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Short Communication

Inhibition of Japanese encephalitis virus entry into the cells by the envelope glycoprotein domain III (EDIII) and the loop3 peptide derived from EDIII

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ABSTRACT

Japanese encephalitis virus (JEV) infection is a major cause of acute viral encephalitis both in humans and animals. The domain III of virus envelope protein (EDIII) plays important roles in interacting with host cell receptors to facilitate virus entry. In this study, recombinant JEV EDIII was expressed and purified. The protein showed the ability to inhibit JEV infection in BHK-21 cells with 50% inhibition at a concentration of 25 μg/ml. Based on NMR structure of JEV EDIII, we chose several loop peptides that were reported to be related to receptor binding to test their possible inhibitory activities on virus infection. Our *in vitro* experiments demonstrated that one of the loop peptides (loop3) can prevent JEV infection with 50% inhibition at concentration of 10 μM by interfering in virus attachment to the cells. Our *in vivo* experiments on mice showed the loop3 was the most protective peptide when administered before virus challenge. Therefore, the loop3 peptide may be served as basis for the development of novel antiviral agents against Japanese encephalitis virus or other flaviviruses infection.

Japanese encephalitis virus (JEV) is an emerging pathogen causing poliomyelitis-like acute flaccid paralysis, aseptic meningitis and encephalitis in humans and animals (Solomon, 2003). The structure of the envelope protein domain III of JEV, DENV-4, TBEV and YFV are determined by NMR spectroscopy (Mukherjee et al., 2006; Volk et al., 2009; Volk et al., 2007; Wu et al., 2003). The domain III (EDIII) of JEV is composed of six antiparallel strands: β1, β2, β3, β4, β5, and β6, which are corresponding to strand A, B, C, E, F, and G in WNV, TBEV, and YFV domain III. It is reported that the EDIII of JEV, WNV and DENV can induce the production of neutralizing antibodies, and many of these antibodies have been shown to block the virus entry by neutralization *in vitro* and *in vivo* (Batra et al., 2010; Bharati et al., 2007; Nybakken et al., 2005; Smouse and Burt, 2010). Therefore, the EDIII protein represents an attractive target for the design of novel antiviral agent (Chávez et al., 2010; Perera et al., 2008).

In order to determine whether the JEV EDIII protein can inhibit JEV infection like other flaviviruses EDIII proteins (Chin et al., 2007; Chiu and Yang, 2003; Chu et al., 2005), we expressed the EDIII protein of JEV using bacterial expression system, and its antiviral activity against JEV infection was determined by plaque assay, Q-PCR and western blot at 48 h post JEV infection (MOI = 0.02). The results showed that the protein (100 μg/ml) can significantly inhibit JE virus infection in a dose-dependent manner (data not shown).

In one study, some peptides are found to possess the inhibitory abilities on dengue virus infection, and one of them cross-inhibits WNV infection in Vero cells (Hrobowski et al., 2005). A peptide against WNV infection was also identified by a murine brain cDNA phage display library assay using WNV envelope protein as probe (Bai et al., 2007). The BC loop of WNV involves in EDIII folding, virus infectivity, virulence and antigenicity (Zhang et al., 2010), and an external loop and the fusion peptide of dengue type virus 2 are involved in binding to different cells receptors and responsible to membrane fusion (Hung et al., 2004). The peptide derived from the "stem" of dengue virus type 2 (DENV-2) envelope protein can specifically bind to the postfusion conformer of sE (Schmidt et al., 2010a). Further study shows the stem peptide from residues 419 to 447 of DENV-2 can inhibit all DENV serotypes, but the corresponding peptides derived from other related flaviviruses can not (Schmidt et al., 2010b). Therefore, envelope protein peptides represent a new direction to pursue potential candidates in therapeutics for flavivirus infection.

In the JEV EDIII structure, some of the β-barrel loops (top loops) project further from the virion surface than any part of the envelope protein, four loop peptides between or before β

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Table 1
Peptides used in the experiments.

