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A high-throughput assay using dengue-1 virus-like particles for drug discovery

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ABSTRACT

Dengue virus (DENV) is a mosquito-borne flavivirus responsible for 50–100 million human infections each year. The development of DENV chemotherapy requires high-throughput screening (HTS) assays. A dengue virus-like particle (VLP) has been constructed using viral structural proteins to package a *Renilla* luciferase reporter replicon. VLP could be produced by either the sequential electroporation of the replicon RNAs and the structural gene RNAs or by electroporating replicon RNA into a stable cell line expressing the structural proteins. In both approaches, the key to produce high titer VLP (3×10^6 foci-forming unit/ml) is to use low temperature (30 °C) in the packaging step. In addition, exogenous expression of host protease furin increased VLP infectivity. The infection could be blocked by antibodies against viral envelope protein and by an inhibitor of viral NS5 polymerase, but not by an inhibitor of host alpha-glucosidase (castanospermine). The VLP infection assay was optimized for HTS in a 384-well format with consistent and robust signal, providing a simple and rapid cell-based assay for screening inhibitors against DENV entry, translation, and replication in an HTS format.

1. Introduction

Dengue virus (DENV) is a significant human pathogen belonging to the genus *Flavivirus* in the family Flaviviridae (Chambers et al., 1990). It is estimated that 50–100 million cases of dengue fever (DF) occur annually worldwide, making it the most common and widespread arthropod-borne viral infection of humans (Gubler and Meltzer, 1999). Currently there is no licensed vaccine or antiviral agent available, highlighting the need for a reliable high-throughput assay in drug discovery.

There are four serotypes of dengue virus (Lindenbach and Rice, 2003). Dengue virion is approximately 50 nm in diameter (Kuhn et al., 2002) and contains a single-stranded, plus-sense RNA genome of about 11 kb in length (Chambers et al., 1990). The genomic RNA contains a single long open reading frame (ORF) that is flanked by a 5′-untranslated region (5′-UTR) and a 3′-UTR. The ORF encodes a polyprotein that is co- and post-translationally processed by viral and cellular proteases into 10 mature proteins: three structural proteins (the capsid [C], premembrane [prM] or membrane [M], and envelope [E] proteins) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Chambers et al., 1990). Structural proteins are primarily involved in viral particle formation. Non-structural proteins are responsible for viral RNA replication.

Sub-genomic replicons, in which the genes encoding the structural proteins of the virus have been replaced with a reporter gene, can be packaged by the structural proteins to form a virus-like particle (VLP). VLP is capable of entry, translation and replication (Ansarah-Sobrinho et al., 2008; Harvey et al., 2004; Puig-Basagoiti et al., 2006). VLP of several different flaviviruses have been constructed and employed in the studies of different aspects of the flavivirus life cycle, such as viral entry (Ansarah-Sobrinho et al., 2008; Davis et al., 2006), replication (Puig-Basagoiti et al., 2005), assembly (Whitby et al., 2005) and secretion (Goto et al., 2005).

Dengue drug discovery requires the development of reliable biological assays. Traditional antiviral assays for flavivirus are based on viral infection of cultured cells, followed by monitoring of compound inhibition of viral infection through observation of cytopathic effects, quantification of viral yields by plaque assay, or measurement of viral RNA by reverse transcription-PCR (Jordan et al., 2000; Morrey et al., 2002). The low-throughput nature of these assays limits their use for the screening of large compound libraries. Alternatively, VLP harboring luciferase-expressing replicon can be applied to infect cells, leading to luciferase expression which could be used to monitor the antiviral activities of potential inhibitors. However, the titer of traditionally produced VLPs of DENV (Qing et al., 2009) is too low for high-throughput screening

Abbreviations: VLP, virus-like particle; DENV, dengue virus; HTS, high-throughput screen; C, capsid protein; prM, premembrane; E, envelope; NS, non-structural protein; FFU, focus-forming unit; VEEV, Venezuelan equine encephalitis virus; SFV, Semliki forest virus; Rluc, Renilla luciferase; FMDV2A, foot-and-mouth disease virus 2A autocleavage site.

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(HTS). In the current study, we developed a high yield production of DENV-1 VLP by packaging at a low temperature (30 °C). This strategy achieved a much higher titer of DENV-1 VLP (upto 3×10^6 FFU/ml). Furthermore, we found that over-expression of furin increased VLPs infectivity, presumably by efficient cleavage of prM to M. We optimized VLP infection parameters and tested various antiviral antibodies and compounds. These data validated the VLP infection assay and showed that it provides a simple and rapid way to screen antiviral compounds against DENV entry, translation, and replication in HTS format.

2. Materials and methods

2.1. Cell lines, viruses, antibodies and compounds

All cell lines were grown at 37 °C in the presence of 5% CO_2. Vero, LLC-MK2 and BHK-21 cell lines were maintained in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin–streptomycin (PS). A549 cell lines were maintained in complete F12 medium supplemented with 10% FBS and 100 U/ml PS. BHK-21 stable cell line expressing DENV-1 structural proteins (CprME) was propagated in complete DMEM (10% FBS and 100 U/ml PS) supplemented with 10 μg/ml puromycin. DENV-2 virus is derived from an infectious clone of DENV-2 New Guinea C strain (GenBank number AF038403, kindly provided by Andrew D. Davidson, University of Bristol, UK). Non-cytopathic Venezuelan equine encephalitis virus (VEEV) replicon was a kind gift from Nancy L. Davis, University of North Carolina. Mouse anti-Myc antibody were purchased from Santa Cruz Biotechnology. Dengue specific monoclonal antibodies 4G2 (Rajamanonmani et al., 2009) were collected from hybridoma cell lines purchased from American Type Culture Collection (ATCC). Compounds NITD008 and castanospermine were made in house. Lycorine was from Apin Chemicals (Abingdon, UK). All compounds were dissolved in 90% DMSO for antiviral experiments.

