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Dengue virus replicons: Production of an interserotypic chimera and cell lines from different species, and establishment of a cell-based fluorescent assay to screen inhibitors, validated by the evaluation of ribavirin's activity

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ABSTRACT

The prevention and treatment of flavivirus infections are public health priorities. Dengue fever is the most prevalent mosquito-borne viral disease of humans, affecting more than 50 million people annually. Despite the urgent need to control dengue infections, neither specific antiviral therapies nor licensed vaccines exist and the molecular basis of dengue pathogenesis is not well understood. In this study we produced a novel dengue virus type 2 (DV2) subgenomic replicon that expresses a fusion protein comprised of Enhanced Green Fluorescent Protein (EGFP) and Puromycin N-Acetyltransferase (PAC). We successfully established BHK, COS and Huh7 cell lines that stably expressed the DV2 replicon. Using EGFP as a reporter of DV replication complex activity, we set up a new HTS assay. The assay was validated using the inhibitor ribavirin, confirmed by flow cytometry analysis and the analysis of NS5 expression by Western-blot analysis. In order to develop a system to test antivirals against the NS5 proteins of all four DV serotypes in a similar cellular environment, the replicon was further modified, to allow easy exchange of the NS5 gene between DV serotypes. As proof of principle, a chimeric replicon in which the DV2 NS5 gene was substituted with that of DV type 3 was stably expressed in BHK cells and used in ribavirin inhibition studies. The assays described in this study will greatly facilitate DV drug discovery by serving as primary or complementary screening. The approach should be applicable to the development of fluorescent cell-based HTS assays for other flaviviruses, and useful for the study of many aspects of DV, including viral replication and pathogenesis.

1. Introduction

Dengue fever is the most prevalent mosquito-borne viral disease of humans, affecting more than 50 million people annually. Dengue virus (DV) is a member of the Flaviviridae family together with other important pathogens such as yellow fever, West Nile, and Japanese encephalitis viruses. The four genetically related but serologically distinct serotypes of DV (types 1–4) cause dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Despite the urgent need to control dengue infections, neither specific antiviral therapies nor licensed vaccines exist, and the molecular basis of dengue pathogenesis is not well understood. DV has a capped positive-sense single stranded RNA genome containing a single open reading frame (ORF), flanked by 5′ and 3′ untranslated regions (UTR). The ORF encodes a polyprotein precursor, which is subsequently cleaved by cellular and viral proteases into three structural proteins (capsid (C), premembrane (prM) and envelope (E)) as well as seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The non-structural proteins assemble with cellular proteins to form a replication complex, where the viral RNA is synthesized (Lindenbach et al., 2007). Two of the 10 viral proteins have key enzymatic activities and are thus potential targets of inhibitors. NS3 acts as a serine protease (with NS2B as a cofactor), 5′RNA triphosphatase, nucleotide phosphatase, and helicase (Lescar et al., 2008). NS5 is a bifunctional protein: its amino-terminal domain carries a methyltransferase (MTase) involved in methylation of the 5′ cap structure of genomic RNA (Dong et al., 2008), whereas its carboxy-terminal domain is the RNA-dependent RNA polymerase (RdRp) responsible for the synthesis of viral RNA (Malet et al., 2008).

Abbreviations: DV, dengue virus; DV2, DV serotype 2; DV3, DV serotype 3; EGFP, Enhanced Green Fluorescent Protein; HTS, high throughput screening; IRES, internal ribosome entry site; MTase, methyltransferase; NS5, non-structural protein 5; PAC, Puromycin N-Acetyltransferase; RdRp, RNA-dependent RNA polymerase.

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To control the spread of DV, new tools are required, both to improve our understanding of the replication complex and provide a cost-effective means of screening antiviral compounds. Subgenomic replicon constructs contain all of the genetic elements needed for genome amplification in permissive cells but lack the major part of the genes encoding the structural proteins. The RNAs consequently replicate but are not packaged into viral particles. Subgenomic replicon can also encode drug selectable markers and/or reporter proteins, that may substitute for the structural protein coding regions. As they are non-infectious, subgenomic replicons are important tools for the study of viral replication, the screening of antiviral compounds and the testing of their inhibitory potential (Khromykh et al., 2001; Lo et al., 2003a,b; Tilgner and Shi, 2004; Alvarez et al., 2005, 2008; Liu et al., 2005; Rossi et al., 2005, 2007; Tilgner et al., 2005; Filomatori et al., 2006; Gu et al., 2006; Ng et al., 2007; Noueiry et al., 2007). They avoid problems associated with the use of infectious virus and are particularly important for the study of BL-3 pathogens, like DV.

In this study, we established stable cell lines expressing a novel DV type 2 (DV2) replicon. In place of the DV structural genes, the replicon contained a gene construct encoding a fusion protein (EGFF–PAC) comprised of the Enhanced Green Fluorescent Protein (EGFP) and Puromycin N-Acetyltransferase (PAC). Cells containing the replicon could be selected by puromycin treatment, whilst EGFP could be used as a reporter to follow the replication level of the subgenomic replicon. First, BHK cells expressing the DV2 replicon were established. The replicon systems were then extended for the first time to the COS monkey cell line and a human hepatic cell line (Huh7). Extension of the replicon system to COS cells is important as these cells are not easily naturally infected (unpublished observations (Rodrigo et al., 2006)). The DV2 replicon Huh7 stable cell line constitutes a suitable tool for studying the interactions between viral proteins and hepatic proteins, some of which may be responsible for the infection-induced liver damage (Ekkapongpisit et al., 2007; Pattanakitsakul et al., 2007; Higa et al., 2008).

Second, we set up a screening assay of inhibitors, based on the use of EGFP as a readout. To validate the assay, we used ribavirin, a broad spectrum antiviral molecule, which inhibits DV replication (Koff et al., 1982; Crance et al., 2003; Takhampunya et al., 2006). The inhibitory effect of ribavirin observed in the DV2 replicon cell lines, was similar to that described using infected cells, establishing a new fluorescent HTS assay in a BL-2 environment. Moreover, the establishment of multiple cell lines carrying the DV replicon enables evaluation of the inhibitory potential of selected compounds in cell lines from different organs and species.

Finally, with the aim to test antivirals against the NS5 proteins of the four DV serotypes and potential NS5 mutants in a similar cellular environment, we designed an original method. We modified the DV2 replicon to allow easy exchange of a NS5 gene cassette. We validated the construct by introducing the DV type 3 (DV3) NS5 gene into the DV2 replicon. The chimeric replicon construct was shown to be viable as it was possible to establish a stable cell line expressing the DV2/3-NS5 replicon. In addition to demonstrating that a replication complex containing the NS1 to NS4B proteins from DV2 and the NS5 protein from DV3 is functional, the chimeric replicon can be used as a tool to test the effectiveness of antivirals specifically against the MTase and the RdRp of the different DV serotypes in a cellular context.

