Antiviral Research 98 (2013) 449–456

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Novel, anionic, antiviral septapeptides from mosquito cells also protect monkey cells against dengue virus

Chaowanee Laosutthipong [a,b], Nipaporn Kanthong [c], Timothy W. Flegel [a,b,d,*]

[a] Dept. Biotechnology, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[b] Centex Shrimp, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[c] Dept. Biotechnology, Faculty of Science and Technology, Rajamangala University of Technology Tawan-ok, Sriracha, Chonburi 20110, Thailand
[d] National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Klong 1, Klong Luang, Pratum Thani 12120, Thailand

ARTICLE INFO

Article history:
Received 18 January 2013
Revised 10 April 2013
Accepted 10 April 2013
Available online 17 April 2013

Keywords:
Antiviral peptides
Dengue virus
Cytokine
Viprolaxikine
C6/36 cells
Vero cells

ABSTRACT

We have shown previously that ultrafiltrates (5 kDa cutoff) of cell-free medium from mosquito cell cultures persistently infected with DENV serotype 2 (DENV-2) contained a novel antiviral agent (called viprolaxikine) that could protect pre-treated, naïve mosquito cells from DENV infection. Here, we show that viprolaxikine also reduced DENV-2 titers by almost 4 logs (>99.9%) when compared to Vero cells mock-treated with ultrafiltrates from cultures of uninfected mosquito cells. Protease treatment removed the anti-DENV-2 activity. Pre-incubation for 48-h was required to obtain the maximum, dose-dependent protection against DENV-2, indicating that the antiviral activity was based on the interaction between Vero cells and viprolaxikine rather than direct action of viprolaxikine on DENV-2. Activity was highest against DENV-2, but there was also significant activity against the 3 other DENV serotypes. LC–MS–MS analysis revealed that the active viprolaxikine fraction contained anionic, antiviral peptides, each comprised of 7 amino acids (DDHELQD, DETELQD and DEVMLQD or DEVLMQD) and with a common sequence motif of D-D/E-X-X-X-Q-D. These sequences do not occur in the dengue virus genome, suggesting that the peptides are produced by the host insect cells when persistently infected with DENV-2. These peptides represent a new class of anionic, insect-derived, antiviral peptides with activity against a flavivirus in both mammalian and insect cells.

1. Introduction

Dengue virus (DENV) is divided into four serotypes (DENV-1 to 4) (Westaway, 1997) any of which may cause disease ranging from acute febrile dengue fever (DF) to life-threatening dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS). Outbreaks of DF and DHF are still a major public health problem worldwide, especially in tropical and subtropical countries (WHO, 2002).

Persistent, *in vitro* virus infections have been established for many flaviviruses in both mosquito and mammalian cell lines (Chen et al., 1994, 1996; Debnath et al., 1991; Lancaster et al., 1998; Randolph and Hardy, 1988; Schmaljohn and Blair, 1977; Vlaycheva and Chambers, 2002). From high passage cultures of persistently infected insect cells, it has been reported that high numbers of defective interfering particles (DIP) are produced and that they can account for the low infectivity of supernatant solutions for challenged mammalian cells (Huang and Baltimore, 1970; Von Magnus, 1951). An alternate explanation to DIP is that some factor(s) produced by persistently infected insect cells can act as an antiviral agent as previously reported for an antiviral protein (AVP) from mosquito cells persistently infected with Sindbis virus (Luo and Brown, 1993, 1994; Riedel and Brown, 1979).

Similarly, an antiviral preparation from mosquito cells persistently infected with DENV-2 (called viprolaxikine) successfully protected naïve mosquito cells against DENV-2 infection (Kanthong et al., 2010). This suggested that previously reported failure to infect Vero cells with DENV-2 produced from high passage cultures of mosquito cells persistently infected with DENV-2 (Sinarachatanant and Olson, 1973) might be due to viprolaxikine rather than DIP. To test this hypothesis, cell-free medium from C6/36 cells persistently infected with DENV-2 was investigated for the presence of antiviral substances. This revealed that viprolaxikine contained a new class of anionic septapeptides that could protect both C6/36 cells and Vero cells against DENV-2 infection.

2. Materials and methods

2.1. Cell lines, viruses and virus titration

Vero cells (ATCC: CRL-81) were cultured at 37 °C in Minimum Essential Medium (Invitrogen) supplemented with 10%

* Corresponding author at: National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency, Klong 1, Klong Luang, Pratum Thani 12120, Thailand. Tel.: +662 201 5870; fax: +662 354 7344.
E-mail address: sctwf@mahidol.ac.th (T.W. Flegel).

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http://dx.doi.org/10.1016/j.antiviral.2013.04.011

heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1.2% antibiotics (Penicillin G and Streptomycin) in a 5% CO_2 incubator. C6/36 cells (*Aedes albopictus*) (ATCC: CRL-1660) were grown in Leibovitz's (L-15) medium containing 10% heat-inactivated FBS, 10% tryptose phosphate broth (TPB), and 1.2% antibiotics (Penicillin G and Streptomycin). Dengue virus serotype-1 (DENV-1) (HAWAII), DENV-2 (NGC), DENV-3 (H87), and DENV-4 (H241) were propagated in C6/36 cells as previously reported (Kanthong et al., 2010) and stored as a stock at −80 °C. Viral infectivity was determined by focus forming units (FFU) and expressed as virus titer of FFU/ml as previously described (Cruz and Shin, 2007) with some modifications (Kanthong et al., 2008).

2.2. Immunofluorescence assays

To determine the proportion of infected cells by flow cytometry, C6/36 cells or Vero cells were washed twice with 1× phosphate buffered saline (PBS) and fixed with 2% formaldehyde in PBS for 1 h. Two additional washings were carried out using 0.1% triton X-100/PBS. Cells were incubated with mouse antibody to DENV at room temperature for 1 h, washed twice with 0.1% triton X-100/PBS and incubated with rabbit anti-mouse IgG conjugated with FITC (F0261, DAKO) for 30 min in the dark. Cells were washed and resuspended followed by FACS analysis (Becton Dickinson). Mock-infected, negative control C6/36 cells were run in parallel.

Immunofluorescence staining of DENV antigen in the cytoplasm of Vero cells was carried out as previously described (Kanthong et al., 2010) using anti-DENV envelope protein antibody labeled with FITC (green fluorescence) while nucleic acids were counterstained with TO-PRO-3 iodide (T-3605, Molecular Probes)(red fluorescence). Results were observed using a confocal laser microscope (FV1000, OLYMPUS).