2.2. DNA construct, RNA transcription, electroporation and selection of puromycin-resistant stable cell line expressing dengue structural protein

DENV-1 replicon (DENV-1-Rluc2A-Rep) construct was reported previously (Puig-Basagoiti et al., 2006). The DENV-1 structural genes were either expressed by a Semliki forest virus (SFV) replicon (SFV-DENV-1-CprME) (Qing et al., 2009) or by a non-cytopathic Venezuelan equine encephalitis virus (VEEV) replicon (VEEV-PAC-2A-DENV-1-CprME). VEEV-PAC-2A-DENV-1-CprME is constructed by replacing the VEEV structural genes with a puromycin resistance gene (puromycin acetyltransferase, Pac) and a 2A autocleavage site of foot-and-mouth disease virus (FMDV2A) fused to DENV-1-CprME genes. Stbl2 cells (Invitrogen) transformed with DNA constructs were grown in LB medium containing 75 μg/ml Ampicilin (Invitrogen) at 30 °C overnight. Plasmid DNA was purified by Maxiprep (Qiagen) and linearized by NotI digestion. For RNA transcription, linearized DNA was extracted from phenol-chloroform and chloroform, precipitated before being used as a template for *in vitro* transcription using the mMESSAGE mMACHINE T7 Kit (Ambion) according to the Manufacturer's protocol. The extracted RNA was resuspended in RNase-free water and quantitated by spectrophotometry. For RNA electroporation, 8×10^6 BHK-21 cells were washed once with cold PBS before being resuspended in 800 μl of cold PBS containing 10 μg of RNA. The cells were pulsed three times with 3-s intervals at 0.85 kV/25 μF in 0.4-cm cuvettes using the Gene Pulser Xcell apparatus (Bio-Rad). Electroporated cells were plated into 10-cm plates and grown in DMEM with 10% FBS. At 24 h post-electroporation, fresh medium containing 10 μg/ml of puromycin was added. The medium was replaced every 3–4 days to select puromycin-resistant cells. After 7 days, individual foci of puromycin-resistant cells were picked and expanded in the presence of puromycin (10 μg/ml). To detect the expression of VEEV-PAC2A-DENV-1-CprME, BHK-21 clone #1.2 containing VEEV-PAC2A-DENV-1-CprME were at seeded 6000 cells per well in 8-well chamber slide and fixed in methanol the next day. Cells were then incubated with 4G2 (mouse polyclonal antibodies against E protein) and detected using FITC labeled anti-mouse antibodies (Jackson Immunolab). Nuclei of the cells were stained with Dapi (Vectashield® Mounting Medium with Dapi, Vector laboratories). Myc-tagged Furin plasmid was purchased from Origene.

2.3. VLP production

For VLP production, we first used sequential electroporation method previously described (Qing et al., 2009). Briefly, 10 μg of replicon RNA (DENV-1-Rluc2A-Rep) was electroporated into 8×10^6 BHK-21 cells and cells were kept at 37 °C. After 24 h, cells were electroporated with 10 μg of structural gene RNA (SFV-DENV-1-CprME) and cultured at 37 °C or 30 °C. The culture supernatant of the sequentially electroporated cells was collected at various time points post-electroporation and stored at −80 °C prior to VLP quantification. The other way of VLP production was by VEEV-PAC-2A-DENV-1-CprME cell line which stably expresses DENV-1-CprME protein. DENV-1-Rluc2A-Rep-RNA (10 μg) was electroporated into VEEV-DENV-1-CprME cell line (8×10^6) and cultured cells at 37 °C for 24 h, followed by 30 °C. The culture fluid of the electroporated cells was collected. We also produced VLP by co-electroporating replicon RNA (10 μg) and Myc-Furin DNA (9 μg), into the BHK-21 cell line stably expressing the structural proteins.

2.4. VLP quantification

VLP infection can be quantified either by luciferase activity or by immunofluorescence staining of the foci (foci-forming units [FFU]/ml). For luciferase assay, EnduRen™ (Promega) was added at 1:2000 dilution (30 μM) and incubated for 3 h before the luminescence was read. Cell viability was measured by CellTiter-Glo® luminescence cell viability assay (Promega) for ATP level detection in live cells according to the instruction of the Manufacturer. For immunofluorescence staining of the foci, anti-NS1 antibody (a kind gift from Dennis R. Burton, Scripps Research Institute) and Alexa Fluor® 594 goat anti-human IgG (Invitrogen) were used as primary and secondary antibodies to stain VLP positive cell. VLP (50 μl) was serially diluted from 1:30 to 1:300,000 for infection in Vero cells (40,000 cells/well in 96-well plate) for 48 h. The titer of VLP = (number of NS1 positive cells × dilution factor)/volume of infection.

2.5. Inhibition of VLP infection by antibodies and antiviral compounds

For VLP neutralization assay, four-fold serial dilution of antibody 4G2 were mixed with different dilutions (1:10, 1:20 and 1:40 dilutions) of VLP (3×10^6 FFU/ml) (Crill and Chang, 2004; Henchal et al., 1982; Rajamanonmani et al., 2009; Summers et al., 1989), and incubated at room temperature for 1 h. Vero cells in 96-well plate (40,000 cells per well) were infected with antibody treated VLPs. At 48 h post-infection, luciferase activity was measured by EnduRen™ (30 μM). The luminescence reading was plotted against the fold dilutions of the antibodies. For antiviral compounds, Vero cells were plated at 40,000 cells per well in a 96-well plate. The next day, 50 μl of VLP (3×10^5 FFU/ml) and serial two-fold dilution of compound was added to the cells. At 48 h post-infection, luciferase activity was measured with Enduren (30 μM) and the

luminescence reading was plotted against the concentration of the compound.

2.6. Western blot

Ten microliters of cell lysis, collected at 72 h after Myc-Furin/Rep-RNA or pcDNA3.1/Rep-RNA transfection, were separated on an SDS-PAGE. The proteins in the SDS-PAGE were transferred to a Hybond-C Super nitrocellulose membrane (Amersham). The nitrocellulose membrane was blocked with 5% skim milk in PBS, incubated with primary antibody (mouse anti-Myc antibody, Santa Cruz Biotechnology) and a secondary antibody (HRP-conjugated goat anti-mouse IgG; Jackson Immuno-Research Laboratories), and developed with chemiluminescence ECL reagents (Amersham).

3. Results

3.1. Production of high titer DENV-1 VLPs

Initially, VLPs were produced by sequential electroporation of DENV-1 replicon RNA (DENV-1-Rluc2A-Rep) and CPrME-expressing RNA (SFV-DENV-1-CprME) at 37 °C in BHK-21 cells (Fig. 1A), with an interval of 24 h between the two electroporations. VLPs were harvested at 32, 48, 72, and 96 h after the first electroporation of DENV-1-Rluc2A-Rep-RNA. The amount of VLPs was quantified by infecting naive Vero cells and the infected cells were then monitored for luciferase activities at 48 h post-infection. Low luciferase signals, $\leq 2.7 \times 10^2$ light units, were obtained from the VLP-infected cells (Fig. 1B), indicating an inefficient VLP production. To improve the VLP yield, we established a stable BHK-21 cell line expressing DENV-1-CprME through a non-cytopathic VEEV replicon (VEEV-PAC-2A-DENV-1-CprME; Fig. 2A) in which the E protein was expressed in all cells of the established cell line (Fig. 2B). A single electroporation of the CprME-expressing cells with DENV-1 replicon RNA generated VLPs. The highest VLP yield was obtained at 32 h post-electroporation, reaching 4×10^4 light units (Fig. 2C). The results suggested that the single electroporation method produces more VLPs than the double electroporation method.

