Antiviral Research 104 (2014) 7–14

Contents lists available at ScienceDirect

Antiviral Research

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

Peptide inhibitor of Japanese encephalitis virus infection targeting envelope protein domain III

Xiangyang Zu [a], Yang Liu [a], Shaobo Wang [a], Rui Jin [a], Zheng Zhou [a], Haibin Liu [a], Rui Gong [b], Gengfu Xiao [a,*], Wei Wang [a,*]

[a] State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, Hubei 430071, China
[b] Center for Emerging Infectious Diseases, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, Hubei 430071, China

ARTICLE INFO

Article history:
Received 4 September 2013
Revised 13 January 2014
Accepted 15 January 2014
Available online 24 January 2014

Keywords:
Japanese encephalitis virus
Phage display peptide library
Envelop protein domain III
Antiviral peptide

ABSTRACT

Japanese encephalitis virus (JEV) is a major cause of acute viral encephalitis in both humans and animals. Domain III of the virus envelope glycoprotein (E DIII) plays an important role in the interaction of viral particles with host cell receptors to facilitate viral entry. Intervention of the interaction between E DIII and its cognate host cell receptor would provide an important avenue for inhibiting JEV infection. A phage display peptide library was therefore panned against E DIII, which resulted in the identification of several peptides. One peptide, named P3, inhibited JEV infection of BHK-21 cells with an IC_{50} of ~1 μM and an IC_{90} at ~100 μM. Further characterization revealed that P3 bound to E DIII with a K_d of 6.06×10^{-6} M and inhibited JEV infection by interfering with viral attachment to cells. Based on in silico prediction by ZDOCK, P3 was found to interact with E DIII via a hydrophobic pocket, which was confirmed by the binding assay of P3 to the V357A mutant. P3 was hypothesized to bind to E DIII by interacting with the sties adjacent to the BC and DE loops, which might interfere with the binding of JEV to cellular receptors, thus impeding viral infection. This newly isolated peptide may represent a new therapeutic candidate for treatment of JEV.

1. Introduction

Japanese encephalitis, caused by the Japanese encephalitis virus (JEV), is one of the most important encephalitides in the world, especially in eastern and southeastern Asia, where it causes 30,000–50,000 infections with 10,000–15,000 deaths per year (Erlanger et al., 2009; Misra and Kalita, 2010; Solomon, 2004). JEV belongs to the genus *Flavivirus* in the family Flaviviridae and is transmitted through a zoonotic cycle among mosquitoes, pigs and water birds. Humans are infected and are a dead-end host because of low-level and transient viremia (Ghosh and Basu, 2009; Medigeshi, 2011). Over the past two decades, JEV has also spread to new geographic locations, such as Australia (Mackenzie et al., 2002; Pyke et al., 2001). This virus is now recognized as an emerging pathogen of global public health significance (Mackenzie et al., 2004; van den Hurk et al., 2009). A WHO-approved JEV vaccine for pediatric use is of limited utility because of potential risks, and there are no clinically approved antiviral drugs specifically targeting JEV infection. Therefore, the investigation and identification of targets and inhibitors of JEV are highly desirable.

Receptor binding is a crucial step for virion internalization into host cells, and the interaction between viral proteins and the host cell via specific receptors provides an important avenue for therapeutic intervention (Geiss et al., 2009). To date, however, JEV receptor molecules have not been conclusively identified. Because it has a diverse set of hosts, JEV utilizes different receptors to complete adhesion to different cells, such as heat shock protein 70 in Neuro2a cells (Das et al., 2009) and vimentin in porcine kidney cells and BHK-21 cells (Das et al., 2011; Liang et al., 2011). Although the receptor for JEV has not yet been clearly identified, the E glycoprotein has been identified as the major antigenic determinant on flavivirus particles and mediates binding and fusion during viral entry (Kuhn et al., 2002). E proteins exist as homodimers on the surface of the virus. Among the three domains of an E protein, DI connects the DII and DIII domains, and DII contains fusion polypeptides that facilitate virus-cell membrane fusion. In addition, DIII has been proposed to act as the binding region for the cellular receptor (Bhardwaj et al., 2001; Heinz and Allison, 2001; Mukhopadhyay et al., 2005). Therefore, blocking the E DIII-receptor binding sites could be critical for inhibiting the initial step of JEV infection.

* Corresponding authors. Address: State Key Laboratory, Wuhan Institute of Virology, Chinese Academy of Science, Xiaohongshan 44, Wuhan 430071, China. Tel.: +86 027 87198685 (G. Xiao). Tel.: +86 027 87198352 (W. Wang).
E-mail addresses: gfxiao@wh.iov.cn (G. Xiao), wangwei@wh.iov.cn (W. Wang).

0166-3542/$ - see front matter © 2014 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.antiviral.2014.01.011

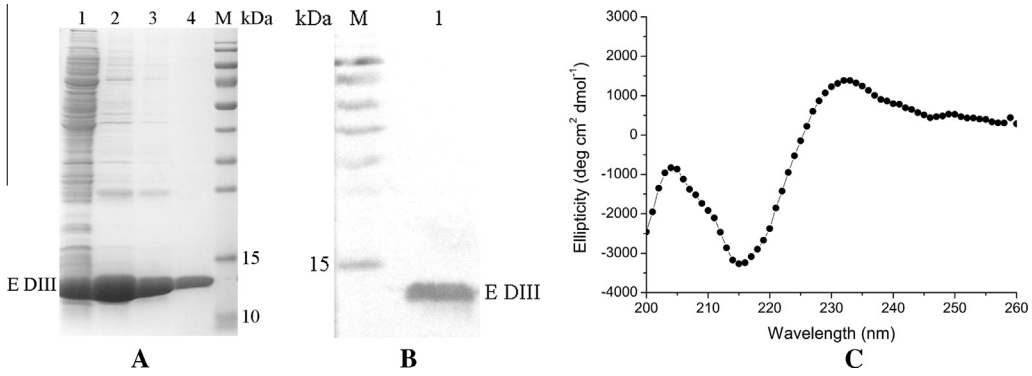

Fig. 1. (A) Purification of E DIII. Lane 1: post-sonication supernatant fraction containing soluble E DIII protein. Lanes 2–4: elution of E DIII with 100, 150 and 200 mM imidazole in elution buffer. Lane M: protein molecular marker; (B) WB detection of E DIII. Lane M: protein molecular marker; Lane 1: purified E DIII. (C) The secondary structure of E DIII measured by CD.