2.3. Real-time RT-PCR

Viral RNA from culture supernatant was extracted using TRIzol reagent (Invitrogen) and RNA yield was determined using spectrophotometry at 260 nm. Primers for a 150 base pair amplicon of the DENV-2 envelope gene 5′- CAC TGT CAC GAT GGA GTG CT -3′ (forward) and 5′-TGA TCC TTG TGT GTC CGC T-3′ (reverse) were used with a SuperScript One-Step RT-PCR (Invitrogen) kit and Platinum *Taq* DNA polymerase. Real-time RT-PCR was carried out using a Rotor-Gene 6000™ (Corbett Research) machine with the protocol: one cycle of 50 °C for 15 min and 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. Relative quantification was achieved by reference to a standard curve prepared using RNA from a DENV-2 stock of known viral titer.

2.4. Persistent dengue virus infections and viprolaxikine preparation

Persistent infections of DENV-2 in C6/36 cells were achieved and viprolaxikine was prepared as previously described (Kanthong et al., 2008, 2010). Before storage at −20 °C, the protein concentration in viprolaxikine preparations was measured using a Bradford protein assay kit (Bio-Rad) and a microplate reader set at OD_{595}.

2.5. Determination of antiviral activity with Vero cells

For time-course activity studies, Vero cell monolayers were incubated with a single addition of persistently infected insect-cell culture filtrate from 0 to 96 h in 96-well plates at 37 °C prior to challenge with serially diluted (10× steps) DENV-2 stock. The filtrate was washed off the cells prior to addition of the viral stock and the exposed cells were assayed for FFU three days later. The negative control consisted of Vero cells pre-incubated in parallel with filtrate from normal insect cell cultures (called naïve cells) followed by washing before challenge with DENV-2.

To compare anti-DENV activity in filtrates, Vero cell monolayers were incubated with a single addition of persistently infected insect-cell culture filtrates from passages 5, 10, 16, and 25 using the optimum filtrate pre-exposure time determined as described above. This yielded the optimum passage number to obtain the highest antiviral activity in the culture supernatant.

To determine the dose dependence of anti-DENV activity, Vero cell monolayers were incubated with a single addition of various quantities (5–200 μl) of persistently infected insect-cell culture filtrates in a total volume of 200 μl for the optimum time determined as described in the previous paragraph. After culture filtrate removal, they were challenged and assayed with DENV-2 as described above. Parallel Vero cell cultures treated with filtrate from uninfected insect cell cultures served as mock-infected cells. The data generated from these experiments were used to define one arbitrary unit of filtrate activity as the volume (μl) or μg protein that resulted in a 50% decrease in viral titer (FFU), and a standard assay was designed where monolayers of naïve Vero cells in a final volume of 100 μl a 96-well microtiter plate (approximately 20,000 cells per well) were prepared and incubated for confluent growth overnight. Then, the old medium was replaced with only 2% FBS-MEM medium or the same mixed with viprolaxikine preparations (final volume 100 μl) and incubated for 48 h before washing and then exposure to DENV-2. The virus titer was determined 3 days later using focus forming assays. Every batch of crude filtrate was calibrated in this way. For convenience, the active filtrate and active fractions of it are referred to here as viprolaxikine.

To determine the antiviral activity of viprolaxikine for other DENV serotypes, Vero cells were treated with filtrates from the 16th passage of C6/36 cells persistently infected with DENV-2 according to the standard assay procedure described above using 50 μl of crude filtrate (1 μg protein) but followed by exposure to each of the 4 DENV serotypes.

2.6. Preliminary purification and mass spectometry of viprolaxikine

Purification of viprolaxikine was based on filtrates from the 16th passage of C6/36 cells persistently infected with DENV-2. Many aliquots (1 ml) of 5 kDa membrane culture filtrates were desalted for removal of low molecular weight contaminants from culture medium using a HiTrap Desalting column (GE Healthcare) and Fast Protein Liquid Chromatography (FPLC) (AKTApurifier). The protein peaks eluted before the salt were collected (2 ml each), pooled and subjected to FPLC using ion exchange chromatography of either the strong cation (HiTrap SP HP) or strong anion (HiTrap Q HP) type eluted using continuous salt gradients ending with 1 M NaCl. Column fractions were tested for antiviral activity by titer with Vero cells. Final purification consisted of reverse phase high performance liquid chromatography (HPLC) (Waters) using a Delta-Pak C_{18} column (2 mm × 150 mm) with buffer A containing 0.1% trifluoroacetic acid (TFA)/H_2O and buffer B containing 0.1% TFA/acetonitrile (ACN). Samples were automatically injected and eluted with a 1–60% ACN gradient over 60 min. Polypeptides were detected by optical density at 256 and 280 nm using a Waters 2487 Dual UV detector, and fractions were dried using a SpeedVac concentrator before re-suspension in culture medium for antiviral testing.

For mass spectrometry, the fraction from the initial HPLC column with highest anti-DENV-2 activity was injected into an Ultimate 3000 Liquid chromatography system (Dionex, USA) that was coupled to an ESI-Ion Trap Mass spectrometry (Bruker, Germany) with electrospray at a flow rate of 300 nl/min to nanocolumn (Onyx, monolithic HDC18, 0.2 mm i.d. × 150 mm). The mobile phase A was 5% ACN in 0.1% formic acid and the mobile

phase B was 80% ACN in 0.1% formic acid. All MS/MS ions obtained were analyzed with Bruker HyStar® Software and de novo sequencing was performed by BioTools program (Bruker Daltonic).

2.7. Testing of synthetic peptides

Four peptides (DDHELQD, DETELQD, DEVMLQD and DEVLMQD) were synthesized, purified and provided at greater than 95% purity by ChinaPeptide® and each preparation was accompanied by a mass spectrum, confirming its mass. In addition, we analyzed the specimens using our LC–MS–MS system to confirm their sequences (Additional Fig. 3). They were re-suspended with sterile distilled water to 5 mg/ml and further diluted to 100 μg/ml using 2% FBS–MEM culture medium as a stock. Tests of synthetic peptide activity were performed using the standard assay system as described in the previous section for the DENV-2 titer assay. In brief, Vero cell monolayers were seeded with 20,000 cells/well on a 96-well microplate followed by overnight incubation. The old medium was removed and replaced with 50 μl of 2%FBS-MEM (control infection) or 10 μl of peptides at appropriate dilutions in 40 μl 2% FBS–MEM medium (Peptide treatment). The incubation time of peptides was 48 h before washing followed by DENV-2 challenge. The anti-DENV-2 activity was determined using virus titration (focus forming assays).