Peptide	Amino acid sequence [a]	Location [b]	MW [c]	GRAVY[c]
Loop1	302-GMCTEKFY-309	Before β1	902.0	−0.263
Loop2	329-SGSDGPCKI-337	β1–β2	862.9	−0.489
Loop3	362-ATSSANSKA-370	β3–β4	863.9	−0.300
Scrambled loop3	SASKAASNT	β3–β4	863.9	−0.300
Loop4	385-VGRGDKQ-391	β5–β6	758.8	−1.714

[a] Numbering from the beginning of the E polyprotein in JEV (NJ 2008 strain), (GenBank Accession ID: GQ918133).
[b] The position in the JEV EDIII domain, based on its NMR structure (PDB ID: 1PJW).
[c] The molecular weight (MW) and grand average of hydropathicity (GRAVY) were predicted with the ProtParam algorithm. (http://expasy.org/tools/protparam.html).

strands in JEV EDIII (Table 1, Fig. 1A) were chosen and chemically synthesized. They are significantly conserved in different JEV strains, and share some similarity with WNV, but little similarity with other flaviviruses (Fig. 1B). The loop peptides did not stimulate significant cytotoxicity in BHK-21 cells even at 200 μM (data not shown). To determine the antiviral activities of the chosen peptides, BHK-21 cells were pre-incubated with peptides (0–100 μM) for 1 h at 4 °C, and then were inoculated with JEV supplemented with different concentrations of peptides. The inhibitory abilities of the peptides to JEV infection were determined by plaque assay and Q-PCR. The plaque assay result (Fig. 2A) showed the loop3 peptide significantly inhibited JEV replication at a concentration of 100 μM ($p < 0.02$), and in a dose-dependent manner. The Q-PCR experiment confirmed the plaque assay result (data not shown). The IC_{50} of loop3 is 10 ± 1.4 μM. The loop1, loop2, and loop4 did not show significant inhibition on virus load ($p > 0.05$). We next synthesized a scrambled peptide of loop3 to address whether the inhibitory ability of the loop3 are sequence-specific. The plaque assay results demonstrated that the scrambled loop3 did not significantly inhibit JEV infection (data not shown). Therefore, we focus on loop3 to determine whether the loop3 peptide treatment inhibited JEV attachment. The virus binding after peptide incubation was measured by ELISA. The significant decrease in virus attachment in the presence of different concentrations of peptide was detected, especially at 100 μM ($p < 0.02$) (Fig. 2B). When the peptide was added to the cells after the virus infection, the plaque assay results showed the JEV titers had no significant changes (data not shown). In conclusion, the loop3 peptide exerts its inhibitory ability by interfering in virus attachment.

As the peptides can significantly inhibit JEV infection *in vitro*, we next determined whether the loop peptides could protect mice from a lethal dose challenge of JEV. BALB/c mice were pre-inoculated intracerebrally with 30 μl of the loop peptides (2 mM) 1 h before challenged with virus (Jones et al., 2006). The mice began to die at about 4 days after virus challenge. Our 4 day survival data suggested that only the loop3 peptide can protect mice from death with the survival rate of 90%. The remaining mice were observed for 3 weeks. The 3 week survival rate of loop1, loop2, loop3 and loop4 treated mice were 10, 20, 80 ($p < 0.02$) and 20%, respectively (Fig. 3A). We randomly sacrificed half of the mice at day 3 to collect brains for virus analysis. The inhibition percent of virus load in the brains were detected by plaque assay. The analyzed result (Fig. 3B) showed that loop3 apparently reduced the JEV load by about 80%, and loop2 and loop4 can not significantly decrease the JEV loads ($p > 0.05$). The Q-PCR result was consistent with the plaque assay (data not shown). Furthermore, we found the scrambled loop3 peptide could not provide significant protection against JEV infection, suggesting the antiviral ability of loop3 peptide is also sequence-dependent in mice (data not shown). Meanwhile, the loop3 peptide failed to treat mice when it was administrated 2 h after JEV challenged (data not shown).

