridae* family, also piqued interest and was selected for downstream evaluation (Kusano-Kitazume et al., 2011). Subsequent assays demonstrated that treatment with ST081006 decreased virus titer by 1.6-log units at 10 μM as compared to ST070252's 0.6-log units at the same concentration. As the effect of ST070252 was both modest and not titratable, ST081006, a synthetic flavan, was the selected as the main compound of choice for the elucidation of its mechanism.

A time-course study was performed to narrow the stage of the virus replication cycle that the compound is likely to affect. A pretreatment assay revealed that incubating the cells to ST081006 for two hours prior to infection did not lower the virus titer at the end of the assay. This suggests that ST081006 is unlikely to act on the entry or early post-entry stages of the virus replication cycle, which includes processes such as the receptor-mediated endocytosis of the virus particle, acidification of the endosomes, and unravelling of the nucleocapsid. Further, the time-of-addition and time-of-removal assays suggest that the presence of ST081006 is necessary for the continued inhibition of the virus replication cycle. Separately, the time-of-addition assay demonstrated that the addition of ST081006 at 36 hpi and after led to a higher virus titer as compared to the addition of ST081006 at earlier timepoints. This suggests that the presence of ST081006 is unable to attenuate the infectivity of the virus that have already replicated and were present during the point of drug addition. On the other hand, the time-of-removal assay revealed that the removal of ST081006 at timepoints earlier than 36 hpi led to a higher virus titer as compared to removal at or after 36 hpi. One possible interpretation is that the presence of ST081006 is necessary for the continued inhibition of the virus replication cycle, and that early removal may afford the virus an opportunity for titer recovery through the successive replication cycles.

qRT-PCR quantification of both positive- and negative-sense viral RNA at 12, 24, and 48 hpi showed a decrease in RNA copy number at 24 and 48 hpi when cells were treated with ST081006. We note that this is a small decrease relative to that observed by the positive control, emetine, which caused a greater decrease in viral RNA copy number of more than 1.5- and 2.5-log units for the negative- and positive-sense viral RNA respectively. One plausible explanation for this observation is that ST081008 may affect viral RNA copy numbers by acting indirectly through a process that is upstream of viral RNA synthesis. A possible example of such a process could be the translation of the viral polyprotein, a process that precedes viral RNA replication. This is further strengthened by the observation that at 12 hpi, treatment with ST081006 did not result in any difference in RNA copy number as compared to the 0.1% DMSO-treated cells. We postulated that at 12 hpi,