2. Materials and methods

2.1. Cells, media and reagents

Cells were grown in Dulbecco's modified eagle medium (DMEM) (PAA) supplemented with penicillin, streptomycin and 5% (BHK cells) or 10% (COS and Huh7 cells) foetal calf serum (FCS). For BHK-, COS- and Huh7-DV EGFP-replicon containing cells, puromycin (Sigma) was added at 3.5, 2 and 1 μg/ml, respectively. Replicon replication inhibition tests were done using a medium without phenol red (Invitrogen), supplemented with penicillin, streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 5% FCS, without puromycin. Ribavirin (Sigma) was resuspended in 100% DMSO at 20 mM and stored at −20 °C.

2.2. Replicon plasmid construction

2.2.1. DV2 (new Guinea C strain) EGFP-PAC replicon plasmid

A DV2 replicon expressing an EGFP-PAC fusion protein was constructed using the replicon pDENΔCprME-PAC2A (Jones et al., 2005) that was previously derived from the genome length DV2 (new Guinea C strain) cDNA clone pDVWS601 (Gualano et al., 1998; Pryor et al., 2001). Initially, the EGFP gene was amplified from pEGFP (Clontech) by PCR, using primers P1 and P2 (all primers are listed in Table 1) and cloned into pCR-Blunt-II-TOPO (Invitrogen) resulting in the plasmid pCR-EGFP. The PAC-1D2A gene cassette was then amplified from pDENΔCprME-PAC2A by PCR as two overlapping fragments (in order to replace an internal XbaI site with a BglII site) using primers P3, P4, P5 and P6 respectively. The PAC and 1D2A gene fragments were then joined by overlap PCR using primers P3 and P6 and the resulting fragment cloned into pCR-Blunt-II-TOPO to produce the plasmid pCR-PAC-1D2A. The PAC-1D2A gene fragment was excised from pCR-PAC-1D2A with NdeI and BamHI and cloned into the corresponding sites of pCR-EGFP resulting in the plasmid pCR-EGFP-PAC-1D2A. The EGFP and PAC genes are separated by a 21 nt linker containing the restriction enzyme sites BglII, SphI and NdeI. The EGFP-

Table 1
List and sequences of the primers used in this study.

Primer	Complete name	Sequence 5′-3′
P1	P1_Sbf_5-GFP	TCGACTGTACAAACTCCTGCAGGTACGCGTACCATGGTGAGCAAGGGCGAGGAGCTG
P2	P2 GFP-3 2r	GTCCATATGGCATGCAGATCTTCGGTACAGCTCG
P3	P3_PAC/G-3	GCATGCCATATGGACATGACCGAGTACAAGCCCACGGTGCGCCTC
P4	P4 PAC/B-2r	CAGATCTGGCACCGGGCTTGCGGGTCATGCACCAG
P5	P5 1D2A/PAC	CCGCAAGCCCGGTGCCAGATCTGTCACCGAGTTGCTTTACCGG
P6	P6_1D2A/Sna/Pme	GTTTAAACGCTACGTACGGGCCCAGGGTTGGACTCGACGTC
P7	P7 F attBl DV2 largeNS5	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAAGTATAGCGGCTAGAGG
P8	P8_R_attB2_DV2_largeNS5	GGGGACCACTTTGTACAAGAAAGCTGGGTGGTTGAAGGCTCTCAAGGGCATCG
P9	P9 F pDON-DV2-largeNS5+AgeI	CGAGAAGGGGAACCGGTAACATAGGAGAGACG
P10	P10_R_pDON-DV2-largeNS5+AgeI	CGTCTCTCCTATGTTACCGGTTCCCCTTCTCG
P11	P11_ F_pDON-DV2-largeNS5+ScaI	GGAAGAGGCAGGAGTaCTGTGGTAGAAGGC
P12	P12_R_pDON-DV2-largeNS5+ScaI	GCCTTCTACCACAGtACTCCTGCCTCTTCC
P13	P13 F attBl DV3 NS5	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGAACCGGTTCACAAGGTGAAACCTTAGGAGA
P14	P14_R_attB2_DV3_NS5	GGGGACCACTTTGTACAAGAAAGCTGGGTCCACAGTACTCCCTCTGACTCCTCCTCCTTCCTGA
P15	P15 F_pDON-DV2/3-largeNS5 VLtoAI	CAGAGGGAGCAATTTGGTAAAAGGCAAAACTAACATG
P16	P16 R_pDON-DV2/3-largeNS5 VLtoAI	CATGTTAGTTTTGCCTTTTACCAAATTGCTCCCTCTG