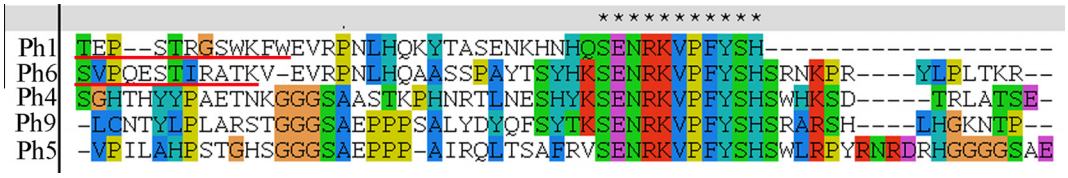

Fig. 2. Amino acid sequence alignment of the displayed peptides that occurred at higher frequencies during E DIII-specific phage bio-panning. *Highly conserved amino acid.

Phage display technology allows rapid high-throughput screening of one billion clone-peptide libraries and has become a powerful approach to identify and improve peptide molecules for pharmaceuticals (Castel et al., 2011; Scott and Smith, 1990; Sidhu, 2000). In this study, we utilized E DIII as bait to screen a phage display peptide library to identify peptides that interact with the JEV E DIII. We obtained and characterized a peptide, designated P3. P3 binds to the N terminus of E DIII, blocks virus attachment to cells and inhibits viral infection with a 50% inhibition concentration (IC_{50}) of ~1 μM. The information obtained in this study may contribute a peptide-based candidate drug for the development of an anti-JEV small molecule drug.

2. Materials and methods

2.1. Cells and virus

Baby hamster kidney (BHK-21) cells and African green monkey kidney (Vero) cells were cultured in Dulbecco's modified Eagle medium (DMEM) (HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA) at 37 °C in a 5% CO_2 incubator. The JEV strain AT31 (GenBank accession No. AB196923, kindly provided by Dr. Bo Zhang, Wuhan Institute of Virology, CAS, China) was propagated in Vero cells and titrated by a plaque assay in BHK-21 cells.

2.2. Expression, mutation and purification of E DIII protein

The sequence encoding the E DIII protein (E protein 292–402) was amplified by PCR from the pPreM-E JEV plasmid (kindly provided by Dr. Shengbo Cao, Huazhong Agricultural University, China) and cloned into the pET-22b(+) vector (Novagen, USA). The E DIII expression plasmid was transformed into the *Escherichia coli* strain BL21 (DE3) and induced by adding IPTG at a final concentration of 1 mM at 25 °C for 16 h. The bacterial pellets were harvested, sonicated and centrifuged. The supernatant was loaded onto a nickel column, and the bound protein was eluted with a gradient concentration of imidazole buffer. The fractions containing soluble E DIII were dialyzed against phosphate-buffered saline (PBS) at 4 °C overnight. E DIII protein was detected using mouse anti-JEV E protein serum as previously described (Li et al., 2010) in Western-blot (WB) analyses.

The L294A/L296A and V357A E DIII variants were constructed from the template pET-22b(+)-E DIII using the East Mutagenesis System Kit (TransGen Biotech, China) with the following primers: forward primer (L294A/L296A), GGCTGACAAAGCGGCTGCGAAA GGCAC; reverse primer (L294A/L296A), CGCAGCCGCTTTGTCA GCCATGGCATG; forward primer (V357A), CGGCTGGTGACAGCG AACCCCTTCG and reverse primer (V357A), CGCTGTCACCAGCCGCC CAACGG. The E DIII variant proteins were expressed and purified as described above.

2.3. Circular dichroism spectroscopy

The secondary structure of the E DIII protein and variants were determined by circular dichroism (CD) spectroscopy. The purified protein was dissolved in PBS at a final concentration of 0.3 mg/ml, and the CD spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics, UK). Wavelength spectra were recorded at 25 °C using a 1 mm path-length cuvette in the range of 200–260 nm. The scan rate was 1 nm/s, and the final spectrum was averaged over three scans. CD spectra of the appropriate buffers were recorded and subtracted from the protein spectra. The mean residue ellipticity $[\theta]$ (deg cm^2 dmol^{-1}) was calculated using the formula:

$$[\theta] = \theta_{obs}/(c \times l \times M)$$

where c is the protein concentration, l is the path length and M is the mean residue molecular mass.