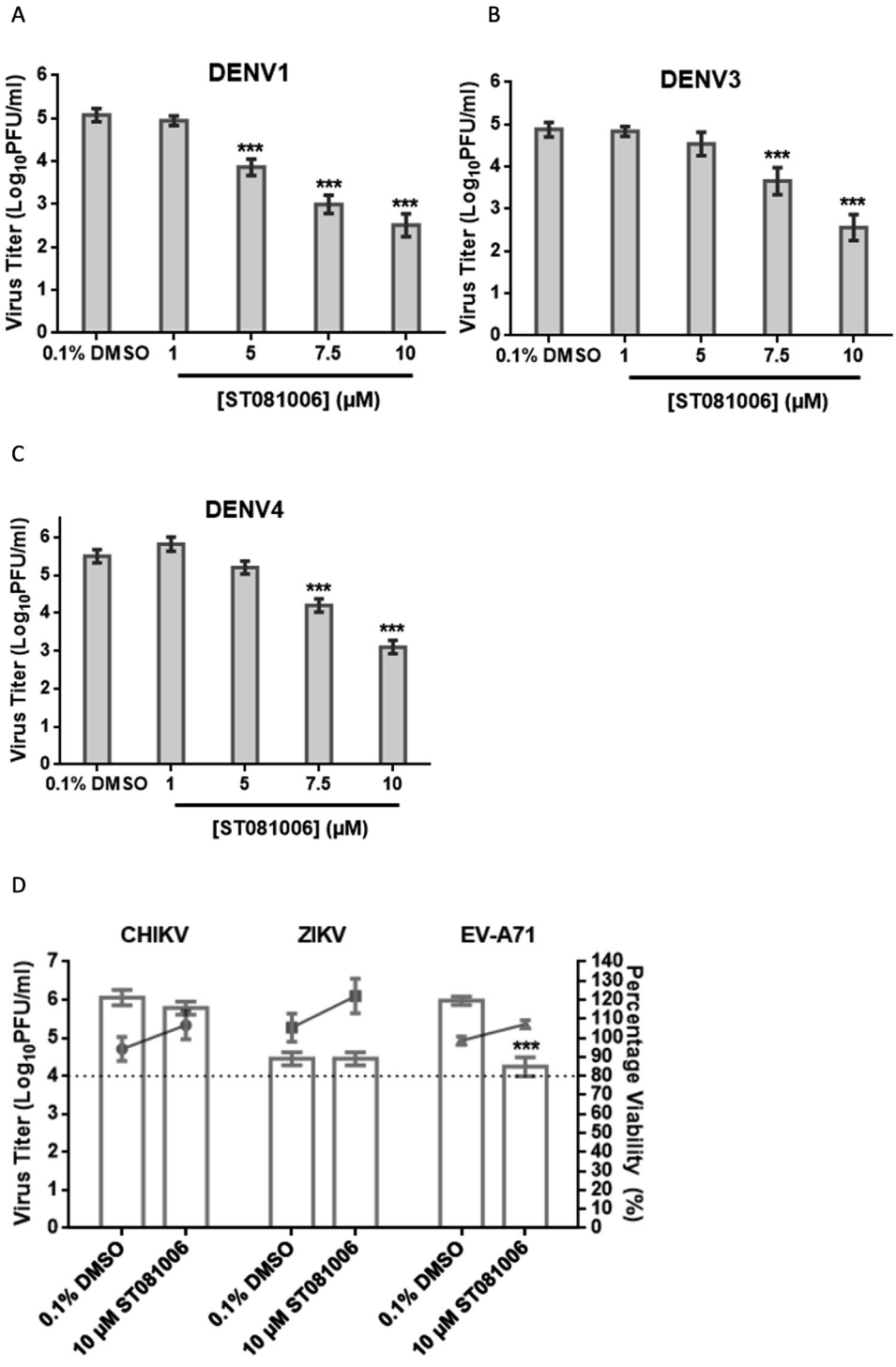

Fig. 8. Effects of ST081006 against other dengue serotypes and other positive-sense RNA virus. A, B, C) Inhibitory profile of ST081006 against DENV1, DENV3, and DENV4 respectively, with HuH-7 as the host cell. A > 2-log unit reduction in virus titer was observed for all serotypes at 10 μM. D) Effects of 10 μM ST081006 on other positive-sense RNA virus. EV-A71 saw a significant reduction in virus titer upon treatment with 10 μM of ST081006, while CHIKV and ZIKV were unaffected by it. Error bars represent standard error of experimental replicates with n = 3. Statistical analysis was performed using one-way ANOVA with Dunnett's posttest. ***$P < 0.001$.

the process that was affected by ST081006 has yet to assert an indirect effect on the viral RNA synthesis.

Western blot analysis of both structural and non-structural viral proteins showed a decrease in both the structural E protein and the non-structural NS4B proteins at timepoints 24 and 32 hpi. As viral protein translation and viral RNA synthesis exert a feedback effect on each other as part of the virus replication cycle, the observation that both processes appear to be affected by the presence of ST081006 is expected. To further determine if viral protein translation is a process targeted by ST081006, we constructed a translation reporter clone lacking the GDD catalytic triad in the NS5 coding region of the DENV2 genome. As the GDD catalytic triad makes up the catalytic core of the viral RNA-dependent RNA polymerase (RdRP) (Hodge et al., 2016), the deletion of the GDD motif renders the virus unable to further progress to RNA synthesis. Because viral replication cannot progress to the viral RNA synthesis stage, viral protein synthesis is limited to the first round of the virus RNA replication cycle. As such, the amount of viral polyprotein produced will be directly proportional to the intensity of the F. luc signal measured. The data obtained from this assay showed that viral protein synthesis decreases in the presence of ST081006, and this is consistent with the data from the Western blot analysis. This indicates that ST081006 has a direct inhibitory effect on DENV protein synthesis.