2.8. Statistics

Virus titers, DENV-2 genome copy numbers and the percentage of immunopositive cells by flow cytometry were determined as means plus or minus standard deviation (SD). Analysis of variance (ANOVA) was used to test among P35 washed or un-washed DENV, and DENV stock (Fig. 2). The student *t*-test (SigmaStat 3.5, Systat Software Inc., Chicago) was used for pairwise group comparisons. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Low DENV-2 titers from mosquito cell culture medium not due to defective virions

As previously reported (Kanthong et al., 2008), cytopathic effects (CPE) and multinucleate giant cells were seen in early but not late passages after C6/36 mosquito cells were infected with DENV-2. When DENV-2 infectivity in culture medium from persistently infected cells was determined in Vero cells, the titer decreased with increasing passage number and reached zero from passage 64 onward as previously reported (Sinarachatanant and Olson, 1973)(Additional file1. Fig. S1). By contrast, the virus remained highly infectious for C6/36 cells as previously reported (Kanthong et al., 2010) and analysis by real-time RT-PCR revealed that mean viral genome copy numbers (±SD) in the supernatant solution remained high ($5.21 \times 10^6 \pm 2.79 \times 10^6$ FFU/ml) from passages 3 to 65.

At the beginning of our work, we carried out a preliminary experiment to determine the presence of an antiviral substance(s) and/or defective virions in the supernatant medium from passage 35 (P35) of C6/36 cells persistently infected with DENV-2. We chose P35 because we had confirmed previously published work that untreated medium from this passage gave very low DENV-2 titer with Vero cells (Sinarachatanant and Olson, 1973). Specifically, 4 ml of supernatant medium from P35 was collected and passed through a membrane filter with 5 kDa cutoff to capture large proteins and viral particles. Virions that collected on the upper side of the filter were washed with ice-cold, sterile 1× PBS before re-suspension in 4 ml of fresh and ice-cold, 2% MEM medium for FFU measurement using Vero cells. The titer obtained was compared to those of the unfiltered supernatant medium (i.e., unwashed virions) and the DENV-2 stock. Cells exposed to unwashed virus gave a significantly lower titer than those exposed to washed virus ($p < 0.01$). The latter gave a titer not significantly different from that of the positive control, stock virus ($p = 0.56$) (Fig. 1). Since washing resulted in major restoration of viral titer, the results suggested that most of the titer reduction from persistently infected mosquito cells was due to a very small component(s) (5 kDa or less) of the culture medium that was removed by washing, and not due to defective viral particles (also called defective interfering particles or DIP). Later, after standardization steps (see next section), it was found that maximum antiviral activity was obtained using cell-free culture medium from cells persistently infected with DENV-2 at passage 16 (P16). The washing experiment was then repeated using cell-free medium from P16 and the results confirmed that washing the virus restored its ability to infect C6/36 cells (not shown). These experiments also revealed that the effect of the antiviral substance was based on its interaction with Vero cells and not on its direct interaction with DENV-2.

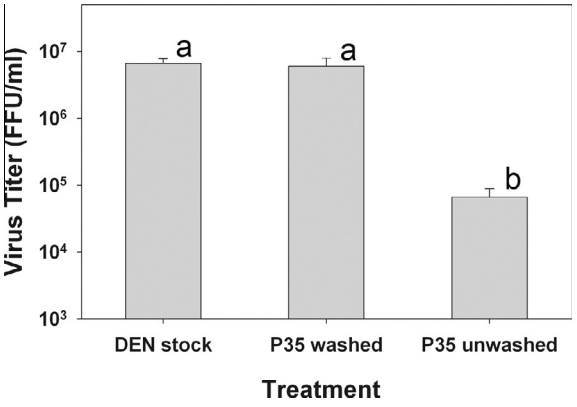

Fig. 1. Restoration of viral titer by washing. Comparison of viral titer for washed and unwashed virions from the supernatant solution of a C6/36 cell culture persistently infected with DENV-2 compared to that of the viral stock. Bars marked with different letters were significantly different ($p < 0.01$).

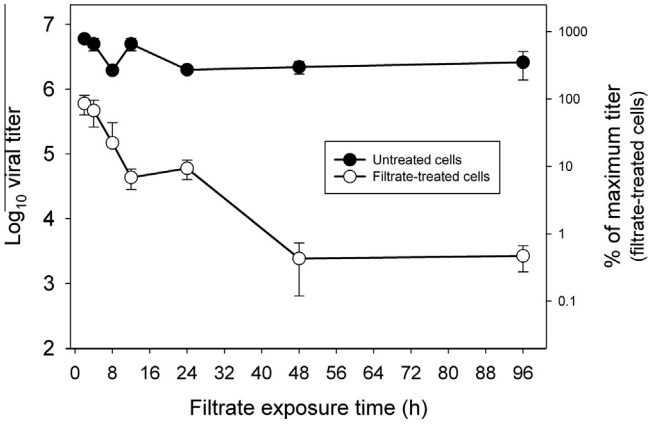

Fig. 2. Optimum incubation time for antiviral activity. Titers measured for DENV-2 (FFU/ml) stock using Vero cells pre-incubated and washed prior to challenge for various times (h) with the 5 kDa filtrate from C6/36 cells persistently infected with DENV-2 (open circles) by the 100 μl standard assay containing 50 μ1 culture filtrate with 1 μg protein = 5 units of viprolaxikine activity. Controls consisted of Vero cells pre-incubated in culture medium for the same periods and washed prior to challenge (black circles). Each point represents the mean virus titer ± SD from three independent treatments.