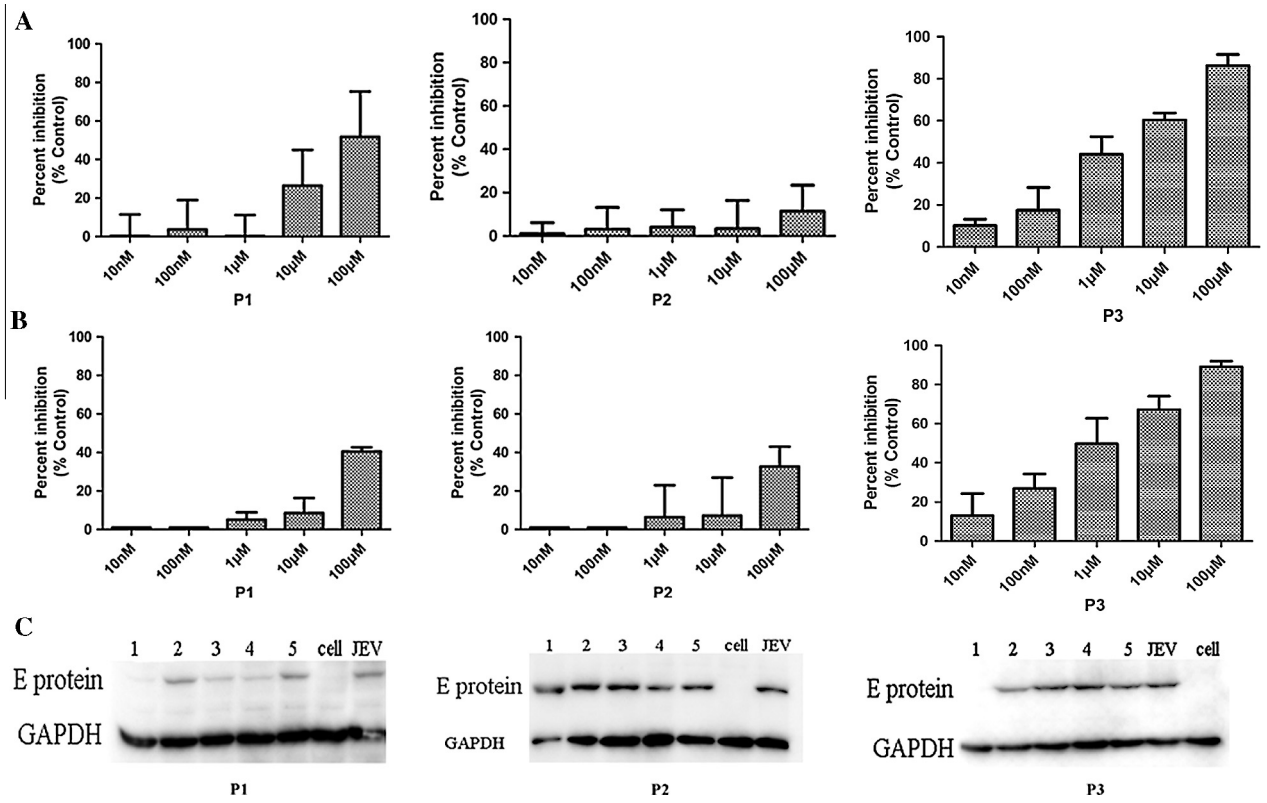

Fig. 3. Peptide-inhibition of JEV infection in BHK-21 cells at 24 h. (A and B) The inhibitory ability of the peptides was detected by plaque formation assay (A) and qRT-PCR (B). (C) Viral infection was detected by WB using E protein antibody. 1–5, respectively, represent the following concentrations of peptides: 100 μM, 10 μM, 1 μM, 100 nM and 10 nM. All data were obtained in triplicate. The results represent the means ± SD of triplicate measurements.

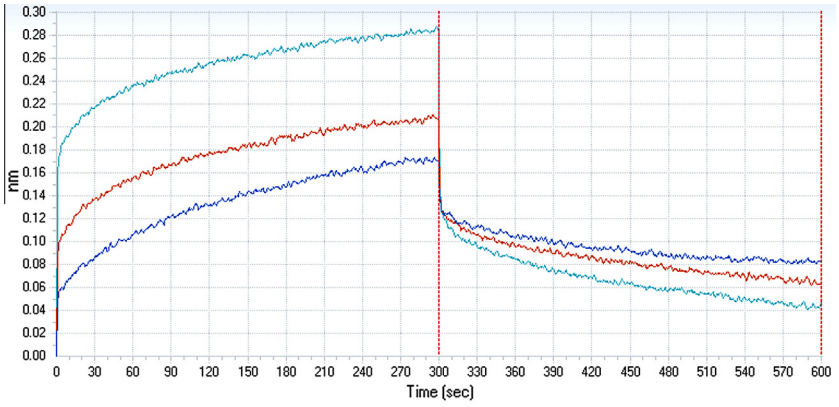

Fig. 4. Binding affinity of P3 to E DIII. P3 molecules were immobilized onto biosensors and the binding of E DIII was assessed at the following concentrations: 10 μM (blue), 20 μM (red) and 40 μM (cyan). The association and dissociation curves under increasing relative concentrations of E DIII to P3 are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Peptide screening against E DIII

The Ph.D.-12™ phage display peptide library was used for screening E DIII-binding peptides using the bio-panning procedure according to the manufacturer's protocol (NEB, USA). After five rounds of bio-panning, 48 plaques were stabbed and amplified. The selected phages, combined with E DIII, were detected by ELISA with BSA used as a control. Single-stranded DNA from the amplified selected phage clones was isolated and sequenced by Sangon Sequencing (Shanghai, China). The selected peptides were synthesized by C-terminal amidation by HD Biosciences (Shanghai, China). The purity of the peptides was 98%. The peptides were dissolved in PBS to obtain a 10 mM stock solution, and the stock solution was stored at −80 °C.

2.5. Antiviral activity

2.5.1. Inhibition of JEV infection of BHK-21 cells by peptides

Peptides at different concentrations ranging from 10 nM to 100 μM were incubated with JEV at a multiplicity of infection (MOI) of 0.1 at 4 °C for 1 h. Cells were subsequently infected with the JEV–peptide mixture at 37 °C for 1 h and with JEV without peptides as control. Then, the JEV–peptide mixture and JEV solution were discarded. The cells were washed three times with PBS and incubated in DMEM medium with 2% FBS at 37 °C (Bai et al., 2007; Hung et al., 2003). At 24 and 48 h post infection (p.i.), supernatants from cells infected with JEV were collected for viral titer determination by plaque assays on BHK-21 cells. The suppression effects of the peptides were determined using the formula given below:

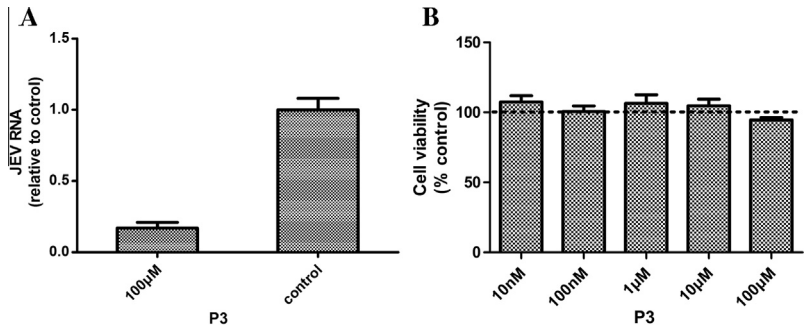

Fig. 5. (A) P3 inhibits JEV attachment to BHK-21 cells. (B) P3 is not cytotoxic. BHK-21 cells were exposed to P3 from 10 nM to 100 μM for 72 h. Each sample was assayed in duplicate, and the data are presented as the mean ± SD.

Table 1
Enrichment and analysis of higher frequency E DIII-specific phage by bio-panning.