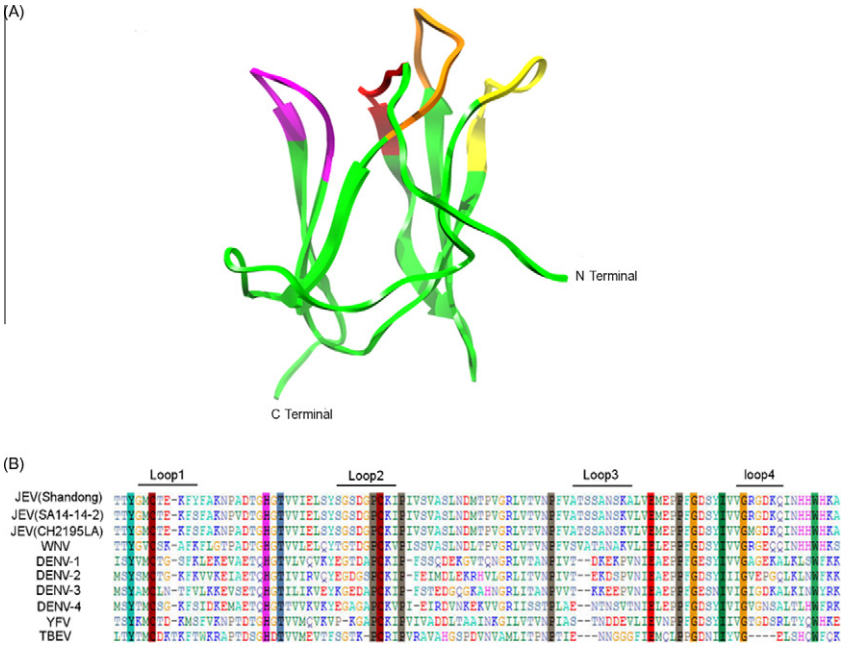

Fig. 1. The location of the four peptides in the JEV EDIII and alignment of the amino acid sequences of EDIII of flaviviruses. (A) The tertiary structure of JEV EDIII protein determined by NMR (PDB ID: 1PJW). The loop peptides are shown in different colors: loop1 (red), loop2 (orange), loop3 (yellow), and loop4 (pink). (B) Alignment of the amino acid sequences of EDIII structures of several flaviviruses. The conserved residues are shaded, the four loop peptides between β strands are labeled. Amino acid sequence accession numbers are JEV (strain NJ2008, GenBank Accession ID: GQ918133, strain SA14–14-2, GenBank Accession ID: AF315119, strain CH2195LA, PDB ID: 1PJW). WNV (PDB ID: 1ZTX); DENV-1 (PDB ID: 3G7T); DENV-2 (PDB ID: 1OAN). DENV-3 (PDB ID: 1UZG); DENV-4 (PDB ID: 2H0P); YFV (PDB ID: 2JV6); TBEV (PDB ID: 1SVB) are obtained from PDB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