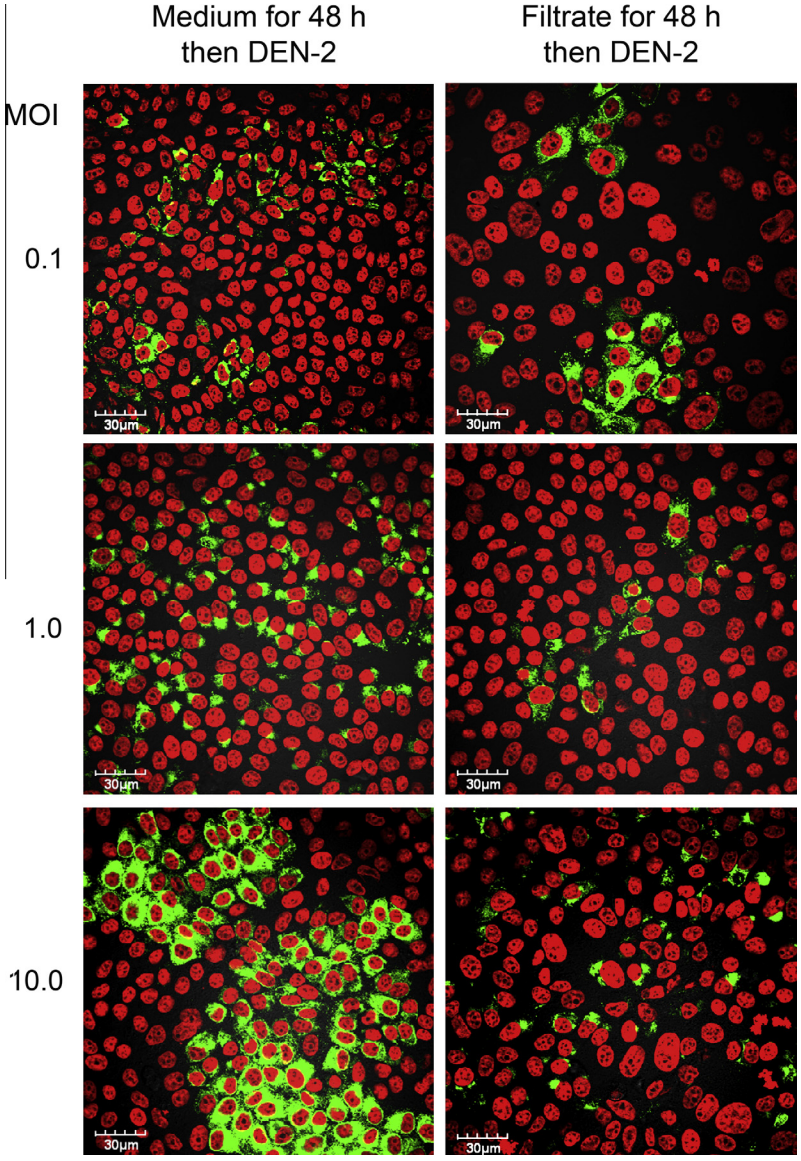

Fig. 3. DENV-2 infection of filtrate-treated Vero cells challenged at various MOI. Photomicrographs showing DENV-2 immunofluorescence (green) of Vero cells pre-treated for 48 h or not and washed before DENV-2 virus challenge (DEN48H) at different multiplicities of infection (MOI 0.1, 1, and 10). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Standardizing filtrate anti-DENV-2 activity for Vero cells

For initial tests using 5 kDa filtrates from the culture medium of C6/36 cells persistently infected with DENV-2, the filtrates and DENV-2 stock were mixed immediately prior to challenge of naïve Vero cells. This resulted in a reduced viral titer from 10^7 FFU/ml to 10^5 FFU/ml (i.e., 2 logs or 99% reduction) when compared to naïve cells challenged with DENV-2 stock alone. For subsequent tests, the Vero cells were pre-treated with the 5 kDa filtrate for various intervals prior to washing followed by DENV-2 challenge, and this resulted in increasing titer reductions with increasing incubation time up to 48 h, at which time a reduction of more than 3 logs (i.e., >99.9%) was obtained (Fig. 2). Thus, for all subsequent tests, a pre-incubation period of 48 h followed by washing prior to DENV-2 challenge was chosen as the optimum to obtain the maximum protective effect.

When Vero cells were treated with filtrate or control medium for 48 h followed by washing and then DENV-2 challenge at multiplicities of infection (MOI) 0.1, 1.0 and 10.0, the numbers of immunopositive cells (FITC-labeled antibody against DENV-2 envelope protein) in all the filtrate-treated groups at 48 h post challenge were visibly lower than those in the respective control groups (Fig. 3). This was confirmed by cell flow cytometry at MOI 0.1 where the number of DENV-2 positive (infected) cells in the filtrate-treated group was significantly ($p < 0.001$) lower (15.7 ± 0.9%) than that in the control group (46.5 ± 3.0%).

The response of Vero cells to viprolaxikine was shown to be dose dependent (Fig. 4), and one unit of viprolaxikine activity was arbitrarily defined as the extract volume that reduced the titer of the DENV-2 stock by 50% after pre-incubation with a monolayer of Vero cells in a total of 100 µl medium for 48 h. With protein quantification, values could also be expressed as units per µg protein. Using this definition with 5 kDa filtrates from various passages of C6/36 cells persistently infected with DENV-2, the culture supernatant of passage 16 (P16) gave higher anti-DENV-2 activity (100 Units for 20 µg protein in 1 ml or 5 Units/µg protein) than those from P5 (0.78 Units/ml), P10 (0.9 Units/ml) and P25 (10 Units/ml). Thus, for subsequent tests, 5 kDa filtrate prepara-

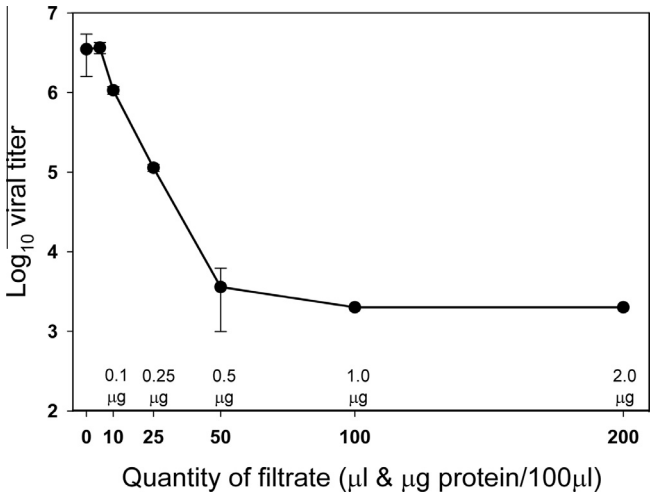

Fig. 4. Dose-dependent response to viprolaxikine. Vero cells treated with filtrate at 5, 10, 25, 50, 100, and 200 µl in a total volume of 200 µl for 48 h before removal and challenge with DENV-2 virus show increasing protection against DENV-2 with increasing amount of filtrate as determined by focus forming assay. Each point represents the mean virus titer ± SD from three independent treatments. Note that these initial experiments were performed in double the volume used in the final standard assay.