Phage	Phage clones	Frequency	Deduced amino acid sequences	No. of amino acid residues
Ph1	51, 52, 53, 58, 514, 519, 521, 62, 64, 613, 620, 622, 623	13/44	TEPSTRGSWKFWEVRPNLHQKYTASENKHNHQSENRKVPFYSH	43
Ph6	511, 516, 520, 523, 69	5/44	SVPQESTIRATKVEVRPNLHQAASSPAYTSYHKSENRKVPFYSHSRNKPRYLPLTKR	57
Ph4	57, 512, 66, 612	4/44	SGHTHYYPAETNKGGGSAASTKPHNRTLNESHYKSENRKVPFYSHSWHKSDTRLATSE	58
Ph9	517, 518, 614	3/44	LCNTYLPLARSTGGGSAEPPPSALYDYQFSYTKSENRKVPFYSHSRARSHLHGKNTP	57
Ph5	59, 510	2/44	VPILAHPSTGHSGGGSAEPPPAIRQLTSAFRVSENRKVPFYSHSWLRPYRNRDRHGGGGSAE	62

Inhibition % = (1 − the number of plaques produced with peptide treatment/the number of plaques produced without peptide treatment) × 100. The infected cells were processed and analyzed by quantitative real-time PCR (qRT-PCR) as previously reported (Jia et al., 2008; Jin et al., 2013). Briefly, total RNA was isolated using TRIzol reagent (Invitrogen, USA) and reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, USA) following the manufacturer's directions. The resulting cDNA was then amplified using Fast SYBR Green Master Mix (ABI, USA) and a quantitative real-time PCR system (ABI Stepone, USA). The following two pairs of primers were used: JEV forward primer, TACAACATGATGGGAAAAAGAGAGAAGAA; reverse primer, CTTGCT TTCCTGCTATGTCACGGAGGA; β-actin forward primer, TCATCAC TATTGGCAACGAGC and reverse primer, AACAGTCCGCCTAGAAG CAC for the β-actin gene of BHK-21 cells. The percentage inhibition of infection was determined using the formula given below: Inhibition % = $(1 - 2^{-\Delta\Delta Ct}) \times 100$. The cell lysates were evaluated by monitoring the amount of viral E protein that reacted with mouse anti-JEV E protein serum in Western-blot analyses.

2.5.2. Blocking of JEV binding to BHK-21 by P3

P3 was incubated with JEV at 4 °C for 1 h with a final concentration of 100 μM, and then the JEV–peptide mixture was added to the cells (MOI of 1) at 4 °C for 1 h, with JEV without the peptide as a control. The unattached viruses were washed away with cold PBS three times at 4 °C, and the samples were immediately processed for total RNA extraction (Bai et al., 2007; Hung et al., 2003). The blocking ratio for JEV binding to BHK-21 cells was quantified by qRT-PCR as described above.

2.6. Cytotoxicity assay

Peptides were incubated with BHK-21 cells for 24, 48 and 72 h in different concentrations from 10 nM to 100 μM. After incubation, the medium was removed, and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (Sigma, USA) was added at a final concentration of 5 mg/ml at 37 °C for 4 h. The supernatant was then removed, and 50 μl of DMSO was added to dissolve the formazan product. The plates were read at 490 nm using a Thermo Multi-skan ELISA reader (MA, USA).

2.7. Peptide: E DIII biolayer interferometry binding assay

Real-time binding assays between peptides and the E DIII protein and each variant were performed using biolayer interferometry on an Octet QK system (Fortebio, USA) according to previously reported methods (Costin et al., 2010). Binding kinetics were calculated using the Octet QK software package, which fit the observation to a 1:1 model to calculate the association and dissociation rate constants. Binding affinities were calculated as the kinetic dissociation rate constant divided by the kinetic association rate constant.

2.8. Molecular modeling and docking of the E DIII/P3 complex

The NMR crystal structure of E DIII (PDB 1PJW) was used to build a model. A homology-based model of P3 was built in http://zhanglab.ccmb.med.umich.edu/I-TASSER (Roy et al., 2010; Zhang, 2008). The model with the lowest objective function of ten models was selected. The ZDOCK 3.0.2 program was employed to model the interaction between the E DIII and P3 proteins. The ZDOCK program can be found in http://zdock.umassmed.edu (Pierce et al., 2011). The resulting model was represented by Chimera 1.8rc.

2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism (version 5; GraphPad Software).

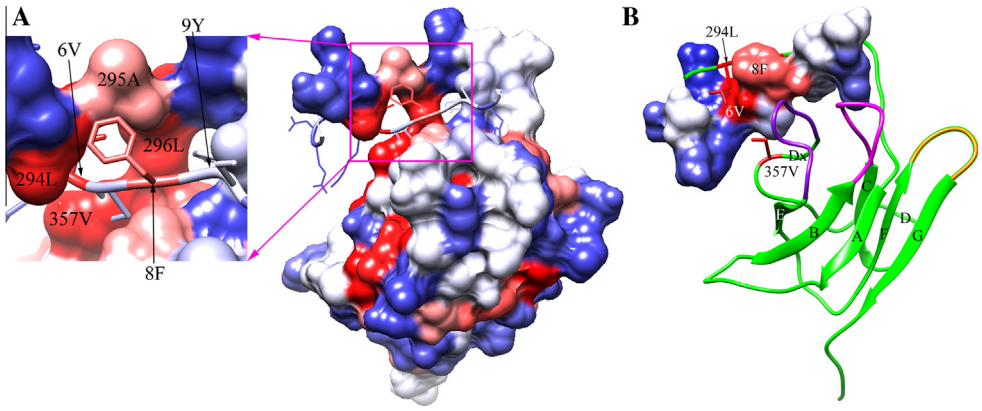

Fig. 6. Molecular modeling and docking of E DIII/P3 complex. (A) The surface representation of E DIII/P3 complex. The E DIII surface and P3 are colored by amino acid hydrophobicity, from blue for the most hydrophilic, to white, to red for the most hydrophobic. (B) The ribbon diagram of the E DIII/P3 complex. The P3 surface is colored by amino acid hydrophobicity, from blue for the most hydrophilic, to white, to red for the most hydrophobic. The BC loop is magenta, the DE loop is purple and the FG loop is orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Peptides synthesized and tested for inhibitory activity.[a]

Peptide	No. of amino acid residues	Amino acid sequence	MW	pI	GRAVY
P1	12	TEPSTRGSWKFW	1481.6	8.41	−1.325
P2	12	SVPQESTIRATK	1316.4	8.46	−0.792
P3	12	SENRKVPFYSHS	1450.5	8.33	−1.408

[a] The molecular weight (MW), pI and grand average of hydropathicity (GRAVY) were predicted with the ProtParam algorithm.

