Virus Research 260 (2019) 142–150

Contents lists available at ScienceDirect

Virus Research

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

In vitro analysis of synthetic peptides in blocking the entry of dengue virus

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ARTICLE INFO

Keywords:
Peptide inhibitor
Dengue virus
Envelope glycoprotein
Domain III
Flavivirus
Synthetic peptide

ABSTRACT

Dengue fever is the most prevalent arthropod-borne viral disease, and no specific therapeutic or promising antiviral drug is available for its treatment. Peptide inhibitors are less toxic than synthetic compounds and have found proven effective against viral infections. Here, three peptides that mimic part of the E protein of the dengue virus (DENV) were synthesized and evaluated for their inhibitory activity against four serotypes of DENV in African green monkey kidney (Vero) and rhesus macaque (*Macaca mulatta*) monkey kidney (LLC-MK2) cell lines. The three peptides, Pep1, Pep2, and Pep3 are located in domains I, II, and III of the E protein respectively. All three peptides effectively reduced > 80% of focus forming units in the virus treated mammalian cell lines than control and exhibited their IC_{50} in the range of 10–33 μM. Pep1 was found effective against DENV-2, DENV-3, and DENV-4 (IC_{80} below 50 μM). Pep2 showed the highest inhibitory activity against all four serotypes (IC_{50} below 20 μM). Pep3 reduced the 80% focus forming units in all serotypes at the concentration of 40 μM. Evaluation of peptides at different time points of viral infection in the mammalian cell lines revealed that the peptides inhibited viral infection by binding to the virus and not by binding to cellular receptors and blocking viral entry. The peptides assumedly exert their inhibitory effects by binding to the E protein and repressing its conformational changes; this prevents the virus from binding to cellular receptors, thereby inhibiting viral entry. Hence, these peptides might limit viral spread and reduce the virus's ability to infect.

1. Introduction

Dengue fever is an arthropod-borne viral disease that has emerged as a significant public health concern owing to its health-related and economic burdens, particularly in the tropical and subtropical regions of Africa, Asia, Australia, and America (Gubler, 2002). Approximately 390 million cases of dengue infection occur each year, and 96 million of those result in severe disease (Bhatt et al., 2013). In the last two decades, several environmental and anthropogenic factors, such as climate change, urbanization, population growth, and population mobility, have contributed to the expansion of dengue infections in not just endemic regions but worldwide (Gubler, 2006). Autochthonous dengue cases have also been reported in non-endemic areas in Europe (Gjenero-Margan et al., 2011; La Ruche et al., 2010; Succo et al., 2016). The causative virus is primarily transmitted by infected *Aedes aegypti* and *A. Albopictus* female mosquitoes.

Dengue virus (DENV) (family, Flaviviridae; genus, *Flavivirus*) is the etiological agent of dengue fever (Gubler, 1998). DENV has antigenically distinct serotypes: DENV-1, DENV-2, DENV-3, and DENV-4 (Henchal and Putnak, 1990). The dengue virion is a spherical particle (diameter, 40–50 nm) with a lipopolysaccharide envelope (Gubler, 1998). Its positive single-stranded RNA genome is approximately 11 kb in length, with a single open reading frame encoding three structural proteins [capsid (C), membrane (M), and envelope glycoprotein (E)] and seven nonstructural proteins [NS1, NS2 A, NS2B, NS3, NS4 A, NS4B and NS5] (Chambers et al., 1990; Kuhn et al., 2002).

Abbreviations: DENV, dengue virus; FFA, focus-forming assay; PBS, phosphate-buffered saline; FFUs, focus-forming units; Aa, amino acid; Pep1, peptide 1; E protein, envelope glycoprotein

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https://doi.org/10.1016/j.virusres.2018.11.016
Received 7 February 2018; Received in revised form 29 November 2018; Accepted 29 November 2018
Available online 30 November 2018
0168-1702/

Developing vaccines against DENV is challenging owing to the circulation of the four serotypes that share 60%–75% sequence identity and are capable of causing disease and death (Guzman and Harris, 2015). Moreover, non-neutralizing antibodies that form immune complexes with viruses increase the viral load in infected cells via "antibody-dependent enhancement" (Daughaday et al., 1981; Gollins and Porterfield, 1984). Vaccines may be ineffective in patients in the viremic phase. Antiviral inhibitors would be beneficial to patients with high viral loads and reduce the development of dengue shock syndrome/dengue hemorrhagic fever (Panya et al., 2014). Therefore, the development of potential antiviral therapeutic agents is a feasible, alternative approach for controlling dengue fever.