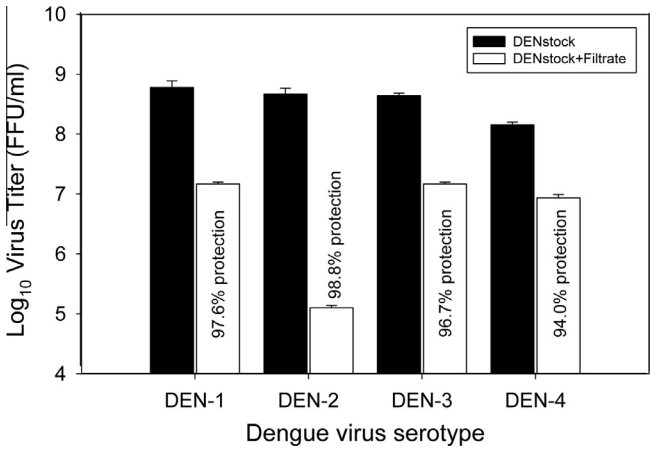

Fig. 5. Comparison of viprolaxikine activity among DENV serotypes. Each bar indicates the mean virus titer ± SD (FFU/ml) from three independent treatments. The difference in titers between filtrate-exposed and unexposed Vero cells for all DENV types were statistically significant ($p < 0.01$). The DENV-2 viral stock used for these experiments was of higher titer than that used for the other experiments described here.

tions were made from the supernatant solution at P16 and units of viprolaxikine activity were calibrated for each batch based on this unit definition. From these results, a standard assay protocol was devised to measure antiviral activity (see Section 2): in a total volume of 100 µl in 96-well microtiter plates, viprolaxikine preparations were added to Vero cells and incubated for 48 h before removal and replacement by DENV-2 challenge solution followed by virus titer determination using focus forming assays. Using this method, a maximum antiviral activity of more than three logs reduction in DEN2-titer (>99.9% reduction) was obtained using the crude culture filtrate from P16 at 1 µg protein (i.e., 50 µl) in a total assay volume of 100 µl.

3.3. Viprolaxikine protects against 4 DENV serotypes

Specificity testing with 4 DENV serotypes using Vero cells treated with the 50 µl viprolaxikine crude filtrate (1 µg protein) by the standard assay revealed that activity was highest for DENV-2 but that there was also significant activity ($p < 0.01$) against DENV serotypes 1, 3 and 4 (94–98% reduction in titer) when compared to the non-treated control (Fig. 5).

3.4. Anti-DENV-2 activity does not persist in exposed cells

A single exposure to an uncharacterized antiviral protein (AVP) from mosquito cells persistently infected with Sindbis virus has been reported to permanently protect naïve mosquito cells from Sindbis virus on serial sub-passage, and also to induce their perpetual production of AVP (Luo and Brown, 1993). Thus, tests were carried out with C6/36 cells and Vero cells to determine whether viprolaxikine protection from DENV-2 has a similar effect on C6/36 cells or Vero cells upon exposure and subsequent passaging. Results showed that protection against DENV-2 diminished with passage number for both C6/36 cells and Vero cells (Fig. 6) indicating that viprolaxikine activity differed in character from that of the AVP associated with Sindbis virus.

3.5. Purification and characterization of viprolaxikine

Cell-free culture medium from passage 16 of C6/36 cells persistently infected with DENV-2 was first passed through a membrane filter (5 kDa cutoff) to remove large proteins and nucleic acids and DENV-2 viral particles before it was passed through a strong anion exchange (Q) column to trap viprolaxikine (determined by preliminary screening to be negatively charged at pH 7). Antiviral activity of HPLC peak fractions (1 ml) measured by DENV-2 FFU in Vero cell cultures gave the highest antiviral activity of approximately 3 logs titer reduction (99.7% reduction) when compared to untreated cells. Table 1 shows the viprolaxikine anti-DENV-2 activity in fractions from various purification steps. Approximately half of the activity was lost at the ion-exchange chromatography step, indicating need for improvement. Although the 7 times increase in specific activity seems to be modest, it must be kept in mind that the initial step of filtration using a membrane filter of 5 kDa cutoff would have removed a major portion of other, larger proteins, as confirmed by the liquid chromatography (LC) step described in the following paragraph.

The final fraction of highest specific activity was subjected to LC–MS–MS analysis. The initial LC step showed a single activity peak (Additional file 2, Fig. S2). When this active fraction was subjected to Ultimate 3000 Liquid chromatography coupled to an ESI-Ion Trap Mass spectrometer (see methods), the mass spectrum revealed that the fraction produced two peaks of approximately 849 and 871 kDa (Additional file 3, Fig. S3a). Further analysis of these two peaks revealed that they originated from 3 to 4 strongly anionic peptides of 7 amino acids each (DDHELQD, DETELQD, DEVMLQD and/or DEVLMQD) and with a common sequence motif of D-D/E-X-X-X-Q-D. (Additional files 3b–d, Figs. S3b–S3d).

3.6. Testing of synthetic peptides

Based on the mass spectrometry results, the peptides DDHELQD, DETELQD, DEVMLQD and DEVLMQD were synthesized and purified by ChinaPeptide®. DENV-2 testing was performed as described above using 1 unit of crude viprolaxikine solution (0.2 µg protein) as the standard (i.e., 50% reduction in viral titer or 50% protection). When the synthetic peptides were serially diluted in 2% MEM and tested with Vero cells in the standard assay, 1 unit of activity was obtained using an equal mixture of the 4 test peptides diluted to