3. Results

3.1. Expression, purification and identification of E DIII

The flavivirus envelope protein domain III (E DIII) was reported to be expressed in bacteria either as a soluble protein (Bhardwaj et al., 2001; Volk et al., 2004) or as a refolded molecule from inclusion bodies (Jaiswal et al., 2004). We expressed the JEV E DIII sequence within the pET22b vector in *E. coli* BL21 (DE3) (Wu et al., 2003). The E DIII protein was found predominantly in the supernatant of the bacterial lysate (Fig. 1A), which indicated that E DIII was soluble. With increasing concentrations of imidazole, E DIII was eluted at 200 mM imidazole with high purity using Ni–NTA affinity chromatography (Fig. 1A). E DIII was detected by WB using anti-JEV E protein serum, which showed a single band with a molecular size similar to the theoretical value (12.9 kDa) of E DIII (Fig. 1B).

The JEV E DIII 3D structure suggests that the protein has a β-barrel type structure composed of six antiparallel β-strands resembling the immunoglobulin-like constant domain (Luca et al., 2012; Wu et al., 2003). The secondary structure of the purified E DIII protein was evaluated by CD spectroscopy (Fig. 1C). There is a small, positive ellipticity at 232 nm and a strong, negative ellipticity at 216 nm, indicating the presence of a large amount of β-strand structure. Furthermore, the spectra of E DIII show a negative ellipticity at approximately 200 nm, indicating the presence of α-turn structures. The CD results indicated that the secondary structure of the purified E DIII protein was similar to the WNV-E DIII and DENV-E DIII (Yu et al., 2004) structures, which suggested E DIII was folded correctly.

3.2. Identification of peptides binding to E DIII by screening of the phage display library

Forty-eight phages panned using the Ph.D.-12™ phage display peptide library showed significant binding to the E DIII in ELIAS (data not shown). The coding sequences of 44 positive clones were determined (four clones failed to be sequenced), and the corresponding peptide sequences were deduced. The lengths of the peptides were varying (data not shown). According to the manufacturer's protocol, the libraries often contain a small percentage (<1%) of clones with multiple inserts of the randomized region. Preferential selection and amplification of these clones may occur when panning against targets whose ligand specificity spans a length greater than that specified by the insert. The five phage sequences encountered at high frequency (Ph) are listed in Table 1.

The principle of the Ph.D.-12™ phage display peptide library is that the peptides displayed at the N terminus of the phage are the sites of binding to the bait protein. Therefore, the 12 amino acids at the N-terminus of the two polypeptides that were displayed by the phages encountered at high frequency (Ph1 and Ph6) were synthesized (Fig. 2, highlighted with a red line) and named P1 and P2 (Table 2). Furthermore, the homologous alignment of peptides displayed by frequently encountered phages was analyzed, resulting in the identification of a highly conserved 12 amino acid peptide sequence (Fig. 2) that was also synthesized and named P3 (Table 2). Thus, three peptides were identified and examined for antiviral activity.

3.3. Inhibition of JEV infectivity by peptides

The inhibitory ability of the peptides against JEV infection were determined by plaque assays, qRT-PCR and WB at 24 h p.i. (Fig. 3). The plaque assay results (Fig. 3A) showed that P3 significantly inhibited JEV infection in a concentration-dependent manner, whereas P1 inhibited JEV infection only at high concentrations. P2 did not show any inhibitory effect. The results obtained by qRT-PCR corroborated the plaque assay results (Fig. 3B). The IC_{50} of P3 against JEV was 1.42 ± 0.41 μM and 1.12 ± 0.38 μM, as measured by the plaque assay and qRT-PCR, respectively, and both assays had a similar IC_{90} of ∼100 μM. The inhibitory ability of P1 was not as strong as that of P3. The IC_{50} of P1 was ∼100 μM, which is 100-fold higher than the IC_{50} of P3. WB showed similar results to the plaque assay and qRT-PCR (Fig. 3C). The results as shown in

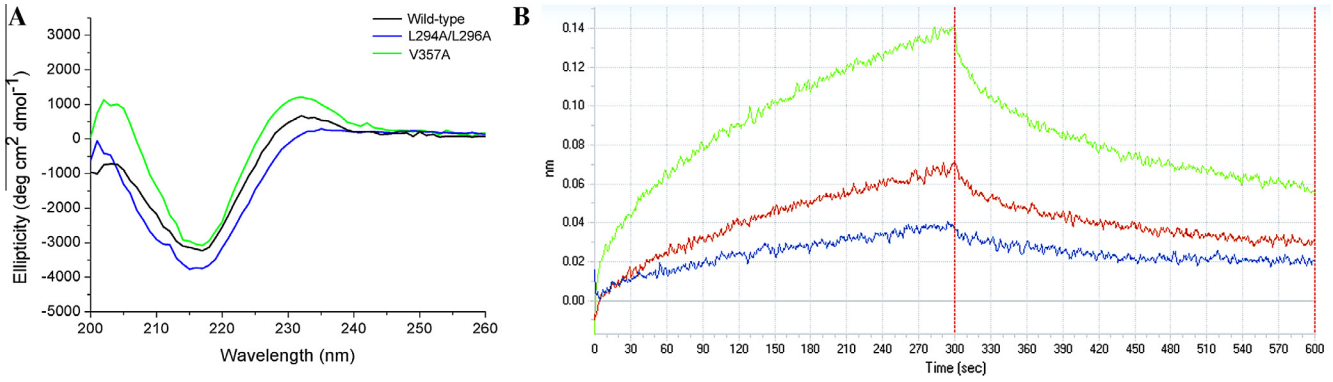

Fig. 7. (A) The secondary structure of E DIII and its variants measured by CD. (B) The binding affinity of P3 to E DIII V357A. P3 molecules were immobilized onto biosensors, and the binding of E DIII V357A was assessed at the following concentrations: 10 μM (blue), 20 μM (red) and 40 μM (green). The association and dissociation curves under increasing relative concentrations of E DIII V357A to P3 are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3 enable us to reasonably conclude that P3 could inhibit JEV infection at the viral, mRNA and protein levels.