During the course of our study, Pierson and colleagues reported that culturing transfected cells at lower temperature increased the yield of DENV VLP (Ansarah-Sobrinho et al., 2008). We observed similar results when producing recombinant DENV-2 NGC (New Guinea C) strain. BHK-21 cells were transfected with genome-length RNA, derived from an infectious cDNA clone. The transfected cells were subjected to three incubation schemes: at 37 °C, 30 °C, or at 37 °C for the first 24 h followed by incubation at 30 °C. Viral titers in culture fluids were determined at 48 to 120 h post-transfection (Fig. 3A). The results showed that the 37 → 30 °C incubation scheme produced highest viral titers, whereas the 37 °C incubation scheme yielded the lowest viral titers. The results demonstrated that the viral yields from the genome-length RNA-transfected cells are temperature-dependent.

The above three temperature schemes were applied to improve the yield of VLP production. For the sequential transfection method, the cells were incubated at 37 °C for 24 h after the replicon RNA electroporation to allow optimum RNA synthesis. The cells were then transfected with structural protein-expressing RNA and incubated at 30 °C for VLP production. Alternatively, the electroporated BHK-21 cells were incubated at 37 °C or 30 °C throughout the VLP production (without temperature shift). The 37 → 30 °C incubation scheme (temperature shifted at 24 h after the first electroporation of DENV-1 replicon RNA) generated the highest VLP yield, as indicated by the luciferase signals derived from the VLP-infected cells (Fig. 3B, bottom left panel). Next, we determined the optimal time point to shift the incubation temperature from 37 to 30 °C. The

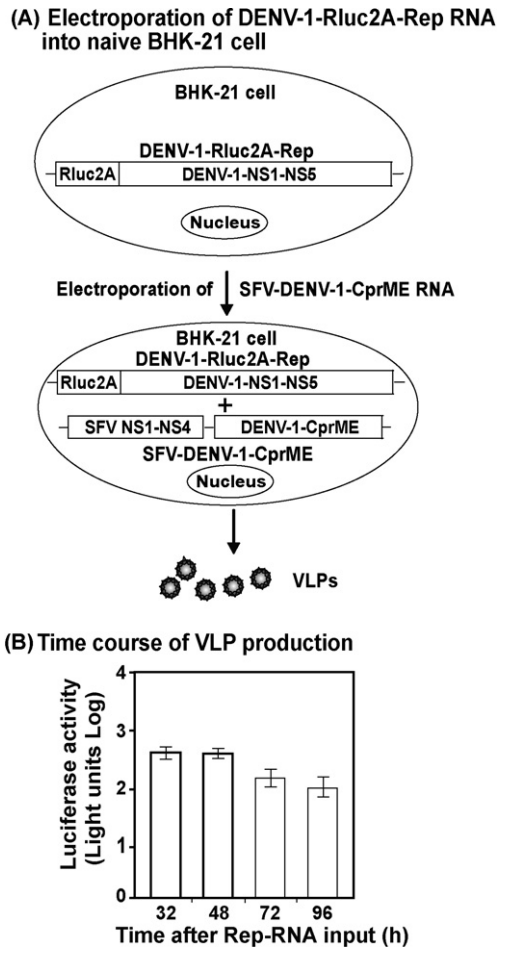

Fig. 1. Production of DENV-1 VLP by sequential electroporation. (A) BHK-21 cells were first electroporated with replicon RNA. Replicon construct (DENV-1-Rluc2A-Rep) was made by fusing a *Renilla* luciferase gene (Rluc) followed by a FMDV2A cleavage site (2A) to all the non-structural genes of DENV-1 (NS1-NS5). Cells were then electroporated with RNA of DENV-1 structural gene expressed by a SFV replicon (SFV NS1-NS4-DENV-1-CprME). (B) Quantification of VLP produced at different time point after replicon RNA (Rep-RNA) electroporation. VLPs were harvested at 32, 48, 72 and 96 h post-electroporation of DENV-1-Rluc2A-Rep-RNA. The amount of VLPs was quantified by infecting 40,000 Vero cells per well in 96-well plate (without dilution of the VLP stock). The infected cells were measured for luciferase activity at 48 h post-infection. Average results of four independent experiments are presented. Error bars represent standard deviations.

double electroporated cells (with a 24-h interval between the two transfections) were incubated at 37 °C for 24, 34, or 48 h before shifting to the incubation at 30 °C (Fig. 3B, top panel). VLPs were harvested at various time points, and quantified by infecting Vero cells. The luciferase results showed that shifting the incubation temperature to 30 °C at 24 h after the first electroporation of DENV-1 replicon generated the highest level of VLP (Fig. 3B, bottom right panel).

We also tested the low temperature procedure in electroporating DENV-1 replicon into the BHK-21 cell line stably expressing structural proteins. Similar to the transfection scheme described in Fig. 3A, shifting the incubation temperature from 37 to 30 °C at 24 h post-electroporation produced the highest yield of VLPs at 72 h post-electroporation (Fig. 3C, left panel). Next, we confirmed that cells incubated for 24 h (instead of 14, 34, or 48 h) at 37 °C before transferring to 30 °C was the optimal time for the highest titer of VLP (Fig. 3C, right panel); 24 h at 37 °C showed much higher production than 48 h at 37 °C. Although 14 and 34 h at 37 °C produced a similar amount of VLP, they are less convenient time points to work with. Finally, immunofluorescence assay (IFA) was performed to