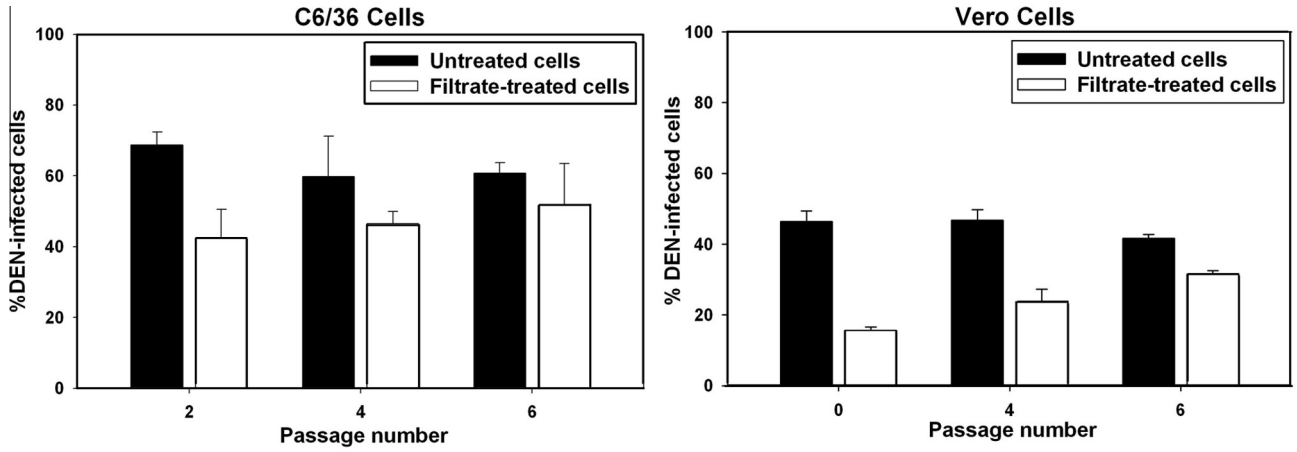

Fig. 6. Persistence of viprolaxikine protection. Cells exposed to viprolaxikine for 48 h and then passaged before DENV-2 challenge progressively lost protection as the passage number increased. Bars show mean % infection ± SD for 3 independent replicates measured by cell-flow cytometry.

Table 1
Specific activity of viprolaxikine at various purification steps.

Source	Total vol. (ml)	Protein (μg/ml)	Total protein (μg)	Activity (U/ml)	Total activity (U)	Specific activity (U/μg protein)
Broth filtrate	10	20	200	100	1000	5
Anion exchange	1	75	75	500	500	7
C18 HPLC	1	15	15	500	500	33

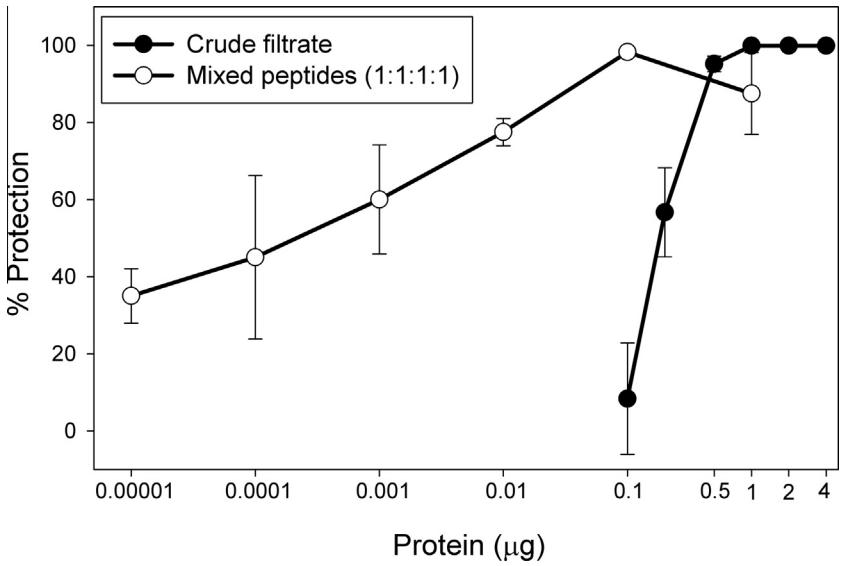

Fig. 7. Protection of Vero cells against DENV-2 by synthetic peptides. By the standard assay, Vero cells were treated with a mixture of the 4 synthetic peptides (DDHELQD, DETELQD, DEVMLQD and DEVLMQD at 1:1:1:1 ratio) at various dilutions and compared to the antiviral activity of the crude P16 filtrate at various dilutions. The graph shows% protection against DENV-2 compared to control Vero cells set at 100%. The difference between the peak activities of 98.3% for the peptide mix and 99.9% for the crude filtrate was significantly different ($p = 0.001$) while that between 98.3% and 87.5% for the peptide mix was not significantly different ($p = 0.154$).

0.001 μg total protein (Fig. 7). At equivalent protein levels, activities for the individual peptides and dual combinations of the same had lower activities (Additional file 4, Fig. S4). Thus, 1 unit of anti-DENV-2 activity was achieved using 200 times less protein than the crude filtrate (0.001 μg vs 0.2 μg, respectively), indicating much higher specific activity for the mix. However, the slope of the dose response curve for the 4-peptide mix was much lower than that of the crude filtrate, so that an increase of 100 times to 0.1 μg gave 98.3% protection compared to 8.3% for 0.1 μg of the crude filtrate. Increasing the protein of the 4-peptide mix by 10 times to 1.0 μg resulted in a decrease in protection to 87.5% while protection from the crude filtrate reached a plateau of 99.9% at 1 μg. The difference between the peak activities of 98.3% for the peptide mix and 99.9% for the crude filtrate was significantly different ($p = 0.001$) while that between 98.3% and 87.5% for the peptide mix was not significantly different ($p = 0.154$).

4. Discussion

We have shown that filtrates from C6/36 cells persistently infected with DENV-2 produce a new class of anionic, antiviral

peptides that can protect both insect (mosquito) cells and mammalian (Vero) cells from dengue virus. This confirms and extends the previous report for mosquito anti-DENV-2 activity of 5-kDa filtrates from C6/36 cells persistently infected with DENV-2 and it explains the loss of activity after proteinase-K treatment (Kanthong et al., 2010). We have also shown that these antiviral peptides have significant but slightly lower activity against 3 other DENV serotypes (i.e., 97.6% protection against DENV-1 and -2 and 94% against DENV-4 compared with 98.8% against DENV-2), suggesting that protection was at least partially serotype specific. This raises the question (remains to be answered) as to whether persistent infections of C6/36 cells with those serotypes would give reciprocal results for each. If the answer turned out to be affirmative, it would raise additional questions as to the reason for the specificity. One possibility might be variation in composition of the antiviral peptide mix (see Section 4).