3.4. P3 binding to JEV E DIII

Because phages were screened for binding to E DIII, biolayer interferometry was conducted to determine the binding affinity of the chemically synthesized peptides to the JEV E DIII protein. Amino-terminally biotinylated peptides were immobilized onto streptavidin biosensors and then the association and dissociation of E DIII with the immobilized peptides were monitored. The interactions of E DIII with peptides at three different concentrations are shown (Fig. 4). The affinities of the peptides for E DIII were calculated using a 1:1 binding model. The results showed that P3 bound to E DIII with a K_d of 6.06×10^{-6} M. The association rate constant was $1.10 \times 10^{3} \pm 4.24 \times 10^{1}$ M^{-1} s^{-1} and the dissociation rate constant was: $6.67 \times 10^{-3} \pm 1.34 \times 10^{-4}$ s^{-1}, indicating that P3 has a higher affinity for E DIII.

3.5. P3 blocking JEV attachment

P3 exhibited strong inhibition at 24 h p.i. but showed little inhibition of viral infection at 48 h p.i. (data not shown), which indicated that P3 may play a role in the early stages of viral infection. Furthermore, P3 had high affinity to E DIII, which plays an important role in the interaction of JEV with the cell receptor. Thus, it was inferred that P3 interfered with JEV binding to the cell receptor. The viruses that bound to the cell surface were quantified by qRT-PCR at 4 °C. The results demonstrated that 100 μM P3 reduced 81.76 ± 7.53% of JEV attachment to BHK-21 cells (Fig. 5A), indicating that P3 could substantially block JEV attachment to cells.

To address whether the peptides were cytotoxic and thereby whether they nonspecifically inhibited JEV entry or replication, BHK-21 cells were exposed to P3. The results of the cell viability assay at 72 h are shown in Fig. 5B, and these data demonstrate that P3 is not cytotoxic.

3.6. Modeling of the E DIII/P3 complex

A model of the E DIII/P3 complex was developed by molecular modeling based on the available E DIII structure (PDB 1PJW) (Wu et al., 2003), and the prediction of the P3 structure is displayed (Fig. 6). According to this model, on the surface of E DIII, three amino acids (294L, 295A and 296L) at the N terminus, together with 357V, form a hydrophobic pocket. Closely adjacent to the pocket, several hydrophilic amino acids and hydrophobic amino acids form a channel. The 6V and 8F residues of P3 bind to the E DIII hydrophobic pocket via a hydrophobic interaction. Notably, the 9Y residue of P3 integrated into the channel precisely. Therefore, P3 bound to the N terminus of E DIII via a hydrophobic interaction near the BC loop (B–C strand) and DE loop (D–E strand) of the protein (Fig. 6B).

To verify the model of the E DIII/P3 complex, the Leu residues at positions 294 and 296 and the Val residue at position 357 of E DIII were mutated to Ala (L294A/L296A and V357A) to destroy the structure of the hydrophobic pocket. The secondary structure of the variants was evaluated by CD spectroscopy (Fig. 7A). All mutations had minor changes compared with wild type E DIII and did not affect overall β-strand content.

The affinities of P3 for the variants were examined by biolayer interferometry. The results showed that the K_d of P3 to E DIII V357A was 2.85×10^{-5} M, the association rate constant was $1.36 \times 10^{2} \pm 6.99$ M^{-1} s^{-1}, and the dissociation rate constant was $3.88 \times 10^{-3} \pm 3.87 \times 10^{-5}$ s^{-1} (Fig. 7B), which showed that P3 had a lower affinity to the V357A mutant than to the wild type (6.06×10^{-6} M). However, P3 had a similar affinity to the L294A/L296A mutant at the same scale of 10^{-6} M (date not shown).

Based on the model of the E DIII/P3 complex, the 294L and 296L residues were distributed in the random coil of the E DIII N terminus with high flexibility and the 357V residue was located in the D β-strand with more stability. The 357V residue might play a key role in forming the hydrophobic pocket. The lower affinity to the E DIII V357A mutant indicated that the V357 residue was the most important site for P3 binding and that P3 could indeed bind to the hydrophobic pocket.

4. Discussion

In addition to targeting viral enzymes indispensable for replication, interference with the viral entry step has become an attractive therapeutic strategy in recent years (Altmeyer, 2004; Perera et al., 2008). JEV infects cells by receptor-mediated endocytic uptake and a low pH-triggered membrane fusion reaction (Heinz and Allison, 2000). The E glycoprotein interacts with cellular receptors and mediates virus-cell membrane fusion (Kuhn et al., 2002). Recently, inhibition of E glycoprotein interactions with cellular proteins has been explored as a strategy for intervention during flavivirus infection. As E DIII is predicted to be involved in receptor binding (Bhardwaj et al., 2001; Heinz and Allison, 2001; Mukhopadhyay et al., 2005), several studies have used E DIII as a cellular receptor antagonist to inhibit viral infection. For instance, expression of recombinant forms of DIII has successfully and

specifically blocked viral entry (Chin et al., 2007; Chiu and Yang, 2003; Chu et al., 2005; Fan et al., 2013; Liao and Kielian, 2005). Additionally, the peptide derived from the "stem" of the dengue virus type 2 (DENV-2) envelope protein can specifically bind to the post-fusion conformation to prevent virus-membrane fusion (Schmidt et al., 2010a). A further study showed the stem peptide from residues 419–447 of DENV-2 can inhibit all DENV serotypes, but the corresponding peptides derived from other related flaviviruses cannot (Schmidt et al., 2010b). The loop3 (DE loop) peptide derived from E DIII can prevent JEV infection by interfering with viral attachment to the cells (Li et al., 2012). Furthermore, some peptides possess inhibitory ability against dengue virus infection, and one of them was found to cross-inhibit WNV infection in Vero cells (Hrobowski et al., 2005).