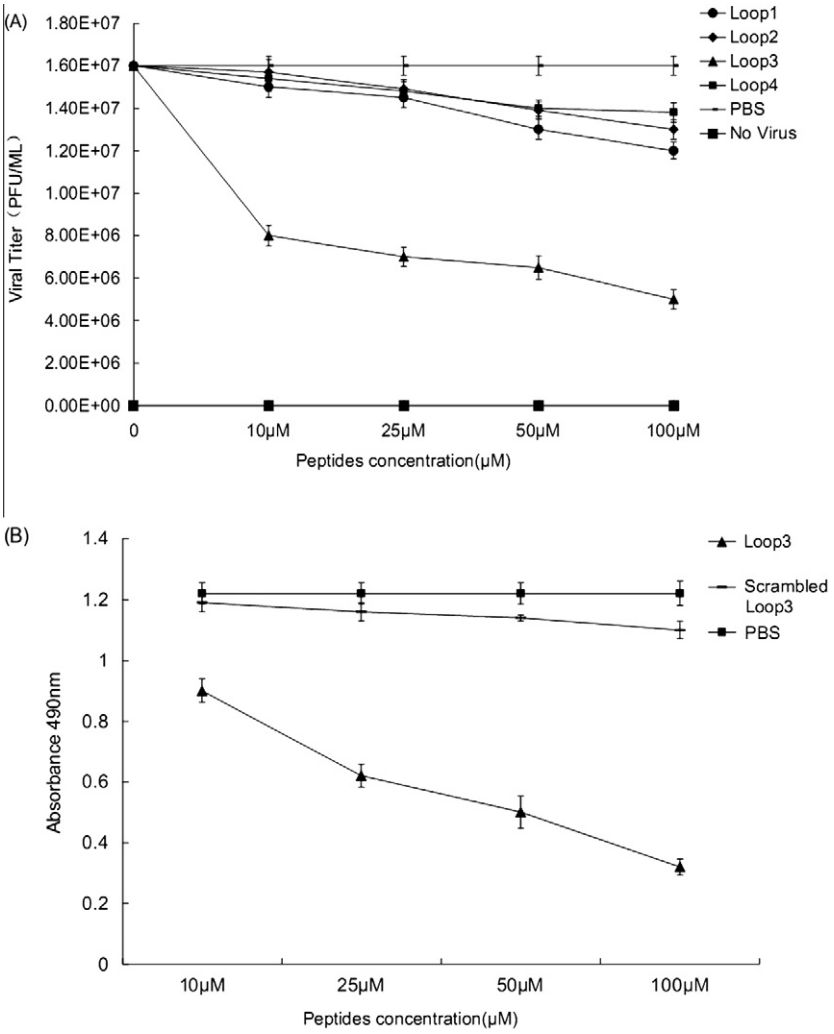

Fig. 2. Antiviral activities of peptides. (A) The loop peptides were inoculated with BHK-21 cells first, and then with JE virus (MOI = 0.02) supplemented with the peptides, viral titers determined by plaque assay on 48 hpi. (B) The loop3 peptide and scrambled loop3 peptide (0–100 μM) were added to BHK-21 cells at 4 °C, then the cells were infected with JEV (MOI = 0.1). The amount of viruses that bound to the cell surface was evaluated using monoclonal anti-JEV antibody by ELISA. All data were determined in triplicate and are representative of at least twice separate experiments. The results represent the means ± SD of triplicate determinations. The differences between means were considered significant at $p < 0.05$.

In summary, the JEV EDIII protein had antiviral effect at microgram range, and the loop3 peptide exerts inhibitory effects on virus infection by interfering in virus attachment to the host cells in a dose-dependent and sequence-specific manner, and it also can significantly increase survival rate in animal models, when administered before lethal JEV challenges. Our results showed JEV EDIII and the loop3 peptide could not inhibit JEV infection in BHK-21 and in mice completely, because we can not exclude any possibility that other peptides of the envelope protein may also be necessary for post-binding process of JEV entry into host cells (Smit et al., 2011). In our experiment, we re-administrate once with the peptide to the mice 2 days after challenge, but we did not observe significant difference between one administration (data not shown). Because many intracerebral injections may increase the chances of injury to the mice and their mortality, we did not pursue more peptide injections. In our studies, the loop3 was given to the mice by intracerebral injection, though it is not the normal route for virus infection. Therefore, other administration methods are necessary for efficiently drug delivery in clinical application. For example, intravenous injection is common route for drug administration, and attempts by using carrier proteins, peptides or drug have been tried to breached brain–blood barrier (BBB) (Bickel et al., 2001; Carman et al., 2011; Demeule et al., 2002; Vogler et al., 2005; Zhang and Pardridge, 2005). There are other possibilities to improve the antiviral efficacy of loop3 peptide. First of all, sustained delivery systems (Bjerregaard et al., 2001; Kim et al., 1998) might be necessary to keep sufficient plasma concentration. Next, previous successful strategies can be tested to improve peptide plasma half-life without decreasing inhibition by modification of the N and C terminus, head, and tail cyclization (Marastoni et al., 1994). In addition, the inhibitory activity of loop3 could be potentially improved by modifying the amino acid residue composition by using computational modeling analysis.