The reason for the difference in the dose response curves for the 4-peptide mix and the crude filtrate is not known. One possible explanation for the decrease in specific activity with increasing concentration might have been that the ratio of the 4 peptides in the mixture was not optimized for maximum activity against DENV-2. For example, various combinations of dual-peptide mixtures at a ratio of 1:1 gave variable levels of anti DENV-2 protection (Additional file 4). Another possibility may have been that the crude filtrate contained other potentiating co-factor(s) that were missing in our synthetic peptide preparations. For example, a very preliminary test (not shown) revealed that addition of 1 mM of EDTA to the crude filtrate removed its antiviral activity. Optimization of both peptide ratios and a co-factor(s) might be required to obtain maximum specific activity from the synthetic peptide mix. Possibly related to this issue of complexity of the crude filtrate is the puzzling phenomenon that Vero cell titers from supernatant medium of C6/36 cells persistently infected with DENV-2 decrease with passage number and can be restored by washing while infectivity for C6/36 cells remains stable without washing (Kanthong et al., 2010). Despite the fact that these questions require further investigation, it is clear that the newly discovered peptides have potent antiviral activity for all 4 dengue serotypes.

Other antiviral compounds previously reported from insects include alloferons (Chernysh et al., 2002) with molecular weights around 1.2 kDa and a hydrophobic antiviral protein (AVP) of 3.2 kDa reported from C6/36 cells persistently infected with Sindbis virus (Luo and Brown, 1993; Riedel and Brown, 1979). Although there are many publications on cationic antimicrobial peptides from insects and insect cells and even some from *Aedes albopictus*, very few of these have been reported to have antiviral activity, and when they do have, the antimicrobial activity is directly against the target microbe rather than indirectly via host immune induction. Therefore, only one of these, a cercropin-like peptide recently described from *Aedes aegypti* will be discussed here (Luplertlop et al., 2011).

Alloferons are slightly cationic, nonglycosylated peptides of 12–13 amino acids that have been isolated from hemolymph of insects (stimulated with dead bacteria) and also have indirect, cytokine-like antiviral and antitumor properties (Chernysh et al., 2002). Synthetic alloferon-1 stimulated natural killer lymphocyte activity and interferon (IFN) production *in vitro* in mammalian cells, and IFN production and antiviral activity for influenza A and B *in vivo* in mice. Although alloferons also originate from Dipterans, their cationic character, sequence length and consensus sequence differ from those of the viprolaxikine peptides.

Like viprolaxikine with DENV-2, an uncharacterized antiviral protein (AVP) reported from C6/36 cells persistently infected with Sindbis virus (family *Togaviridae*) had the ability to protect C6/36 cells (U4.4 clone only) from Sindbis virus infection (Luo and Brown, 1993). Curiously, the cells remained protected from Sindbis virus for 10 months of continual subculture during which time they also produced AVP continuously, indicating that the AVP originated from a host gene rather than a Sindbis virus gene. Thus, the cells became permanently altered after exposure to AVP, much in the manner of cells whose fates are altered by exposure to developmental peptides. Unlike viprolaxikine, AVP did not protect mammalian cells (BHK-21 hamster kidney cells) against Sindbis virus (Condreay and Brown, 1988). A substance similar to this AVP has also been reported from mosquito cells infected with Semliki Forest virus (SFV) (family *Togaviridae*) but not from mosquito cells infected with Kunjin virus (family *Flaviviridae*) or Bunyamwera virus (family *Bunyaviridae*), leading to the suggestion that AVPs were unique to the *Togaviridae* (Newton and Dalgarno, 1983). The occurrence of the viprolaxikine peptides counters this suggestion. Since these two AVPs have not yet been characterized, they cannot be easily compared with the viprolaxikine peptides, but their specificity in protecting only insect cells and the unique property of permanent alteration of treated C6/36 cells suggest that they differ from the viprolaxikine peptides. However, confirmation must await their characterization.

More recently, two antiviral peptides have been reported from mosquito salivary glands. One is a cercropin-like, 59 amino-acid peptide (AAEL000598) from *Aedes aegypti* reported to have antiviral activity against both DENV and Chikungunya virus (Luplertlop et al., 2011). Its larger size and cationic nature distinguish it from the viprolaxikine peptides. The other is a cysteine-rich, anionic peptide of 113 amino acids (*Cx*Vago) from the mosquito (*Culex quinquefasciatus*) that has been shown to be an orthologue of the *Drosophila* peptide *Dm*Vago (Paradkar et al., 2012). The evidence suggests that it functions as an IFN-like antiviral cytokine. Given its molecular weight, size and structure (including eight conserved cysteine residues forming a VWC domain), this peptide is also unlike the viprolaxikine peptides, except for its anionic nature and indirect cytokine-like function.

A final question is whether the viprolaxikine peptides are the product of the DENV genome or the host mosquito cells. This is not a trivial question in the light of reports that the DENV envelope protein or portions of it can protect BHK-21 (Baby Hamster Kidney) cells from DENV-2 infection. Specifically, synthesized peptides of 29 amino acids (419–447) derived from the "stem" of dengue virus type 2 (DENV-2) envelope (E) 34 protein inhibited DENV-2 infectivity by interfering with fusion between the virus and the host cell membrane (Schmidt et al., 2010). The 29-aa fragments had molecular weights in the range of approximately 3000–4000 kDa (actual for DENV-2 = 2940) which would fit with the membrane filter size used to produce our product from C6/36 cells. Further, they found that the key portion of the molecule for activity consisted of 7 amino acids at the C terminal end (441–447), although they did not test 7-amino-acid peptides for activity. For DENV-2 the 7 C-terminal amino acids are GAIYGAA (gly–ala–ile–tyr–gly–ala–ala) which would give a molecular weight of 620 Da, so that neither the sequence or molecular weight or charge correspond to those of the viprolaxikine peptides. In addition, a BLASTn search of dengue virus group sequences revealed no significant similarity to the viprolaxikine peptides. Further, the activity of viprolaxikine did not correspond to that described for the synthetic envelope peptides, since the latter required only 18 min rather than 48-h host-cell exposure to obtain maximum antiviral activity. In summary, it is clear that the peptides from the viprolaxikine fraction were not derived from dengue virus but from the host cell genome.