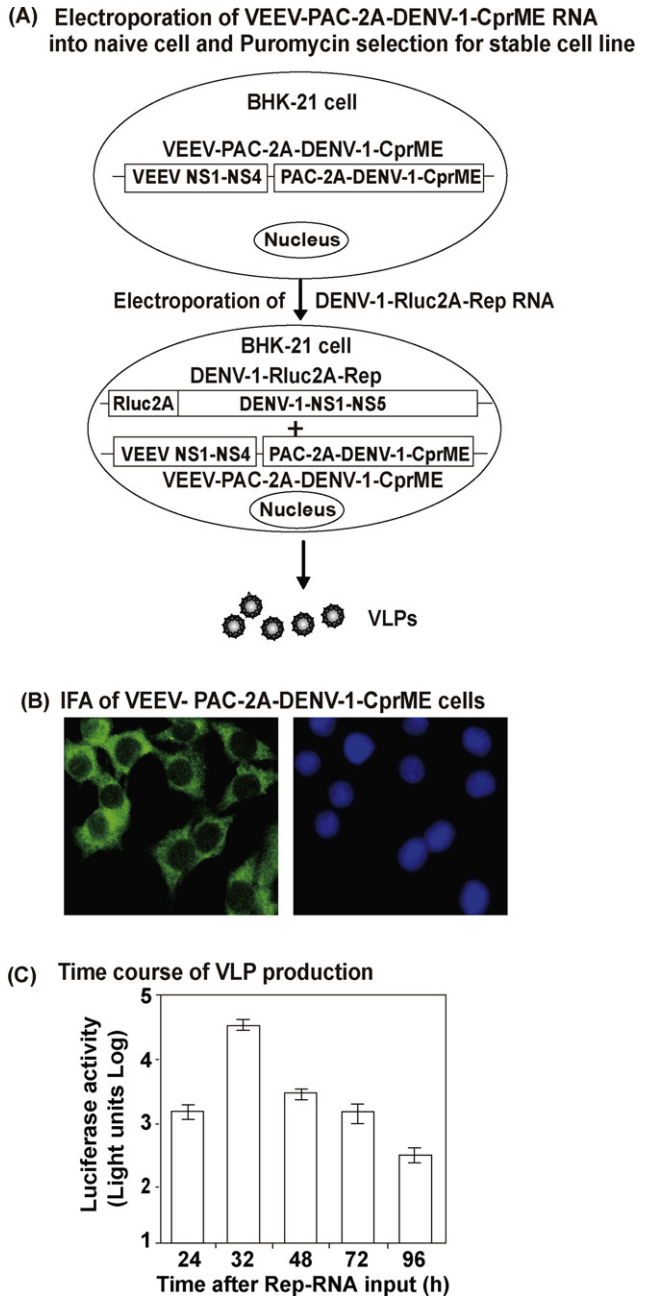

Fig. 2. Production of DENV-1 VLP by electroporating cell line expressing CprME proteins with replicon RNA. (A) VLP is generated by one round of electroporation of DENV-1 Replicon RNA into the puromycin-resistant BHK-21 cell line stably expressing DENV-1-CprME by VEEV-PAC-2A-DENV-1-CprME. (B) Immunofluorescence labeling of the E protein by 4G2 monoclonal antibody and FITC anti-mouse IgG in the puromycin-resistant VEEV-PAC-2A-DENV-1-CprME cell line (left panel). The right panel shows the DAPI staining of the nucleus. Photos were taken with a 40× objective. (C) Quantification of VLP produced at indicated time point after replicon RNA (Rep-RNA) electroporation by luciferase activity as in Fig. 1B. Error bars indicate the standard derivations from three independent experiments.

estimate the VLP titer by staining cells infected with 50 μl of serially diluted VLPs (see Section 2). The highest VLP titer, collected at 72 h post-transfection from the 37 → 30 °C scheme, was estimated to be 3×10^6 FFU/ml (Fig. 3D). This titer is much higher than the VLPs produced by the conventional method (2×10^3 FFU/ml).

Prior to the release of flavivirus particles from infected cells, the viral surface protein prM is cleaved to M by the host protease furin (Zybert et al., 2008). To increase the infectivity of VLP, replicon RNA (Rep-RNA) and Myc-tagged Furin DNA were co-electroporated into the BHK-21 cell line stably expressing structural protein, following the 37 → 30 °C scheme (Fig. 4A). The empty vector pcDNA3.1 was used as a control and the expression of furin was confirmed by Western blot (Fig. 4C). Replication kinetics of DENV-1 replicon in electroporated cells were monitored by Renilla luciferase activity at 2, 24, 48 and 72 h post-electroporation. The result indicates that the replication kinetics of DENV-1 replicon in furin expressing cells are not changed (Fig. 4B). However, VLPs harvested from the furin expressing cells at 48 and 72 h post-electroporation showed 1.6- and 2.4-fold increase of infectivity in Vero cells (Fig. 4D). This result demonstrated that exogenous expression of furin can improve DENV-1 VLP infectivity.

3.2. Optimization of VLP infection assay for compound screening

With the high titer VLP produced, we went on to develop a VLP infection assay for HTS. To identify an appropriate cell line for VLP infection, 50 μl of diluted VLPs (3×10^5 FFU/ml) were added to infect 10,000–60,000 Vero, LLC-MK2, BHK-21 or A549 cells grown in DMEM plus 10% FBS for 48 h in a 96-well plate. Infection of Vero and LLC-MK2 cells with DENV-1 VLP yielded the highest luciferase signal and BHK-21 and A549 were the lowest. The signal in Vero and LLC-MK2 cells were cell number-dependent; 40,000–60,000 cells per well generated the highest signal of luciferase at 48 h post-infection (Fig. 5A).

To explore the mechanism that controls the difference in VLP infection among different cell lines, we stained the infected Vero, BHK-21 and A549 cells using anti-NS1 antibody with concentrated VLP (50 μl of 1×10^6 FFU/ml). The result showed that more NS1-expressing cells were detected in Vero cells than in BHK-21 and A549 cells (Fig. 5B). Since VLP is only capable of one round of entry, this result clearly showed that the VLP entry was more efficient in Vero cells than in the two other cell lines.

We then performed a time course study of VLP infection of different cell lines. VLPs (50 μl at 3×10^5 FFU/ml concentration) were used to infect 40,000 cells of each cell line; the infected cells were assayed for luciferase activities at 24, 48, and 72 h post-infection. A steady increase of luciferase signal was observed over 72 h of infection in Vero cells (Fig. 5C, left panel) and decreased at 96 h post-infection (data not shown), indicating that replication of DENV-1 replicon lasted for at least 3 days in Vero cells. In contrast, the luciferase signals peaked at 48 h and decreased at 72 h post-infection in BHK-21 and LL-CMK2 cells; A549 cells showed the fastest decrease of signal after 24 h (Fig. 5C, left panel). These results suggest that the VLP replicates at different kinetics among different cell lines.

To examine whether serum concentration affects VLP infection, we tested VLP infection in the presence of 2%, 5%, and 10% of Fetal Bovine Serum (FBS). VLP infection in 2% FBS showed the highest signal for BHK-21, Vero, LLC-MK2 and A549 cells (Fig. 5C, right panel). In order to stay in the linear range of replication, we decided to take 48 h post-infection point as standard assay time point in Vero cells with 2% FBS.

Since the purpose of the assay is to screen compounds which are usually dissolved in DMSO, we tested the effect of DMSO on VLP infection in 96-well plate. Increasing amount of DMSO decreased luciferase signals (Fig. 6A). Upto 0.1% DMSO is well tolerated, but 0.1% DMSO showed 30–40% inhibition of the VLP infection. Given the solubility of compounds and DMSO concentration of compounds stock in library, we chose 0.5% DMSO as our final assay concentration.