Nevertheless, based on our studies, JEV EDIII protein and one inhibitory peptide (loop3) derived from JEV EDIII that can inhibit JEV infection have been identified. The inhibitory loop3 peptide may be served as the lead to develop new therapeutics for the Japanese encephalitis virus of different genotypes or other flaviviruses infection due to the conservation between envelope proteins domain III and the loop3 peptide.

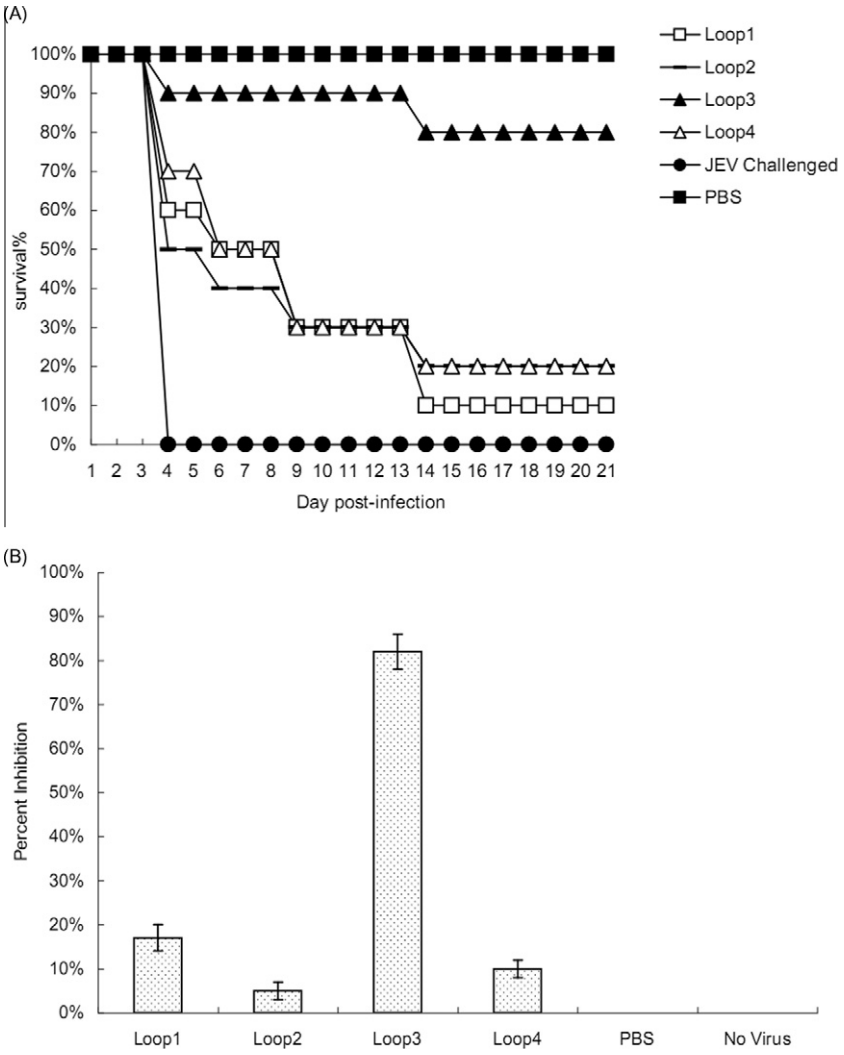

Fig. 3. The loop peptides inhibited JEV infection *in vivo*. The mice were inoculated either with PBS, loop peptides, then challenged with JEV, while others were inoculated with PBS only (not challenged with JEV), and the mice were monitored for morbidity. (A) The morbidity of mice during 21 day observation period. (B) On day 3 post-infection, half of the mice from each group were sacrificed and brains were harvested, the viral load was determined by plaque assay. The inhibition percent of virus load in the brains were analyzed based on plaque assay result. Animal use was in compliance with the Nanjing Agricultural University Institutional Animal Care and Use Committee. All data were determined in triplicate and are representative of at least twice separate experiments. The results represent the means ± SD of triplicate determinations. The differences between means were considered significant at $p < 0.05$.

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