In conclusion viprolaxikine filtrates from C6/36 cells persistently infected with DENV-2 virus contained 3–4 related anionic peptides of 7 amino acids each that could protect both insect cells and Vero cells from DENV-2 infection via a cytokine-like Vero cell induction response. These characteristics differ from those of other known, insect-derived, antiviral substances sufficiently to

constitute a new class of anionic, antiviral peptides. Given their small size (i.e., probable lack of antigenicity) and lack of visible negative effects on Vero cell cultures, it may be of interest to explore the possibility of using viprolaxikine peptides for anti-dengue therapy. Finally, the existence of these peptides suggests that it may be fruitful to screen insect cells persistently infected with other viruses for similar, very low molecular weight antiviral peptides. In these assays, it might be important to include a pre-exposure step before viral challenge to reveal activity or to obtain maximum activity.

Acknowledgements

This work was supported by a scholarship to CL from the Development and Promotion of Science and Technology Talented Project, Ministry of Education, Government of Thailand, by Mahidol University and by the Thai National Center for Genetic Engineering and Biotechnology (BIOTEC), Thai National Science and Technology Development Agency.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.04.011.

References

Chen, W.J., Chen, S.L., Fang, A.H., 1994. Phenotypic characteristics of dengue 2 virus persistently infected in a C6/36 clone of *Aedes albopictus* cells. Intervirology 37, 25–30.

Chen, L.K., Liao, C.L., Lin, C.G., Lai, S.C., Liu, C.I., Ma, S.H., Huang, Y.Y., Lin, Y.L., 1996. Persistence of Japanese encephalitis virus is associated with abnormal expression of the nonstructural protein NS1 in host cells. Virology 217, 220–229.

Chernysh, S., Kim, S.I., Bekker, G., Pleskach, V.A., Filatova, N.A., Anikin, V.B., Platonov, V.G., Bulet, P., 2002. Antiviral and antitumor peptides from insects. Proc. Natl. Acad. Sci. USA 99, 12628–12632.

Condreay, L.D., Brown, D.T., 1988. Suppression of RNA synthesis by a specific antiviral activity in Sindbis virus-infected *Aedes albopictus* cells. J. Virol. 62, 346–348.

Cruz, D.J., Shin, H.J., 2007. Application of a focus formation assay for detection and titration of porcine epidemic diarrhea virus. J. Virol. Methods 145, 56–61.

Debnath, N.C., Tiernery, R., Sil, B.K., Wills, M.R., Barrett, A.D., 1991. In vitro homotypic and heterotypic interference by defective interfering particles of West Nile virus. J. Gen. Virol. 72 (Pt. 11), 2705–2711.

Huang, A.S., Baltimore, D., 1970. Defective viral particles and viral disease processes. Nature 226, 325–327.

Kanthong, N., Khemnu, N., Sriurairatana, S., Pattanakitsakul, S.N., Malasit, P., Flegel, T.W., 2008. Mosquito cells accommodate balanced, persistent co-infections with a densovirus and dengue virus. Dev. Comp. Immunol. 32, 1063–1075.

Kanthong, N., Laosutthipong, C., Flegel, T.W., 2010. Response to dengue virus infections altered by cytokine-like substances from mosquito cell cultures. BMC Microbiol. 10, 290.

Lancaster, M.U., Hodgetts, S.I., Mackenzie, J.S., Urosevic, N., 1998. Characterization of defective viral RNA produced during persistent infection of Vero cells with Murray Valley encephalitis virus. J. Virol. 72, 2474–2482.

Luo, T., Brown, D.T., 1993. Purification and characterization of a Sindbis virus-induced peptide which stimulates its own production and blocks virus RNA synthesis. Virology 194, 44–49.

Luo, T., Brown, D.T., 1994. A 55-kDa protein induced in *Aedes albopictus* (mosquito) cells by antiviral protein. Virology 200, 200–206.

Luplertlop, N., Surasombatpattana, P., Patramool, S., Dumas, E., Wasinpiyamongkol, L., Saune, L., Hamel, R., Bernard, E., Sereno, D., Thomas, F., Piquemal, D., Yssel, H., Briant, L., Misse, D., 2011. Induction of a peptide with activity against a broad spectrum of pathogens in the *Aedes aegypti* salivary gland, following infection with dengue virus. PLoS Pathog. 7, e1001252.

Newton, S.E., Dalgarno, L., 1983. Antiviral activity released from *Aedes albopictus* cells persistently infected with Semliki forest virus. J. Virol. 47, 652–655.

Paradkar, P.N., Trinidad, L., Voysey, R., Duchemin, J.B., Walker, P.J., 2012. Secreted Vago restricts West Nile virus infection in *Culex* mosquito cells by activating the Jak-STAT pathway. Proc. Natl. Acad. Sci. USA 109, 18915–18920.

Randolph, V.B., Hardy, J.L., 1988. Phenotypes of St Louis encephalitis virus mutants produced in persistently infected mosquito cell cultures. J. Gen. Virol. 69 (Pt. 9), 2199–2207.

Riedel, B., Brown, D.T., 1979. Novel antiviral activity found in the media of Sindbis virus-persistently infected mosquito (*Aedes albopictus*) cell cultures. J. Virol. 29, 51–60.

Schmaljohn, C., Blair, C.D., 1977. Persistent infection of cultured mammalian cells by Japanese encephalitis virus. J. Virol. 24, 580–589.

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

Sinarachatanant, P., Olson, L.C., 1973. Replication of dengue virus type 2 in *Aedes albopictus* cell culture. J. Virol. 12, 275–283.

Vlaycheva, L.A., Chambers, T.J., 2002. Neuroblastoma cell-adapted yellow fever 17D virus: characterization of a viral variant associated with persistent infection and decreased virus spread. J. Virol. 76, 6172–6184.

Von Magnus, P., 1951. Propagation of the PR8 strain of influenza A virus in chick embryos. III. Properties of the incomplete virus produced in serial passages of undiluted virus. Acta Pathol. Microbiol. Scand. 29, 157–181.

Westaway, E., 1997. Taxonomy and evolutionary relationships of flaviviruses. In: Gubler, D.J. (Ed.), Dengue and Dengue Hemorrhagic Fever. CAB International, New York, pp. 160–162.

WHO, 2002. Dengue and Dengue Hemorrhagic Fever. World Health Organization, Geneva, Switzerland, p. Fact sheet no.117.