In contrast to cellular receptor antagonists that have been previously reported, in our study, we used E DIII to screen against a phage display peptide library for amino acid sequences. The peptides could bind E DIII and block the key sites of the virus that interact with cellular receptors to inhibit viral infection. Three peptides were chemically synthesized and examined for antiviral activity, among which P3 most notably achieved levels of inhibition of JEV infection in BHK-21 cells that reached 90% at ~100 μM, with an IC_{50} at ~1 μM. Based on the structure of the P3/E DIII complex, P3 bound to the N terminus of E DIII via a hydrophobic interaction near the BC loop and DE loop regions. According to previous reports, the N terminal external loop region of E DIII is involved in serotype-specific binding to mosquito, but not mammalian, cells for dengue virus type 2 (Hung et al., 2003). The BC loop in the WNV clearly plays a significant role in E protein domain III folding, virus infectivity, virulence and antigenicity (Zhang et al., 2010). The loop3 (DE loop) from the JEV E DIII protein is a protective peptide when administered before virus challenge (Li et al., 2012). Therefore, the BC and DE loops are the most important sites involved in E DIII binding to the cellular receptor. We have reason to believe that P3 binds to the N terminus of E DIII near the BC loop and DE loop, blocking JEV attachment and inhibiting viral infection. Moreover, many of the most potently neutralizing monoclonal antibodies recognize the lateral ridge and "A" strand of E DIII. Antibodies E3 (G302) and E3.3 (I337, F360, R387) recognize the DIII lateral ridge of the BC loop, DE loop and FG loop (Goncalvez et al., 2008; Luca et al., 2012), especially F360 near by V357. P3 may inhibit JEV infection in the same way as the neutralizing monoclonal antibodies, as they both blocked the antigenic epitope on E DIII.

In conclusion, to our knowledge, this is the first report that a peptide binds to sites on JEV E DIII, but not cellular receptors, to interfere with JEV infection. Non-cytotoxic inhibitory P3 that binds adjacent to the BC loop and DE loop of E DIII can block JEV attachment to host cells and inhibit JEV infection. We believe that the inhibitory P3 peptide may contribute to the development of new therapeutics for JEV of different genotypes or other flavivirus infections based on the conservation of E DIII. The findings in this study have relevance for the development of antiviral drugs and for understanding the interaction between the virus and its cellular receptor.

Acknowledgements

This project was funded by the National Key Scientific Program (973) – (No. 2010CB530100) and a Grant from the National Natural Science Foundation o f China (No. 31000089).

The authors would like to thank Dr. Shengbo Cao for providing pPreM-E JEV plasmid, Dr. Bo Zhang for providing JEV and Liangzhen Zheng for statistical analysis and technical assistance.

References

Altmeyer, R., 2004. Virus attachment and entry offer numerous targets for antiviral therapy. Curr. Pharm. Des. 10, 3701–3712.
Bai, F., Town, T., Pradhan, D., Cox, J., Ashish Ledizet, M., Anderson, J.F., Flavell, R.A., Krueger, J.K., Koski, R.A., Fikrig, E., 2007. Antiviral peptides targeting the west nile virus envelope protein. J. Virol. 81, 2047–2055.
Bhardwaj, S., Holbrook, M., Shope, R.E., Barrett, A.D., Watowich, S.J., 2001. Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. J. Virol. 75, 4002–4007.
Castel, G., Chteoui, M., Heyd, B., Tordo, N., 2011. Phage display of combinatorial peptide libraries: application to antiviral research. Molecules 16, 3499–3518.
Chin, J.F., Chu, J.J., Ng, M.L., 2007. The envelope glycoprotein domain III of dengue virus serotypes 1 and 2 inhibit virus entry. Microbes Infec./Institut Pasteur 9, 1–6.
Chiu, M.W., Yang, Y.L., 2003. Blocking the dengue virus 2 infections on BHK-21 cells with purified recombinant dengue virus 2 E protein expressed in *Escherichia coli*. Biochem. Biophys. Res. Commun. 309, 672–678.
Chu, J.J., Rajamanonmani, R., Li, J., Bhuvanakantham, R., Lescar, J., Ng, M.L., 2005. Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. J. Gen. Virol. 86, 405–412.
Costin, J.M., Jenwitheesuk, E., Lok, S.M., Hunsperger, E., Conrads, K.A., Fontaine, K.A., Rees, C.R., Rossmann, M.G., Isern, S., Samudrala, R., Michael, S.F., 2010. Structural optimization and de novo design of dengue virus entry inhibitory peptides. PLoS Negl. Trop. Dis. 4, e721.
Das, S., Laxminarayana, S.V., Chandra, N., Ravi, V., Desai, A., 2009. Heat shock protein 70 on Neuro2a cells is a putative receptor for Japanese encephalitis virus. Virology 385, 47–57.
Das, S., Ravi, V., Desai, A., 2011. Japanese encephalitis virus interacts with vimentin to facilitate its entry into porcine kidney cell line. Virus Res. 160, 404–408.
Erlanger, T.E., Weiss, S., Keiser, J., Utzinger, J., Wiedenmayer, K., 2009. Past, present, and future of Japanese encephalitis. Emerg. Infect. Dis. 15, 1–7.
Fan, J., Liu, Y., Xie, X., Zhang, B., Yuan, Z., 2013. Inhibition of Japanese encephalitis virus infection by flavivirus recombinant E protein domain III. Virologica Sinica 28, 152–160.
Geiss, B.J., Stahla, H., Hannah, A.M., Gari, A.M., Keenan, S.M., 2009. Focus on flaviviruses: current and future drug targets. Future Med. Chem. 1, 327–344.
Ghosh, D., Basu, A., 2009. Japanese encephalitis – a pathological and clinical perspective. PLoS Negl. Trop. Dis. 3, e437.
Goncalvez, A.P., Chien, C.H., Tubthong, K., Gorshkova, I., Roll, C., Donau, O., Schuck, P., Yoksan, S., Wang, S.D., Purcell, R.H., Lai, C.J., 2008. Humanized monoclonal antibodies derived from chimpanzee Fabs protect against Japanese encephalitis virus in vitro and in vivo. J. Virol. 82, 7009–7021.
Heinz, F.X., Allison, S.L., 2000. Structures and mechanisms in flavivirus fusion. Adv. Virus Res. 55, 231–269.
Heinz, F.X., Allison, S.L., 2001. The machinery for flavivirus fusion with host cell membranes. Curr. Opin. Microbiol. 4, 450–455.
Hrobowski, Y.M., Garry, R.F., Michael, S.F., 2005. Peptide inhibitors of dengue virus and West Nile virus infectivity. Virol. J. 2, 49.
Hung, J.J., Hsieh, M.T., Young, M.J., Kao, C.L., King, C.C., Chang, W., 2003. An external loop region of domain III of dengue virus type 2 envelope protein is involved in serotype-specific binding to mosquito but not mammalian cells. J. Virol. 78, 378–388.
Jaiswal, S., Khanna, N., Swaminathan, S., 2004. High-level expression and one-step purification of recombinant dengue virus type 2 envelope domain III protein in *Escherichia coli*. Protein Expr. Purif. 33, 80–91.
Jia, H., Ge, X., Guo, X., Yang, H., Yu, K., Chen, Z., Chen, Y., Cha, Z., 2008. Specific small interfering RNAs-mediated inhibition of replication of porcine encephalomyocarditis virus in BHK-21 cells. Antiviral Res. 79, 95–104.
Jin, R., Zhu, W., Cao, S., Chen, R., Jin, H., Liu, Y., Wang, S., Wang, W., Xiao, G., 2013. Japanese encephalitis virus activates autophagy as a viral immune evasion strategy. PLoS ONE 8, e52909.
Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., Baker, T.S., Strauss, J.H., 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108, 717–725.
Li, C., Zhang, L.Y., Sun, M.X., Li, P.P., Huang, L., Wei, J.C., Yao, Y.L., Isahg, H., Chen, P.Y., Mao, X., 2012. Inhibition of Japanese encephalitis virus entry into the cells by the envelope glycoprotein domain III (EDIII) and the loop3 peptide derived from EDIII. Antiviral Res. 94, 179–183.
Li, Y., Hou, L., Ye, J., Liu, X., Dan, H., Jin, M., Chen, H., Cao, S., 2010. Development of a convenient immunochromatographic strip for the diagnosis of infection with Japanese encephalitis virus in swine. J. Virol. Methods 168, 51–56.
Liang, J.J., Yu, C.Y., Liao, C.L., Lin, Y.L., 2011. Vimentin binding is critical for infection by the virulent strain of Japanese encephalitis virus. Cell. Microbiol. 13, 1358–1370.
Liao, M., Kielian, M., 2005. Domain III from class II fusion proteins functions as a dominant-negative inhibitor of virus membrane fusion. J. Cell Biol. 171, 111–120.
Luca, V.C., AbiMansour, J., Nelson, C.A., Fremont, D.H., 2012. Crystal structure of the Japanese encephalitis virus envelope protein. J. Virol. 86, 2337–2346.
Mackenzie, J.S., Gubler, D.J., Petersen, L.R., 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat. Med. 10, S98–109.