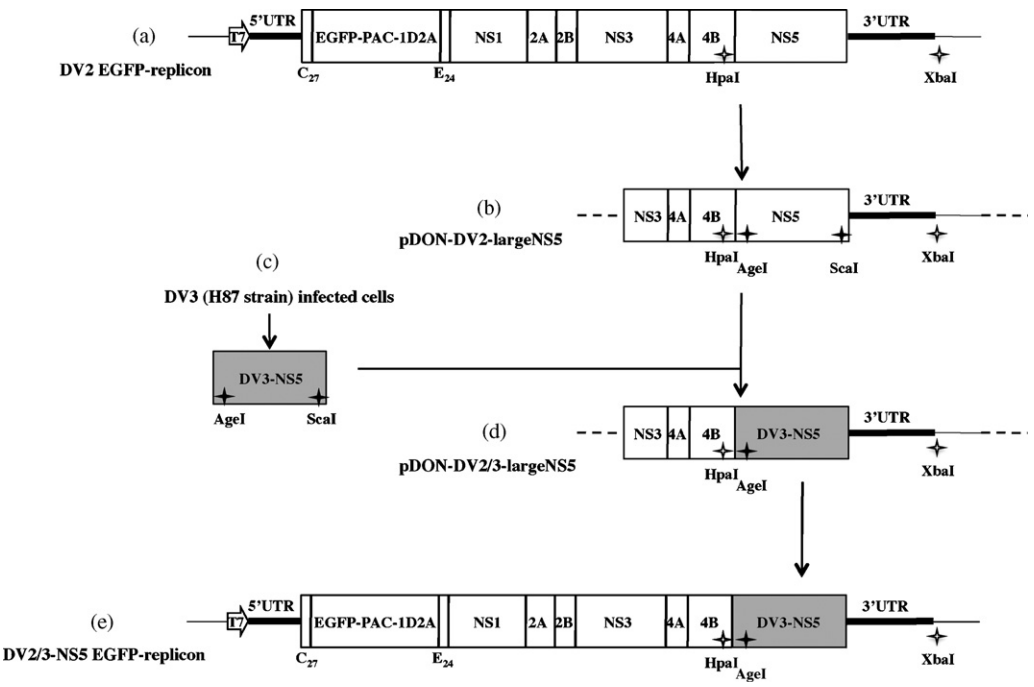

Fig. 1. Schematic representation of the subgenomic DV2 and DV2/3-NS5 EGFP-replicon plasmids. (a) From the full length DV2 NGC infectious cDNA clone pDVWS601, a large part of the C-prM-E coding sequence was deleted, leaving sequence encoding the N-terminal 27 amino acids of the C protein (C_{27}) and the C-terminal 24 amino acids of the E protein (E_{24}). The deleted sequence was replaced by an EGFP-PAC-1D2A gene fusion. This replicon is under the control of a T7 promoter. (b) The pDON-DV2-largeNS5 plasmid was obtained by recombination of the pDON201 vector with a PCR product obtained from the DV2 EGFP replicon plasmid. (c) The DV3 (H87 strain) NS5 sequence was obtained by RT-PCR. (d) The pDON-DV2/3-largeNS5 plasmid was obtained by exchange of the NS5-containing AgeI/ScaI fragments between the DV3 PCR product and the pDON-DV2-largeNS5. (e) The DV2/3-NS5 EGFP-replicon was obtained by exchange of the NS5-containing HpaI/XbaI fragments between the pDON-DV2/3-largeNS5 and the DV2 EGFP replicon. For all plasmids, white and black stars indicate restriction enzyme sites initially present or added by PCR, respectively.

PAC-1D2A gene fusion was excised from pCR-EGFP-PAC-1D2A by digestion with BsrGI and SnaBI and cloned into the corresponding sites of pDENΔCprME-PAC2A resulting in the production of the replicon plasmid pDENΔCprME-EGFP-PAC-1D2A (hereafter termed DV2 EGFP-replicon). The replicon plasmid contains a T7 promoter upstream of the DV sequence (such that transcription adds a single G nt at the 5′ end of the DV 5′UTR) and retains DV2 sequences coding for the first 27 amino acids of the C protein (including the first methionine) and the last 24 amino acids of the E protein. The remainder of the sequence coding for the viral structural proteins was replaced by the EGFP-PAC-1D2A gene fusion (Fig. 1a).

2.3. Construction of a DV2/3-NS5 EGFP-replicon plasmid

Using the DV2 EGFP-replicon plasmid as a template, a sequence including the NS5 gene (extending from the middle of the NS3 gene to the plasmid sequence downstream of the viral 3′UTR) and containing two pre-existing unique enzyme restriction sites near the 5′ and 3′ termini of NS5 (HpaI and XbaI, respectively) was amplified by PCR with primers P7 and P8. They contained attB1 and attB2 sequences, allowing recombination with the pDON201 vector using the Gateway® (Invitrogen) cloning technology. Unique enzyme restriction sites were then added by site-directed mutagenesis at the exact 5′ (AgeI site, with primers P9 and P10) and exact 3′ (ScaI site, with primers P11 and P12) termini of the NS5 gene resulting in the production of the plasmid pDON-DV2-largeNS5 plasmid (Fig. 1b).

The NS5 gene of DV3 (H87 strain) was amplified by RT-PCR using RNA from DV3-infected cells, with primers P13 and P14 introducing the AgeI and ScaI sites at the exact 5′ and exact 3′ termini (Fig. 1c). Primers P13 and P4 also contained the attB1 and attB2 sequences, allowing recombination with a pDON201 vector. By restriction (with AgeI and ScaI) and ligation (with T4 ligase), the DV2 NS5 sequence was removed from the pDON-DV2-largeNS5 plasmid and replaced with the DV3 NS5 sequence, producing the plasmid pDON-DV2/3-largeNS5 (Fig. 1d). The amino acid substitutions created by the introduction of the ScaI site into the DV3 NS5 sequence was restored (Val.Leu to Ala.Ile) by site-directed mutagenesis (primers P15 and P16).

The HpaI-XbaI fragment from either the pDON-DV2-largeNS5 plasmid (control) or the pDON-DV2/3-largeNS5 plasmid were then used to replace the equivalent fragment in the DV2 EGFP-replicon plasmid. The new EGFP-replicon plasmids were named DV2-NS5 and DV2/3-NS5, respectively (Fig. 1e). All plasmids were sequenced. pDON201-derived plasmids and replicon plasmids were amplified in *E. coli* DH5α at 37 °C and Stbl2 at 30 °C, respectively.

2.4. In vitro RNA transcription and transfection

DV2, DV2-NS5 and DV2/3-NS5 EGFP-replicon plasmids were linearized by XbaI, extracted with phenol/chloroform and precipitated with ethanol. The linearized plasmids were used as templates (2.5 μg in a total reaction volume of 50 μl) for the production of replicon RNAs in the presence of 6 m ^{m7}GpppA cap analog (NEBiolabs), with the T7-dependent MEGAscript® kit from Ambion. Replicon RNA was purified with the RNeasy® kit (Qiagen). Replicon RNA (2 μg) was transfected with the TransMessenger® kit (Qiagen) into BHK and COS cells previously plated in a 6-well plate. Huh7 cells (8×10^6 cells resuspended in 800 μl of cytomix buffer (Liang et al., 2005) were electroporated with 3 μg of replicon RNA (1 pulse, 950 μF, 270 V). After 48 h, transfected and electroporated cells were selected for 7 days with puromycin, at the concentrations described above. Cells expressing high levels of EGFP were sorted with a Becton-Dickinson FACSVantage™ and then propagated in the presence of puromycin.

2.5. Inhibition of the replication of the replicon by ribavirin

Two methods were developed to assess the effects of compounds on the replication of the DV replicon based on either 24 or 96 well formats. Control cells, DV2 and DV2/3-NS5 EGFP-replicon cells were seeded either in 24-well plates or in black 96-well plates at densities of 5×10^4 or 1×10^4 cells per well, respectively, in complete uncolored medium supplemented with 0.5% DMSO (v/v). After 24 h, the medium was removed and cells were incubated with 400 μl (24-well plate) or 100 μl (96-well plate) of the same medium containing a range of ribavirin concentrations (1 μM to 100 μM). In each 96-well plate, incubations were done in triplicate and 6 control wells were also included. The media were renewed after 24 h, as cells were incubated with ribavirin for a total of 48 h.

For the 96-well plate assay, the inhibitory effect of ribavirin was defined according to its cytotoxic effect as follows. In addition to the ribavirin treated and control cells, a range of cell numbers, from 1×10^4 to 8×10^4 were freshly seeded, in triplicate, into each 96 well plate and incubated for 5 to 6 h. The media was then removed from all wells and replaced by 100 μl of PBS. The EGFP fluorescence from each well was read with a Tecan SafireII® fluorimeter at 490 nm (excitation) and 510 nm (emission). Then 20 μl of Celltiterblue® reagent (Promega) was added per well, the plates incubated for a further 75 min at 37 °C and 5% CO_2 and the fluorescence read at 560 nm (excitation) and 590 nm (emission). A 590 nm fluorescence curve produced using the range of cell numbers was then used to define an equation to calculate the number of cells present in each well. The 510 nm fluorescence values were divided by the calculated cell number and mean values for each point of concentration were reported as a percentage of the mean control value.