In order to test the stability of VLP, we left VLP at 4, 22, or 37 °C for upto 6 h. Incubation at 4 °C or 22 °C did not significantly affect the VLP infectivity, but incubation at 37 °C significantly reduced the infectivity (Fig. 6B). We also found that the phenol red in the culture media interfered with luciferase detection. When luciferase

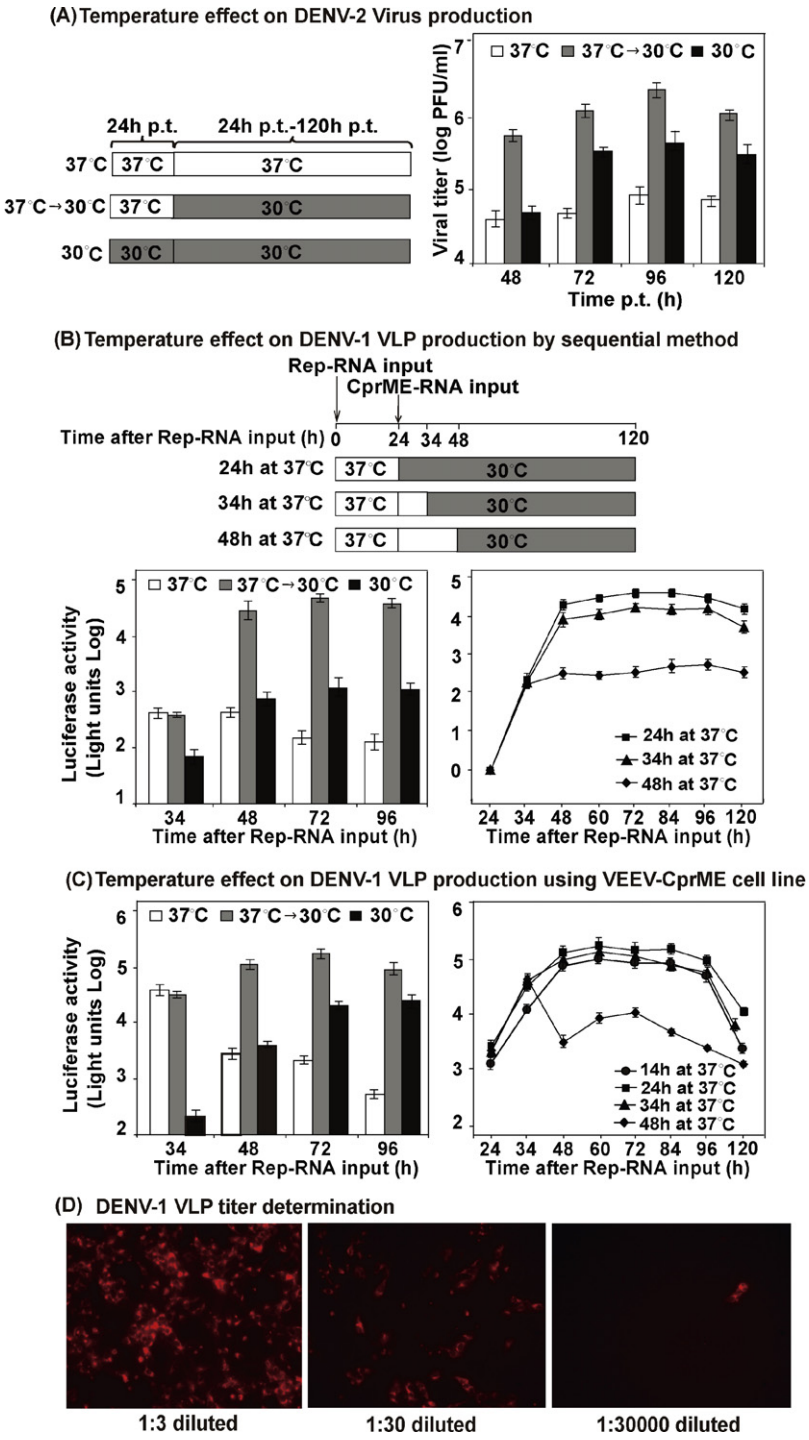

Fig. 3. Temperature-dependent high titer VLP production. (A) Left panel: The schematic representation of the temperature-dependent production of DENV-2 virus. Virus was produced by electroporation of DENV-2 NGC full length infectious clone RNA into BHK-21 cells for 24 h. Cells were then kept at the indicated temperatures for the remaining times. Right panel: Culture supernatants were collected at the indicated time points post-replicon RNA electroporation. Virus titer was measured by plaque assay. 37 °C: cells were incubated at 37 °C throughout the experiment. 30 °C: cells were incubated at 30 °C throughout the experiment. 37–30 °C: cells were cultured at 37 °C for the initial 24 h after electroporation, followed by 30 °C incubation for the rest of the time. Error bars indicate the standard derivations from three independent experiments. (B) Bottom left panel: Culture supernatants were collected at the indicated time points post-replicon RNA electroporation (Rep-RNA input) as in (A). VLP activity was quantified by measuring luciferase activity (light units) in VLP-infected Vero cells. Top panel: Temperature scheme for bottom right panel experiment. Cells were incubated at 37 °C for 24, 34 h or 48 h (24 h at 37 °C/34 h at 37 °C/48 h at 37 °C) after replicon RNA electroporation (Rep-RNA input) followed by 30 °C incubation for the remaining time. CprME RNA was electroporated always at 24 h post-replicon RNA electroporation. Bottom right panel: Culture supernatants were collected at the indicated time points post-replicon RNA electroporation (Rep-RNA input) as indicated in the top panel. VLP activity was quantified by measuring luciferase activity in VLP-infected Vero cells. Error bars indicate the standard derivations from three independent experiments for both the left and right panels. (C) Left panel: VLP production by electroporating replicon RNA into BHK-21 line expressing CprME using a different temperature as in (A). VLP activity was quantified by measuring luciferase activity (light units) in VLP-infected Vero cells. Right panel: VLP produced after different time of incubation at 37 °C (14, 24, 34 or 48 h after electroporation of replicon RNA) followed by 30 °C incubation as described in (B) top panel. VLPs were harvested at indicated time point and quantified by measuring luciferase activity (light units) in infected Vero cells. Error bars reflect the standard error of two independent production experiments for both panels. (D) VLP titer quantification by immunofluorescence staining of the foci. Three-fold to 30,000-fold dilution of VLP were used to infect 40,000 Vero cells per well in a 96-well plate. After 2 days of infection, cells were stained by NS1 antibodies and Alexa fluor 594 labeled goat anti-human IgG (H + L). Photos were taken with a 10× objective.