Mackenzie, J.S., Johansen, C.A., Ritchie, S.A., van den Hurk, A.F., Hall, R.A., 2002. Japanese encephalitis as an emerging virus: the emergence and spread of Japanese encephalitis virus in Australasia. Curr. Top. Microbiol. Immunol. 267, 49–73.
Medigeshi, G.R., 2011. Mosquito-borne flaviviruses overview of viral life-cycle and host–virus interactions. Future Virol. 6, 1075–1089.
Misra, U.K., Kalita, J., 2010. Overview: Japanese encephalitis. Prog. Neurobiol. 91, 108–120.
Mukhopadhyay, S., Kuhn, R.J., Rossmann, M.G., 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. 3, 13–22.
Perera, R., Khaliq, M., Kuhn, R.J., 2008. Closing the door on flaviviruses: entry as a target for antiviral drug design. Antiviral Res. 80, 11–22.
Pierce, B.G., Hourai, Y., Weng, Z., 2011. Accelerating protein docking in ZDOCK using an advanced 3D convolution library. PLoS ONE 6, e24657.
Pyke, A.T., Williams, D.T., Nisbet, D.J., van den Hurk, A.F., Taylor, C.T., Johansen, C.A., Macdonald, J., Hall, R.A., Simmons, R.J., Mason, R.J., Lee, J.M., Ritchie, S.A., Smith, G.A., Mackenzie, J.S., 2001. The appearance of a second genotype of Japanese encephalitis virus in the Australasian region. Am. J. Trop. Med. Hyg. 65, 747–753.
Roy, A., Kucukural, A., Zhang, Y., 2010. I-TASSER: a unified platform for automated protein structure and function prediction. Nat. Protoc. 5, 725–738.
Schmidt, A.G., Yang, P.L., Harrison, S.C., 2010a. Peptide inhibitors of dengue-virus entry target a late-stage fusion intermediate. PLoS Pathog. 6, e1000851.
Schmidt, A.G., Yang, P.L., Harrison, S.C., 2010b. Peptide inhibitors of flavivirus entry derived from the E protein stem. J. Virol. 84, 12549–12554.
Scott, J.K., Smith, G.P., 1990. Searching for peptide ligands with an epitope library. Science 249, 386–390.
Sidhu, S.S., 2000. Phage display in pharmaceutical biotechnology. Curr. Opin. Biotechnol. 11, 610–616.
Solomon, T., 2004. Flavivirus encephalitis. N. Engl. J. Med. 351, 370–378.
van den Hurk, A.F., Ritchie, S.A., Mackenzie, J.S., 2009. Ecology and geographical expansion of Japanese encephalitis virus. Annu. Rev. Entomol. 54, 17–35.
Volk, D.E., Beasley, D.W., Kallick, D.A., Holbrook, M.R., Barrett, A.D., Gorenstein, D.G., 2004. Solution structure and antibody binding studies of the envelope protein domain III from the New York strain of West Nile virus. J. Biol. Chem. 279, 38755–38761.
Wu, K.P., Wu, C.W., Tsao, Y.P., Kuo, T.W., Lou, Y.C., Lin, C.W., Wu, S.C., Cheng, J.W., 2003. Structural basis of a flavivirus recognized by its neutralizing antibody: solution structure of the domain III of the Japanese encephalitis virus envelope protein. J. Biol. Chem. 278, 46007–46013.
Yu, S., Wuu, A., Basu, R., Holbrook, M.R., Barrett, A.D., Lee, J.C., 2004. Solution structure and structural dynamics of envelope protein domain III of mosquito- and tick-borne flaviviruses. Biochemistry 43, 9168–9176.
Zhang, S., Bovshik, E.I., Maillard, R., Gromowski, G.D., Volk, D.E., Schein, C.H., Huang, C.Y., Gorenstein, D.G., Lee, J.C., Barrett, A.D., Beasley, D.W., 2010. Role of BC loop residues in structure, function and antigenicity of the West Nile virus envelope protein receptor-binding domain III. Virology 403, 85–91.
Zhang, Y., 2008. I-TASSER server for protein 3D structure prediction. BMC Bioinform. 9, 40.