For the 24 well based assay, the EGFP-fluorescence intensity of the cells in each well was analyzed by flow cytometry as described below. Values for each ribavirin concentration were reported as a percentage of the control value.

2.6. Protein expression analysis

2.6.1. Flow cytometry

Cells were washed with PBS and trypsinized. Pelleted cells were washed twice with PBS containing 2% FCS and the EGFP-fluorescence intensity was analyzed by flow cytometry with a Becton-Dickinson FACScan™.

2.7. Western-blot

Total proteins were extracted with cell lysis buffer (50 mM Tris Cl pH 7.4, 150 mM NaCl, 0.5% Na deoxycholate, 1% NP40, 100 μg/ml PMSF, protease inhibitor cocktail from Sigma®) and loaded on a SDS-PAGE (10%) gel. Purified recombinant DV2 NS5 protein (Selisko et al., 2006) was also loaded as a control. Proteins were transferred to a PVDF membrane (Millipore), which was then blocked with PBS/0.1% Tween20/5% milk, and then incubated with primary and secondary antibodies. The following antibodies were used; a mouse monoclonal anti-GAPDH coupled to peroxidase (clone GAPDH-71.1, from Sigma®, diluted at 1/25,000), a mouse monoclonal anti-EGFP (clone JL-8, from Clontech, diluted at 1/4000), a rat monoclonal anti-NS3 helicase domain (culture supernatant of the clone 31F9.11, diluted at 1/10, produced in house), a rat monoclonal anti-NS5 RdRp domain (clone 19A8.2, diluted at 1/500, produced in-house), a goat anti-rat IgG (Jackson Immunoresearch, diluted at 1/10,000) and a goat anti-mouse IgG (from Sigma, diluted at 1/4000), both coupled with horse-radish peroxidase. Western-blots were detected by chemiluminescence with ECL (Amersham™) and Biomax Light Films (Kodak).

3. Results

3.1. Construction of the DV2- and chimeric DV2/3-NS5 EGFP-replicon plasmids

As an initial step towards the development of a cell-based fluorescent screening assay for DV inhibitors, a DV2 (New Guinea C strain) replicon expressing the drug selectable marker puromycin N-acetyl-transferase (PAC) gene (Jones et al., 2005) was modified to express an EGFP-PAC gene fusion (Fig. 1a). Previous DV replicons that express both a reporter gene and a drug selectable marker gene have used internal ribosome entry sites (IRES) to initiate translation of the DV non-structural proteins (Puig-Basagoiti et al., 2006; Ng et al., 2007). However, translation from IRES sequences can be much less efficient than cap-dependent translation (Mizuguchi et al., 2000; Ibrahimi et al., 2009) and influenced by positional (Hennecke et al., 2001) and cell type specific effects (Hellen and Sarnow, 2001). Therefore, we expressed the Enhanced Green Fluorescent reporter Protein (EGFP) as a fusion with the PAC gene product. The 1D2A peptide derived from foot-and-mouth disease virus was engineered after the PAC gene. The 1D2A peptide induces a translation 'skip' (Doronina et al., 2008) which served to separate the EGFP-PAC-1D2A fusion protein from the remainder of the DV polyprotein. The EGFP-PAC-1D2A gene fusion replaced the major part of the DV2 structural genes. The sequence coding for the first 27 amino acids of the C protein was retained, as it contains the 5′ cyclization sequence (5′CS) important for replication (You and Padmanabhan, 1999; Khromykh et al., 2001; Alvarez et al., 2005). In addition, the sequence coding for the last 24 amino acids of the E protein were retained because the C-terminal uncharged hydrophobic sequence of the E protein acts as a signal sequence for translocation of NS1 across the endoplasmic reticulum (Rice et al., 1985; Falgout et al., 1989).

With the aim to test inhibitors against the NS5 protein of the different DV serotypes in a cellular environment, we established a DV2 replicon carrying an NS5 gene cassette. To produce chimeric DV EGFP replicon plasmids, we optimised a method designed to avoid the generation of recombination events, frequently observed when working with long viral sequences. We produced a plasmid (pDON-DV2-largeNS5) that can be used to reliably exchange the NS5 sequence in the DV2 EGFP-replicon plasmid in two cloning steps. The DV3 (H87 strain) NS5 sequence was first amplified by RT-PCR from total RNA extracted from infected cells. The NS5 gene cassette in the DV2 replicon was replaced with the DV3 NS5 sequence, producing the DV2/3-NS5-EGFP-replicon plasmid. Details of these constructions are described in Section 2 and in Fig. 1.

3.2. Production of hamster, simian and human cell lines supporting the DV2 EGFP-replicon

The DV2 EGFP-replicon plasmid was used as a template to produce a ^{m7}GpppA-capped replicon RNA that was introduced into hamster BHK, simian COS, and human Huh7 cells. The replicon RNA would be translated as a unique polyprotein by the cellular machinery. The fusion protein EGFP-PAC-1D2A would be separated from the remainder of the polyprotein by the ribosome skip induced by the 1D2A peptide. The DV non-structural proteins would be cleaved either by cellular proteases or by the NS2B/NS3 protease complex (Lindenbach et al., 2007) and assemble into a replication complex. The puromycin concentration used for selection of replicon containing cells was set according to the differential sensitivity level of each cell line: BHK cells were less sensitive than COS cells, which were less sensitive than Huh7 cells. BHK cells transfected with the replicon RNA were resistant to puromycin at 3.5 μg/ml, whereas non-transfected BHK cells were highly sensitive and died in 2 days at 1 μg/ml of puromycin. COS- and Huh7- DV2 cells trans-