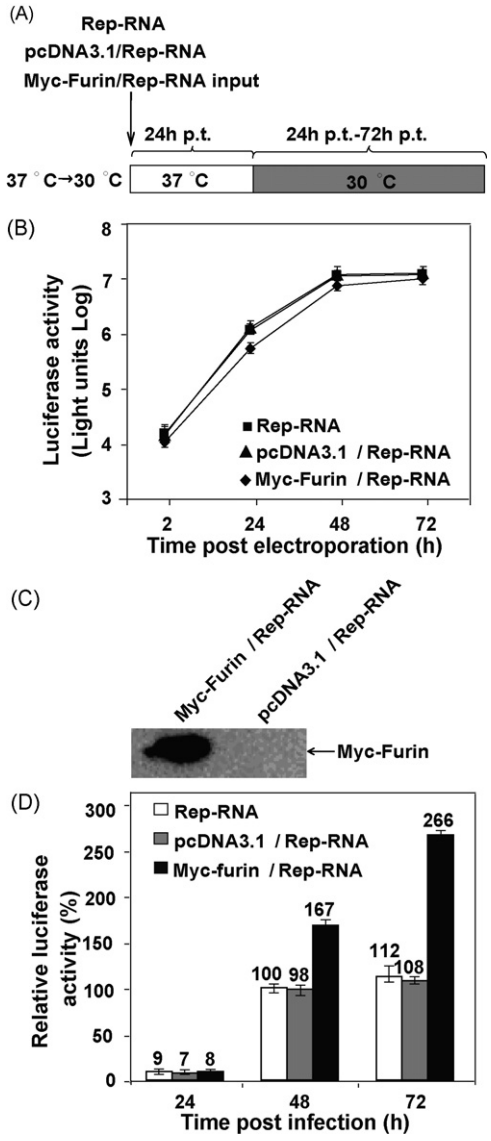

Fig. 4. The effect of overexpressed furin in VLP infectivity. (A) The schematic representation of the temperature-dependent production of DENV-1 VLP in the presence of furin. Empty vector (pcDNA3.1) or Myc-tagged furin (Myc-Furin) plasmid were electroporated into VEEV-CprME cell line together with the replicon RNA (Rep-RNA) into the VEEV-PAC2A-CprME cell line. (B) Transient replication of DENV-1 replicon RNA (Rep-RNA) in VEEV-PAC2A-CprME cells in the presence of overexpressed furin (Myc-Furin/Rep-RNA) or empty vector (pcDNA3.1/Rep-RNA). At 2, 24, 48, 72 h post-electoporation, luciferase activity (light units) was measured. Error bars indicate the standard derivations from three independent experiments. (C) The electroporated cells described in panel (B) were lysed at 72 h post-electroporation, and the expression of furin was detected by Western blotting using an anti-Myc antibody. (D) VLPs were collected at the indicated time points post-electroporation as in (B). VLP infectivity was quantified by measuring luciferase activity in infected Vero cells for 48 h. Vero cell luciferase activity using VLP harvested from cells with replicon alone was set as 100%. Error bars indicate the standard derivations from three independent experiments.

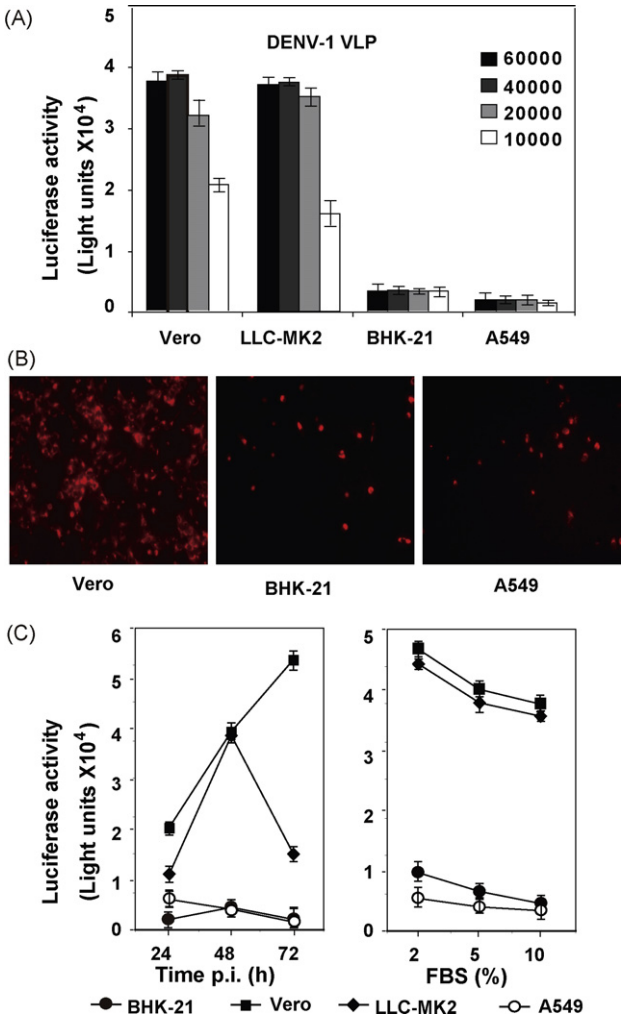

Fig. 5. VLP infection in different cell lines, time course of infection and FBS. (A) VLPs (3×10^5 FFU/ml) were used to infect indicated cell lines at indicated cell numbers. Infection was scored as a function of luciferase activity at 48 h post-infection. Error bars indicate the standard derivations from three independent experiments. (B) 50 μl VLPs (1×10^6 FFU/ml) were used to infect each cell line (4×10^4 per well) in 96-well plate for 48 h. Immunofluorescence staining of VLP-infected cells was done by NS1 antibodies and Alexa fluor 594 labeled goat anti-human IgG (H + L). Photos were taken with a 10× objective. (C) Time course of infection of VLP in different cell lines. VLP infection was done as in (A). Left panel: At each indicated time post-infection (p.i.), luciferase activity was measured. Right panel: different concentration of FBS (2%, 5% or 10%) in the medium was used for VLP infection in indicated cell lines as in (B). Luciferase activity was measured. Error bars represents standard errors of three independent experiments for both panels.

detection reagent Enduren was added in the media without phenol red, a 2-fold enhancement in signal was observed (Fig. 6C).

3.3. Validation of VLP assay by DENV inhibitors

We then went on to test whether known anti-dengue antibodies and inhibitors could block the VLP infection. The antibody 4G2, which recognizes the fusion loop at the extremity of domain II of E protein from all four serotypes of DENV and prevents syncytium formation (Rajamanonmani et al., 2009; Summers et al., 1989), was used to validate the feasibility of DENV-1 VLP for screening viral entry inhibitor. To examine whether VLP infection could be inhibited by 4G2 (Ansarah-Sobrinho et al., 2008; Klasse and Sattentau, 2001), VLPs (1:10, 1:20, and 1:40 dilutions) were incubated with a serial four-fold dilutions of the 4G2 neutralizing antibodies at room temperature for 1 h. The VLPs were then used to infect 40,000 Vero cells in a 96-well plate. The luciferase activities at 48 h post-infection were measured to assess the infection. Fig. 7A showed that 4G2 inhibited VLP infection in a dose-dependent manner, and that all three dilutions of VLP could be efficiently neutralized. Almost 100% inhibition of all dilutions of VLPs was observed at 1:128-fold 4G2 dilution. An average of 77% and 30% inhibitions were, respectively, observed at 1:513- and 1:2000-fold 4G2 dilutions for all VLP dilutions used. These results indicate that VLP behaves similarly to DENV-1 viral particles in Vero cells and could be used as a tool to screen entry inhibitors of DENV-1.