The protein synthesis and RNA replication stages of the DENV replication cycle are tightly coupled together. Therefore, the direct

inhibitory effect that ST081006 has on viral protein synthesis will also have an indirect inhibitory effect on DENV RNA replication. This does not preclude that ST081006 also has a direct inhibitory effect on DENV RNA replication, but we are unable to prove such a direct effect at the moment.

To delve further into the exact mechanism of ST081006, we recommend the use of assays that may further elucidate potential drug targets. The use of high resolution microscopy to visualize changes in subcellular structures in the presence of ST081006 can be used to narrow down potential targets of ST081006. Subsequent characterisation of drug-protein interactions may be used to identify the interacting partner of ST081006 in the subcellular environment.

A common strategy employed to identify the drug target of antiviral is to generate a drug-resistant mutant by repeatedly passaging the same strain of virus in the presence of the compound. To ensure that the virus titer does not hit fixation, we allowed for a period of titer recovery following a significant decrease in virus titer by incubating the infected cells in the presence of ST081006 till approximately 50% CPE was visually observed. Despite so, the strain was unable to acquire resistance against ST081006 and crashed sharply at passage 14. The inability to acquire mutations that confer resistance against ST081006 could point towards the possibility of the compound targeting a host, rather than a virus factor, that is responsible for a critical process in the virus replication cycle.

The potential usage of ST081006 as a DENV antiviral is contingent on its inhibitory property not just on DENV2, but on all serotypes of the dengue virus. When titer reduction assay was repeated for the other dengue serotypes, they were shown to be inhibited by the presence of ST081006. The same assay, repeated on the other positive-sense RNA viruses, demonstrated that ST081006 was able to inhibit the replication of EV-A71, but not CHIKV and ZIKV. Interestingly, despite not affecting the replication of a fellow flavivirus ZIKV, ST081006 was able to affect the replication of the enterovirus EV-A71. This hints at a possibility of ST081006 targeting separate targets that are unique to the replication of DENV and EV-A71, or, on a host factor that is common between DENV and EV-A71. Another possibility is that as the level of drug-metabolising enzymes of an *in vivo* placenta fluctuates throughout gestation, it results in differential metabolism from other tissues (Syme et al., 2004). It follows then that JEG-3, the host cell for ZIKA with a placental origin, could have metabolised ST081006 before it was able to exert its inhibitory influence. Therefore, to establish the influence of ST081006 on other positive-sense RNA virus, the essay could be repeated with other host cells, such as HeLa for CHIKV and HuH-7 for ZIKV.

In conclusion, we screened the flavonoid-derivative library for anti-DENV compounds using a high-throughput screening platform that we have established. We identified ST081006 as a strong inhibitor of DENV, and time-course studies demonstrated that it is likely to act on a stage of the virus replication cycle that occurs after entry or early post-entry events. Mechanistic studies suggest that ST081006 is likely to affect DENV2 replication by its effect on the two closely intertwined processes, viral RNA replication and viral protein synthesis. DENV2 was also unable to acquire resistance against ST081006 even after repeated passage. This, along with its inhibitory effect on EV-A71, but not on ZIKV, points at host factor as a possible drug target. With its inhibitory profile against all dengue serotypes, ST081006 shows great promise for its development as a DENV antiviral. Further studies should seek to fully establish its mechanism and push it towards animal studies.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104589.

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