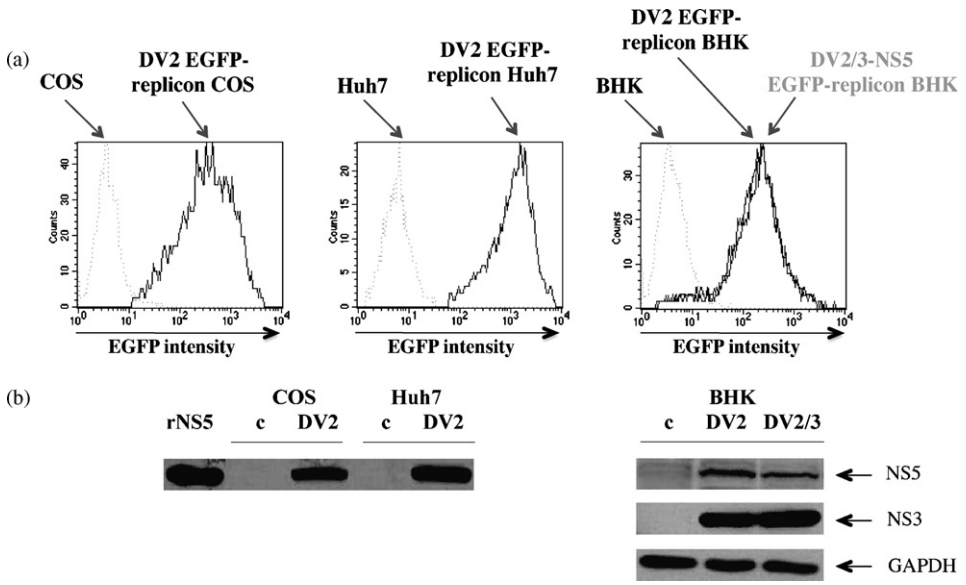

Fig. 2. Analysis of the expression of EGFP, NS3, NS5 and GAPDH proteins in replicon containing cells. (a) Analysis by flow cytometry of the fluorescence level of nontransfected (dotted line) or stable established EGFP-replicon containing (solid line) cells. (b) Analysis by Western-blot of the NS5 (higher panel), NS3 (middle panel) and GAPDH (lower panel) expression in non-transfected cells (lanes 'c') and in EGFP-replicon containing cells. Recombinant NS5 (rNS5) was loaded as a control. DV2: DV2 EGFP-replicon; DV2/3: DV2/3-NS5 EGFP-replicon.

fected with the DV EGFP-replicon were selected using 2 μg/ml and 1 μg/ml of puromycin, respectively.

Transfected puromycin-resistant cells, but not non-transfected cells, also expressed EGFP, as verified by fluorescent microscopy and flow cytometry (data not shown). Cells expressing high levels of EGFP were sorted and amplified. Replicon RNA was extracted and sequenced, and no mutations observed. EGFP expression was maintained at the same level for at least a period of 2 months in the presence of the respective puromycin concentration. BHK-, COS- and Huh7-DV2 EGFP-replicon containing cells were shown to express EGFP (Fig. 2a) and the NS5 protein (Fig. 2b, higher panels). EGFP expression was monitored by flow cytometry, whilst NS5 protein expression was revealed by Western-blot with an in-house produced and well-characterized monoclonal antibody directed to the DV2 NS5 RdRp domain.

3.3. Production of a BHK cell line supporting a chimeric DV2 EGFP-replicon with full length NS5 from DV3

The DV2-NS5- and the DV2/3-NS5-EGFP-replicon plasmids were linearized and *in vitro* transcribed. BHK cells, transfected with one or the other replicon RNA, were resistant to puromycin at 3.5 μg/ml and expressed EGFP. Cells expressing high levels of EGFP were sorted and amplified. In order to detect any adaptive mutations, replicon RNA was once again extracted and sequenced. No mutations were observed. Besides the EGFP protein (Fig. 2a), BHK DV2/3-NS5 EGFP-replicon containing cells expressed the NS5 protein (Fig. 2b, higher right panel). The NS5 signal, as revealed with an anti-NS5 monoclonal antibody directed to the DV2 NS5 RdRp domain, was greater in BHK DV2 cells than in the BHK DV2/3-NS5 cells. However, the levels of expression of the viral NS3 protein

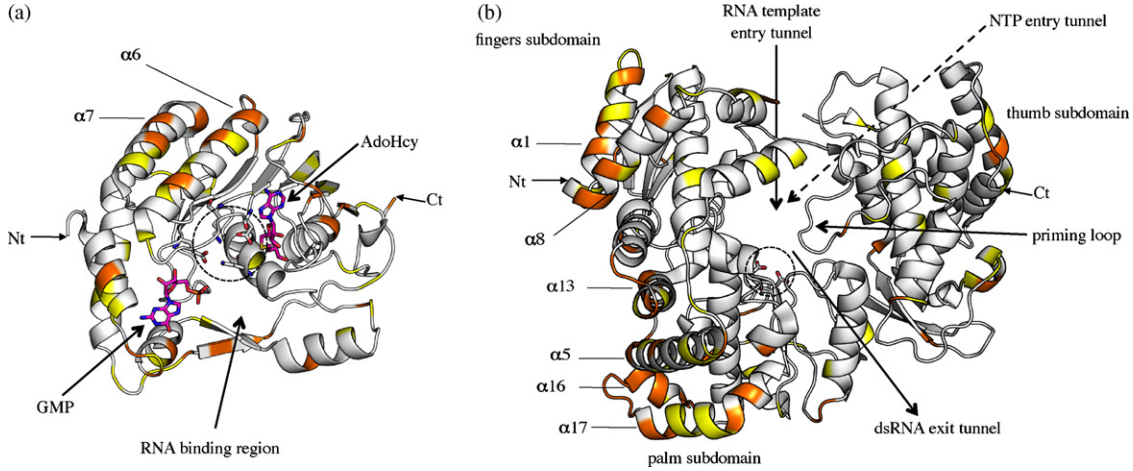

Fig. 3. Localization of solvent-accessible aminoacids differing between DV serotypes 2 and 3 on the NS5 MTase and RdRp domains. On the 3D ribbon views, non-conserved, similar and conserved amino acids are colored in orange, yellow and white, respectively. (a) MTase (left; PDB code: 2P41): the catalytic tetrad (K61-D146-K182-E218), the cofactor S-adenosyl-homocysteine (AdoHcy), the RNA binding region, and the GMP in the cap-binding site are indicated. (b) RdRp (PDB code: 2J7U): fingers, palm, thumb subdomains, tunnels of NTP entry, RNA entry, RNA exit, the catalytic residues (D533-D663), and the priming loop, are indicated. (a) and (b) Dotted circles represent active sites. Amino acids side chains are shown as sticks with the following color code: grey: C, blue: N, red: O. AdoHcy and GMP are shown as sticks with the following color code: magenta: C, blue: N, red: O, yellow: S, orange: P. Nt: amino-terminal, Ct: carboxy-terminal. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(middle right panel) and the cellular protein GAPDH (Fig. 2b, lower right panel) were similar between the BHK DV2 and BHK DV2/3-NS5 EGFP-replicon containing cells, indicating that both replicons replicated at similar levels. The lower NS5 signal in the BHK DV2/3-NS5 EGFP-replicon containing cells may result from a lower affinity of the monoclonal antibody for the DV3-NS5 protein than the DV2-NS5 protein.

The exchange of the DV2 NS5 gene with that of DV3, allowed the assembly of a functional replication complex, as evidenced by the long term maintenance of EGFP and NS5 expression, indicative of replicon replication. The DV2 (NGC strain) and DV3 (H87 strain) NS5 proteins have 79% identity and 91% homology. The 3D structure of the NS5 MTase (Egloff et al., 2002) and RdRp (Yap et al., 2007) domains are shown in Fig. 3. The catalytic tetrade Lys-Asp-Lys-Glu, the Adenosyl-Homocysteine (AdoHcy) cofactor, the cap binding site and the RNA binding region are indicated on the MTase 3D view (Fig. 3a). The palm, thumb and fingers subdomains, the tunnels of NTP entry, RNA entry, RNA exit, the catalytic Asp residues, and the priming loop, are indicated on the RdRp 3D view (Fig. 3b). The amino acids potentially involved in protein-protein interactions are solvent-accessible. Based on an alignment of the DV2 NS5 and DV3 NS5 amino acid sequences (see Supplementary Fig. I), we distinguished three types of solvent-accessible amino acids: identical residues are labeled in white, similar residues in yellow and non-conserved residues in orange. Surface residues that differ between the DV2 and DV3 NS5 MTase domain are mainly observed on the α6 and α7 helices. In the case of the RdRp domain, the main differences are localized on six α-helices: α1 and α8 on the thumb subdomain, and α5, α13, α16 and α17 on the palm subdomain. As shown in Fig. 3, these helices are outside of the catalytic sites, and this suggests that they are not the major determinants of interactions with viral or cellular proteins crucial for a functional DV replication complex.