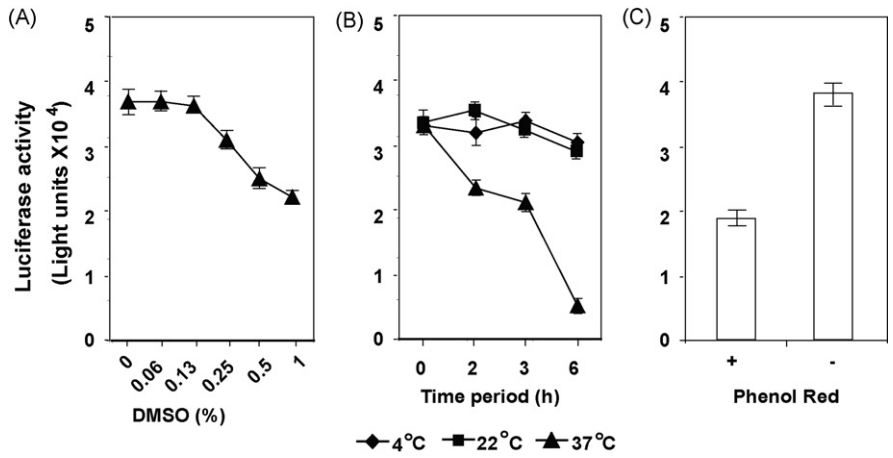

Fig. 6. The effect of DMSO, temperature and phenol red in VLP infection. 50 μl of undiluted VLP (3×10^5 FFU/ml) was used to infect 40,000 Vero cells per well in 96-well plate for 48 h. Luciferase activity was measured and expressed as light unit. (A) Different concentrations of DMSO were added in VLPs infection. (B) VLPs were kept at 4, 22 and 37 °C for 0, 2, 3 and 6 h before the infection. (C) At 48 h post-infection, medium in the wells were replaced with phenol red free or phenol red plus medium. Error bars reflect the standard error of two independent production experiments for all panels.

We then checked whether VLP infection could be inhibited by known replication inhibitors. Vero cells in 96-well plate (40,000 cells per well) were infected by VLP (1:10 dilution) in the presence of various concentrations of NITD008, an nucleoside inhibitor of DENV RdRp (Yin et al., 2009). The VLP infection was measured by luciferase activity at 48 h post-infection, while the cell viability was measured by CellTiter-Glo®. NITD008 showed a clear dose-dependent inhibition of VLP infection without affecting cell viability (Fig. 7B, left panel). Next, we tested lycorine, a natural product inhibitor of flavivirus replication which targets WNV 2K peptide (Zou et al., 2009). At 48 h post-infection, the luciferase signal was inversely correlated with the concentrations of lycorine without detectable cytotoxicity (Fig. 7B, middle panel). The EC_{50} values of NITD008 and lycorine were approximately 1.75 and 0.2 μM, respectively. The VLP-derived results correlated well with the EC_{50} values derived from the live DENV infection results, with EC_{50} of 1 and 0.1 μM for NITD008 and lycorine, respectively (data not shown). These results validated that luciferase reporter activity carried in VLP infection could be used as a true reporter of the viral replication. In contrast, castanospermine, an alpha-glucosidase inhibitor, did not inhibit VLP activity upto 10 μM. Castanospermine strongly affects N-linked oligosaccharide trimming of prM, E, and NS1, leading to protein misfolding and reduction in glycoprotein secretion. These defects contribute to an unproductive virion assembly and secretion (Courageot et al., 2000; Goto et al., 2005; Whitby et al., 2005; Wu et al., 2002). Since VLP lacks assembly and secretion steps, castanospermine did not have any effect on VLP infection (upto 10 μM) as expected. The activity of castanospermine was confirmed in inhibiting DENV-2 live virus infection (EC_{50} < 1 μM, data not shown). All together, we conclude that the inhibition of VLP infection by antibody 4G2 and replication inhibitors but not assembly or secretion inhibitor validated the use of VLP to screen entry and replication inhibitors for DENV.

3.4. Assay robustness for HTS

In order to miniaturize the assay for high-throughput screen, we transformed the assay into 384-well format. The time course of infection and DMSO sensitivity are not affected by the miniaturization (data not shown). We then assessed the assay robustness and test the EC_{50} of our reference compound NITD008 in 384-well plate under different cell numbers and VLP concentrations. VLPs (50 μl at 3×10^5 FFU/ml or at 1×10^4 FFU/ml concentration) were added to 10,000 or 20,000 cells per well along with different concentrations of the compound, and the luciferase activity was measured after 48 h post-infection. The EC_{50} of the reference compound NITD008 under all four conditions was calculated and listed in Table 1. They all closely correlated with the EC_{50} previously measured in a life virus infection assay. The signal to noise ratio (S/N) was calculated using the absolute positive signal (VLP-infected cells) divided by

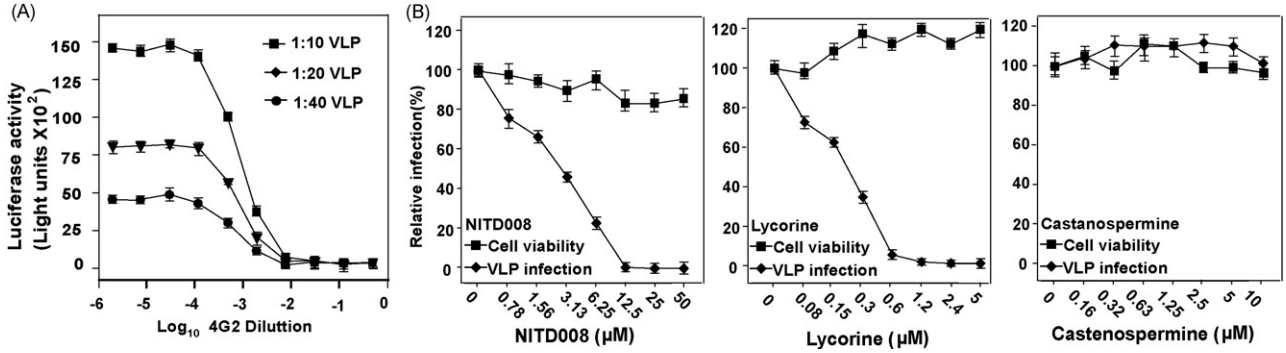

Fig. 7. Neutralizing antibodies and antiviral compounds in VLP infection. (A) Ten serial four-fold dilutions of 4G2 were incubated with VLPs (1:10, 1:20 and 1:40 dilutions) for 1 h at room temperature. VLP-antibody complexes were then added to Vero cells (4×10^4) in triplicate in a 96-well plate. Luciferase activity was measured at 48 h post-infection as described above. (B) Vero cells (4×10^4 cells per well) in a 96-well plate were infected with VLPs (50 μl at 1:10 dilution) in the presence of eight concentrations of NITD008 (left panel), lycorine (middle panel), and castanospermine (right panel). At 48 h post-infection, luciferase activity was measured and untreated VLP infection was set as 100% (100% is equal to approximately 35,000 light units depending on individual experiment). Cell viability was measured in parallel with CellTiter-Glo® (as described in Section 2). The error bars represent the standard deviations derived from three independent experiments for all panels.