3.4. Set-up and validation of a DV inhibitor EGFP-based screening assay

In stable cell lines supporting autonomously replicating subgenomic replicons, RNA replication involves multiple viral and cellular proteins which form the replication complex. These cells provide multiple targets whose functions could be inhibited or interactions disrupted by small molecule inhibitors of viral replication. We therefore examined whether the replicon cell lines described above could be used as the basis of an antiviral screening assay. The EGFP protein is cotranslated from the replicon RNA with the DV non-structural proteins, its level of expression is dependent on a functional replication complex. Thus, the inhibition of the replication and/or the translation of the replicon RNA would lead to a decrease in the level of EGFP expression, that would reflect a similar decrease in the expression of the viral non-structural proteins.

The decrease in EGFP expression is easily measurable in a high throughput assay using a plate-carrying fluorimeter. Identification of inhibitors requires quantification of an EGFP expression decrease in the steady-state level of RNA replicon. The cytotoxicity and the inhibitory potential of the compounds were tested on the same plate. To do so, cells were maintained with the molecules for 48 h, the solution being renewed after 24 h. The initial cell number per well and the duration of the assay are thus critical since the cell density could affect the replication efficiency of the RNA replicon. The cell monolayer may be close to the subconfluent state at the end of the assay, 1×10^4 cells per well in 96-well plates were initially seeded and the readout measurements were done 3 days later. Since phenol red could interfere with fluorescence measurements, to varying degrees depending on the cell density, we decided to use an uncolored culture medium without phenol red during the experiment, and to replace media by PBS before measuring EGFP fluorescence. As chemical compounds have to be dissolved in DMSO, the effect of DMSO concentration on cell viability was examined. Replicon containing cells were fully viable at 0.5% DMSO, but stopped dividing at 1% DMSO and died in less than 24 h above 1% DMSO. The inhibition assays were therefore carried out in 96-well plates, with media containing a final concentration of 0.5% DMSO. Under these conditions, the signal-to-noise ratio (EGFP fluorescence level from replicon-containing cells divided by the background signal from naïve BHK cells) was around 20 to 25. At the end of a 2-day treatment with potential inhibitors, the Celltiterblue® reagent was used to calculate the cell number in each well. Total EGFP fluorescence was then correlated to this cell number. With only these two fluorescence readouts, we could quantify inhibitory activity and toxicity and measure the EC_{50} (the effective concentration that led to 50% of the control fluorescence per replicon cell) and the CC_{50} (the concentration that led to 50% of the control cell number) of selected compounds.

To validate our screening assay, we used ribavirin for two reasons: its antiviral activities and its moderate cellular toxicity (Graci and Cameron, 2006), which are the two characteristics quantified with our assay. Ribavirin, a structural analog of GTP, is an inhibitor of the replication of a broad spectrum of viruses (Sidwell et al., 1972; Streeter et al., 1973). Ribavirin has been shown to inhibit the replication of DV in infection assays (Koff et al., 1982; Crance et al., 2003; Takhampunya et al., 2006) and in a DV subgenomic replicon assay (Ng et al., 2007). Five distinct mechanisms have been proposed to explain the antiviral properties of ribavirin (for a review, see Parker (2005), Graci and Cameron (2006)). These include both indirect mechanisms (inosine monophosphate dehydrogenase inhibition, immunomodulatory effects) and direct mechanisms (interference with RNA capping, polymerase inhibition, lethal mutagenesis). At least one direct mechanism has been reported for a DV protein: the *in vitro* inhibition of the 2′-O MTase activity of the DV NS5 protein (Benarroch et al., 2004).

Experiments carried out in the 96-well plate format, with BHK DV2 EGFP-replicon containing cells, allowed us to calculate a reproducible EC_{50} value of 15.5 ± 1.1 μM for ribavirin (Fig. 4a, solid line). This value is representative of 4 experiments and is consistent with values published previously using other cellular systems (Koff et al., 1982; Crance et al., 2003; Takhampunya et al., 2006; Ng et al., 2007). We calculated a Z' factor, according to the mean values and the standard deviations of the EGFP fluorescence levels of BHK DV2 EGFP-replicon containing cells, either not treated or treated with 100 μM or 50 μM of ribavirin. The Z' factor was 0.76 and 0.73, respectively, indicating that the assay is reliable (Zhang et al., 1999). Similar reproducible EC_{50} values were calculated with the other DV2 EGFP-replicon cell lines (Table 2): 12.7 ± 1.7 μM for the COS DV2 EGFP-replicon containing cells and 11.9 ± 0.7 μM for the Huh7 DV2 EGFP-replicon containing cells. As for the BHK DV2 EGFP-replicon containing cells, we tested the effect of ribavirin on BHK DV2/3-NS5 EGFP-replicon cells and measured a reproducible EC_{50} value of 12 ± 1.2 μM (Table 2).

An alternate assay was developed to directly quantify the fluorescence of EGFP in individual cells treated with ribavirin, independently of the cell count number. Replicon containing cells seeded in 24-well plates were treated with ribavirin following the same protocol used for 96-well plates but analysed by flow cytometry. Fluorescence of individual cells was measured for each ribavirin concentration and the mean values of the fluorescence intensities were directly compared to the control value. Using this assay, the EC_{50} value calculated for BHK DV2 EGFP-replicon containing cells was 18.4 ± 1.8 μM (Fig. 4a, dotted line, representative of 3 individual experiments), which is similar to the EC_{50} value obtained with the high throughput 96-well assay. With the 24-well format method, EC_{50} values were 5.8 ± 0.6 μM, 19.2 ± 1.8 μM and 20 ± 1.5 μM, for COS- and Huh7- DV2-, and BHK DV2/3-NS5-

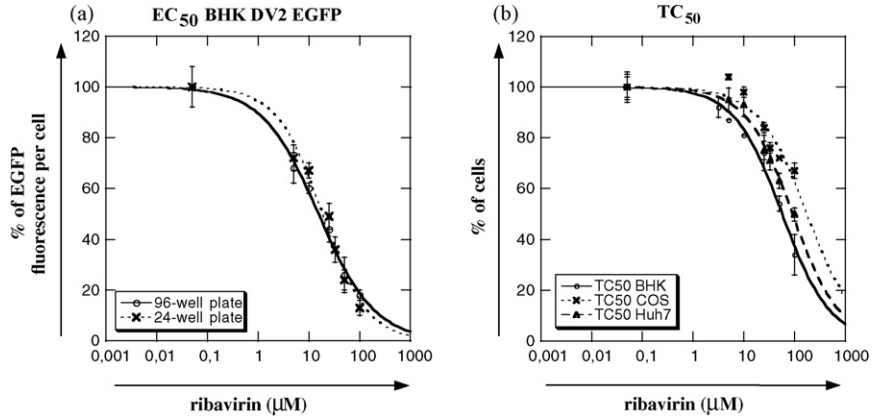

Fig. 4. Measurement of CC_{50} and EC_{50} values of ribavirin on DV EGFP-replicon cells. (a) Ribavirin EC_{50} values were calculated on BHK DV2 EGFP-replicon containing cells, from plate-carrying fluorimeter measurements (o, solid line) or obtained from flow cytometry measurements (×, dotted line). (b) From plate-carrying fluorimeter measurements, ribavirin CC_{50} values were calculated on BHK cells (solid line), COS cells (large dotted line) and Huh7 cells (thin dotted line).

Table 2
EC50, CC50 and SI values of ribavirin on DV EGFP-replicon containing cell lines.

	EC_{50} (μM)		CC_{50} (μM)[c]	SI[d]
BHK DV2 EGFP	15.5 ± 1.1[a]	18.4 ± 1.8[b]	57.2 ± 5.6	3.7
BHK DV2/3-NS5 EGFP	12 ± 1.2[a]	20 ± 1.5[b]		4.7
COS DV2 EGFP	12.7 ± 1.7[a]	5.8 ± 0.6[b]	>100	>7.9
Huh7 DV2 EGFP	11.9 ± 0.7[a]	19.2 ± 1.8[b]	91.9 ± 7.6	7.7

[a] EC50 values determined with the 96-wells plate assay.
[b] EC50 values determined with the 24-well plate assay.
[c] CC50 values were calculated with non-transfected cells.
[d] SI: selectivity index.

EGFP-replicon containing cells, respectively (Table 2). The high throughput fluorescence assay was validated as EC_{50} values are similar to those obtained with the flow cytometry assay. These results demonstrated unequivocally that combining the total EGFP fluorescence intensity measurement and cell counting using a fluorescent reagent is an appropriate method to rapidly calculate inhibitory activity and EC_{50} values of selected compounds.

Moreover, with the same assay, we could measure CC_{50} values and calculate the selectivity index ($SI = CC_{50}/EC_{50}$). For BHK cells, the ribavirin CC_{50} was 57.2 ± 5.6 μM (Fig. 4b, solid line). This gave SI values of 3.7 and 4.7, for BHK DV2- and BHK DV2/3-NS5- EGFP-replicon containing cells, respectively. Ribavirin CC_{50} values were higher than 100 μM for COS cells (Fig. 4b, large dotted line) and 91.9 ± 7.6 μM for Huh7 cells (Fig. 4b, thin dotted line), giving SI values above 7.7. Even though the EC_{50}/CC_{50} and SI values of ribavirin showed only minor variation between different cell types (Table 2), the availability of a number of different DV replicon containing cell lines should prove useful for screening inhibitors that may have different cell type specific characteristics.

3.5. The decrease in ribavirin-induced EGFP expression correlates with replicon NS5 protein expression

To confirm that the decrease in EGFP expression reflected a decrease in the expression of the viral non-structural proteins, we analyzed the amounts of DV NS5 protein following ribavirin treatment. Equal quantities of total protein extracted from ribavirin-treated cells were loaded on a SDS-PAGE gel and transferred to a PVDF membrane. The level of expression of the cellular protein GAPDH was not significantly affected by ribavirin treatment (Fig. 5, lower panel). As expected, the EGFP protein expression level decreased in BHK DV2 EGFP-replicon containing cells treated with ribavirin (Fig. 5, middle panel). Furthermore, the DV2 NS5 protein expression level decreased with increasing ribavirin concentrations (Fig. 5, upper panel). These results demonstrated that ribavirin inhibited the DV replication complex in the replicon containing cell lines and that its antiviral effect can be determined by monitoring the EGFP expression level correlated to the cell number. In conclusion, our Western-blot analysis confirmed that the high-throughput fluorescence measurements on the DV EGFP replicon-containing cells could be used to identify new inhibitory compounds.

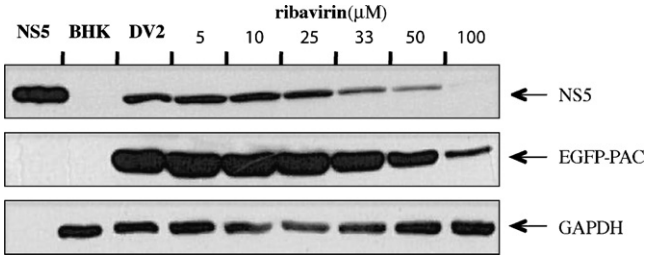

Fig. 5. Analysis by Western-blot of the expression of DV-NS5, EGFP and GAPDH proteins upon ribavirin treatment. Upper panel: NS5, middle panel: EGFP, lower panel: GAPDH. From left to right: purified recombinant NS5 protein (NS5), BHK cells extract (BHK), BHK DV2 EGFP-replicon containing cells extracts, untreated (DV2) or treated with ribavirin, 5, 10, 25, 33, 50 and 100 μM.

4. Discussion

The goal of this study was to extend current genetic systems for the development of HTS assays for anti-flavivirus drug discovery. Using DV2 as a model, we describe the construction of subgenomic DV EGFP-replicon plasmids and the production of several subgenomic replicon containing cell lines.

Self-replicating, non-infectious flavivirus subgenomic replicons have been widely used in studies of adaptive mutations (Liu et al., 2005; Rossi et al., 2005, 2007), viral assembly and packaging

(Harvey et al., 2004; Fayzulin et al., 2006; Leung et al., 2008), coupling between replication and packaging (Khromykh et al., 2001), viral RNA sequence and/or structure requirements for translation or replication (Lo et al., 2003a; Tilgner and Shi, 2004; Alvarez et al., 2005, 2008; Tilgner et al., 2005; Filomatori et al., 2006) and the antiviral immune response (Jones et al., 2005; Hershkovitz et al., 2008). Among these replicons, WNV-derived replicons have also been used for the screening of inhibitors, utilizing the luciferase reporter protein (Lo et al., 2003b; Rossi et al., 2005; Gu et al., 2006; Ng et al., 2007; Noueiry et al., 2007), resulting in several screening successes (Puig-Basagoiti et al., 2005; Goodell et al., 2006; Gu et al., 2006; Noueiry et al., 2007; Puig-Basagoiti et al., 2009). Two reports have described an HTS assay based on a DV replicon, both in BHK cells, with a luciferase reporter detection (Puig-Basagoiti et al., 2006; Ng et al., 2007). The assays described in this study demonstrate the establishment of a new DV replicon system based on an EGFP readout.