Table 1
Comparison of EC_{50} values of NITD008 from different conditions of VLP infection assay in 384-well plate.

Cell number	VLP concentration (FFU/ml)	*Z*	S/N	NITD008 EC_{50} (μM)
20,000	3×10^5	0.75	1443	1.75
20,000	1×10^4	0.59	166	1.95
10,000	3×10^5	0.59	852	2.95
10,000	1×10^4	0.33	108	2.33

absolute negative signal (cell with no VLP). It ranged from a minimum of 108-folds to upto 1443-fold. The assay variability was measured by *Z* factor (Zhang et al., 1999). With different cell numbers and VLP concentrations, the *Z* factor ranged from 0.33 to 0.75. Using $Z \geq 0.5$ as a criteria for a robust HTS assay, we could either use high cell number (20,000) per well with low titer VLP or low cell number (10,000) per well with high titer VLP ($Z = 0.59$ for either condition), but not the low cell number (10,000) with low titer VLP ($Z = 0.33$). These data showed that VLP infection assay is a robust assay with strong *Z* factor and a large window between signal and noise, well suited for anti-dengue compound HTS.

4. Discussion

The VLP production and infection assay described in this report provides a new way of measuring antiviral compounds in a cell-based assay for DENV. Promising targets for flavivirus drug development include key processes and activities of the virus life cycle such as entry, RNA capping, protease cleavage, and replication. Using low temperature packaging, we could produce high titer DENV-1 VLP. We found that the VLP produced could infect Vero cells and the infection mimics closely the natural infection process at the entry and replication steps. We optimized the assay conditions in 384-well plate and validated the assay with a neutralizing antibody, an NS5 nucleoside inhibitor NITD008, and a replication inhibitor lycorine. All the data showed that the VLP assay is robust and reliable for testing anti-DENV compounds. The generation of VLP is not only applicable to DENV-1 VLP, using the same methods and parameters, but DENV-2 VLP could also be generated using TSV01 strain (data not shown).

In agreement with a previous study (Ansarah-Sobrinho et al., 2008), we also found that DENV-1 VLPs was strongly temperature-dependent. The production of VLPs was inefficient at either 37 or 30 °C by either sequential electroporation of replicon RNA and structural gene RNA into BHK-21 cells or by electroporation of replicon RNA into BHK-21 cell line expressing structural proteins. Inefficient production of VLPs at 37 °C was probably due to a rapid decay of infectious VLPs at this temperature and a significant reduction in the rate of infectious VLPs released over time (Ansarah-Sobrinho et al., 2008). On the other hand, preparation of VLP at 30 °C throughout the procedure yielded low titer, most likely due to the slow kinetics of gene expression and replicon replication in cells. Interestingly, by incubating the cells at 37 °C for the first 24 h and then transferring to 30 °C, we could solve the problems of rapid decay at 37 °C and slow kinetics of initial gene expression at 30 °C (Fig. 3B and C). High titer WNV VLP could be produced at 37 °C (Ansarah-Sobrinho et al., 2008), while high titer DENV-1 VLP has to be produced using a combination of 37 °C replication and 30 °C packaging. We believe that the reason lies with the dengue capsid protein. We have observed that the titer of the chimeric VLP produced from WNV replicon RNA packaged by WNV Capsid (and DENV1-prME) was as high as WNV VLP at 37 °C (data not shown). But the titer of chimeric VLPs produced by WNV replicon packaged by DENV-1 Capsid (and DENV1-prME) was as low as DENV-1 VLP at 37 °C. These results suggest that DENV-1 VLP production is limited by an inefficient packaging of the capsid protein at 37 °C, and that the packaging efficiency could be improved by lowering the incubation temperature to 30 °C.

Several studies have shown that both mammalian cells (BHK21 or Vero) and insect cells (C6/36) infected with DENV type 2 release high number of particles containing unprocessed prM, which are immature virions with poor ability to infect cells (Cherrier et al., 2009; Yu et al., 2009; Zybert et al., 2008) In an attempt to solve this problem, we investigated the DENV-1 VLP infectivity from VEEV-PAC2A-CprME BHK-21 cell line with exogenous expression of furin. We found that VLP produced in the presence of furin showed higher infectivity, demonstrating that the prM to M cleavage is a limiting factor in the generation of high titer infectious particles.

Among different cell lines, Vero gave the highest infection signal compared with BHK-21, A549 and LLC-MK2 cells. The difference in infection is unlikely due to translation and replication since electroporated replicon RNA replicated well in Vero, BHK-21, and A549 cells (Fig. 5B). The difference was most likely due to entry as shown by the immunofluorescence staining of the VLP-infected cells. The VLP infection of Vero cells can last for upto 3 days and decays after 72 h, similar to the kinetic of DENV-1 replicon. The infection was the best in the presence of 2% FBS instead of 10% FBS. It is interesting to note that phenol red free media produce higher light emission using Enduren live cell luciferase detection reagent. This helps to improve the signal of VLP infection.

We have tested DENV-1 VLPs with neutralizing antibody 4G2. VLPs were extensively used in investigating the action of neutralizing antibodies (Ansarah-Sobrinho et al., 2008; Klasse and Sattentau, 2001; Mehlhop et al., 2007). Here, we wanted to use the well-characterized neutralizing antibody 4G2 to validate the entry process of VLP. In our study, we performed neutralization assay with three different concentrations of DENV-1 VLP with the antibody. Significant differences in neutralization titer were not observed with increasing concentration of VLP and close to 100% neutralization could be obtained with 1:4 to 1:128 dilution of 4G2. These results showed that the entry of VLP is the same as the virus, which could be neutralized by 4G2. We also tested different antiviral inhibitors using VLP. The VLP infection measured by luciferase activity could be completely inhibited by the in-house developed NS5 nucleoside inhibitor NITD008 and the replication inhibitor lycorine. The EC_{50} values of both compounds were similar to those obtained with live virus infection. VLP infection could not be inhibited by alpha-glucosidase inhibitor Castanospermine (upto 10 μM), confirming the lack of packaging and secretion step in VLP infection. These results further validated the use of VLP for screening of replication inhibitors.

When the assay was miniaturized in a 384-well format, the assay quality remained high. The *Z* factor is 0.6 and the signal to noise ratio is about 800-folds with 10,000 cells and 3×10^5 FFU/ml VLP. The assay is suitable for HTS for a large amount of compound, increasing the chance of identifying anti-dengue hits. In summary, we believe that the development of high titer dengue VLP production and HTS assay using VLP will greatly facilitate the compound screening and anti-dengue drug discovery.

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