The establishment of replicon containing cell lines that can be used for HTS assays requires the expression of genes coding for a drug selectable marker and a reporter protein. A number of approaches have been used to produce the two individual proteins, including the use of self cleaving protein sequences to process polyproteins containing the two proteins and the use of an IRES element to either initiate translation of the viral non-structural proteins or a heterologous protein encoded by a gene cassette inserted in the viral 3′UTR. In the DV2 EGFP replicon, the EGFP and PAC proteins were produced as fusion protein that was cleaved from the DV2 non-structural proteins by the 1D2A peptide sequence from foot-and-mouth-disease virus. Previous studies have shown that translation initiated by an IRES sequence downstream from a cap-dependent translation start site is often inefficient (Mizuguchi et al., 2000; Ibrahimi et al., 2009) and can be subject to cell type specific effects. By contrast, the separation of heterologous gene product/s from the viral non-structural proteins by the 1D2A peptide essentially results in equimolar amounts of products (de Felipe et al., 2006). As the EGFP fluorescence from DV2 EGFP replicon containing cells was taken as a measure of non-structural protein expression, it may be beneficial to have the EGFP-PAC fusion protein separated from the non-structural proteins through the use of the 1D2A sequence rather than an IRES sequence. Both the EGFP and PAC proteins were active in the context of a fusion protein with the EGFP-PAC protein giving similar resistance to puromycin as the PAC gene product alone in previous studies (Jones et al., 2005). Although a gene construct containing an IRES element and a selectable marker gene have been successfully introduced into the 3′ UTR of a number of flavivirus replicons (Khromykh et al., 2001; Shi et al., 2002; Rossi et al., 2005; Gu et al., 2006), including DV type 1 (Suzuki et al., 2007), we and others have found it difficult to reliably insert similar gene cassettes into the DV2 genome (unpublished data (Ng et al., 2007)). The ability to insert a unique gene cassette encoding an EGFP-PAC fusion protein in place of the structural protein genes circumvents this potential difficulty in DV replicon production.

The DV replicon containing cell lines produced in this study will serve as useful tools for high throughput screening of DV replication inhibitors. Our assay is robust as the Z' factor is greater than 0.7 and the signal-to-noise ratio is 20 to 25, values well suited for a HTS assay. The EC_{50} and CC_{50} values determined for ribavirin with the new DV replicon containing cell lines are similar to the values described using virus infected cell lines (Koff et al., 1982; Crance et al., 2003; Takhampunya et al., 2006; Ng et al., 2007). The effectiveness of the inhibition of the DV replication complex, based on the EGFP readout, was confirmed by examining the DV NS5 expression level with a specific monoclonal antibody. Our results demonstrate unequivocally that the determination of replicon replication levels using a fluorescence-based method that correlates the total EGFP fluorescence with total cell numbers is appropriate for the measuring inhibitory activity and for calculating the EC_{50} and CC_{50} values of selected compounds. In a HTS, in 96- or 384-wells plates, the process would be divided in two parts. A single EGFP readout will select compounds with both antiviral activity and/or cytotoxic effect. The selected compounds, decreasing the EGFP level, will be tested for their cytotoxicity, thus allowing to keep the non-cytotoxic compounds with antiviral activity.

Two types of approaches are routinely used for antiviral assay development. One approach is biochemistry-based, in which the enzymatic activity of purified viral protein is assayed. For flaviviruses, enzymatic HTS assays have been developed for the multifunctional NS3 (protease, helicase, nucleoside triphosphatase, and 5′-RNA triphosphatase) and NS5 (MTase and RdRp) proteins (Johnston et al., 2007; Lim et al., 2008). The principal advantage of the biochemistry-based assay is that the targets of the identified inhibitors are known. The other approach is replicon-based, a biosafe alternative to using infectious virus and involves multiple targets in the viral life cycle. The assays described in this study provide additional means for DV drug discovery. For example, inhibitors obtained from anti-MTase and anti-RdRp based screens can now be tested using the DV2 EGFP replicon-containing cells. In addition, with the use of the DV2 or DV2/3-NS5 replicon-containing cells it will be possible to determine the potential of the MTase/RdRp inhibitors in the context of different DV serotypes. Moreover, with the replicon carrying the NS5 gene cassette, it will be possible to characterize NS5 mutants derived from biochemical/structural analysis or resistance studies, in a cellular environment.

A range of cell types were used in this study to stably express the DV replicons. As in a number of previous studies we initially established the DV2 EGFP replicon in BHK cells which were then used to optimize the assays for HTS. The DV inter-serotypic chimeric replicon, expressing the NS1 to NS4 genes from DV2, and the NS5 gene from DV3 was also established in BHK cells. In addition to its use for drug discovery, the chimeric replicon system provides an interesting tool to study the determinants of interactions which occur in the viral replication complex.

Once established in BHK cells, the replicon systems were then extended to a human hepatic cell line (Huh7) and the COS monkey cell line. DV can infect many cell types and causes diverse clinical and pathological effects. Although the main target cells in humans are believed to reside in the reticuloendothelial system, both clinical and experimental observations suggest that there is also liver damage during DV infection. Clinical evidence of liver involvement in DV infections includes the presence of hepatomegaly and increased levels of serum liver enzymes (Seneviratne et al., 2006). DV is able to replicate in both hepatocytes and Kupffer cells (Huerre et al., 2001). Thus, the DV2 replicon Huh7 stable cell line constitutes a suitable tool for studying the interactions between viral proteins and hepatic proteins, some of which may be responsible for the infection-induced liver damage (Ekkapongpisit et al., 2007; Pattanakitsakul et al., 2007; Higa et al., 2008). Extension of the replicon system to COS cells demonstrated that even cells that are poorly infected with DV *in vitro* (unpublished observations (Rodrigo et al., 2006)) can be used for maintaining DV replicons if they are permissive for genome replication. Monkey kidney-derived cells have been previously used for DV protein interaction studies *in vitro* (Kapoor et al., 1995). We therefore produced a COS cell line containing the DV replicon, allowing studies of the DV replication complex with antibodies directed to viral or cellular proteins. Compared to BHK cells, COS cells have the advantage that they are less likely to be susceptible to cross reactivity with mouse or rat monoclonal antibodies, commonly used for immunoprecipitation studies. Overall the production of a range of DV replicon cell lines provides new opportunities for the study of DV replication in a cellular context. At

the same time, the establishment of multiple cell lines carrying the DV replicon demonstrates the feasibility of directly evaluating the potential of selected compounds in cell lines from different organs and species.

In summary, the assays described in this study will greatly facilitate DV drug discovery by serving as primary or complementary screening assays. The approach should be applicable to the development of cell-based HTS assay for other flaviviruses, and should also be useful for the study of DV replication and pathogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.03.010.

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