Both structural and nonstructural viral proteins are considered potential targets for antiviral agents. The E protein, in particular, is a promising target because it is involved in receptor binding and membrane fusion, thereby facilitating viral entry into host cells (Allison et al., 2001; Crill and Roehrig, 2001; Rodenhuis-Zybert et al., 2010). The E protein has three functional domains (I, II, III) apart from a membrane proximal stem and a transmembrane anchor. Structurally, domain I is the central domain; it functions as a molecular hinge for the low-pH-catalyzed reorganization of the E protein (Modis et al., 2003). Domain II functions as a dimerization domain (Modis et al., 2004), whereas domain III functions as a receptor-binding domain (Crill and Roehrig, 2001; Modis et al., 2005) facilitating viral entry and fusion. Recent studies have confirmed that the E protein is a promising target for anti-DENV inhibitors (Alhoot et al., 2013; Poh et al., 2009; Wang et al., 2009).

Peptide inhibitors have been evaluated for their anti-DENV activity because they are less toxic than synthetic compounds and have diverse chemical and biological properties (Alhoot et al., 2013; Costin et al., 2010; Hrobowski et al., 2005; Panya et al., 2014; Schmidt et al., 2012). Further, mimotopes, short peptides comprising parts of viral proteins, reportedly exert antiviral activity. For instance, enfuvirtide (T20), the first commercially available drug for HIV, is a peptide mimic of part of the C-terminal region of the HIV gp41 glycoprotein (Lalezari et al., 2003). In DENV too, several mimotopes from the E protein have been evaluated and reported as antiviral inhibitors (Costin et al., 2010; De La Guardia and Lleonart, 2014; Hrobowski et al., 2005; Lalezari et al., 2003; Lok et al., 2012; Panya et al., 2014; Poh et al., 2009; Schmidt et al., 2010a, 2010b, 2012; Wang et al., 2009).

The present study aimed to evaluate the synthetic peptides that mimic parts of the E protein of DENV (Fig. 1) in terms of their inhibitory activity against four serotypes of DENV. We have previously reported that NGR motif (348–350 amino acids) is involved in serotype specific entry into DENV-1 and DENV-3 by computational analysis (Mary et al., 2016). Subsequently, it was demonstrated that the anti-peptide antibody produced against the peptide has neutralizing activity and inhibited viral entry (Mary et al., 2018). We have also carried out bioinformatic analysis of E protein of DENV in predicting conserved, immunodominant B and T-cell epitopes using BcPred, Bepipred, ProPred I, and ProPred tools (unpublished data). Therefore, we chose the following regions of the E protein for preparing synthetic peptides: amino acid (aa) 339–354 including NGR motif from domain III (because it is involved in dengue virus–host interaction) and two sites corresponding to aa 25–41 of domain I and aa 98–109 of domain II (because these are conserved and immunodominant epitopes). The three synthetic peptides were evaluated for their inhibitory activities at different time points relative to viral infection. We observed that all the three peptides were effective against dengue infection in cells and proposed a possible mechanism behind this phenomenon.

2. Materials and methods

2.1. Cells, virus, and synthetic peptides

African green monkey kidney (Vero), Rhesus macaque (*Macaca mulatta*) kidney (LLC-MK2), and C6/36 mosquito cell lines at fewer than 20 passages were used. The Vero and LLC-MK2 cell lines were maintained in MEM supplemented with 10% FBS (Gibco, NY, USA), 100 U/ml penicillin G, and 100 mg/ml streptomycin incubated at 37°C with 5% (v/v) CO_2. The C6/36 cell line was maintained in RPMI supplemented with 10% FBS, 1% penicillin G and streptomycin, 1% glutamine, and 1% non-essential amino acids and incubated at 28°C. The four serotypes of DENV [DENV-1 (Hawaii), DENV-2 (NGC), DENV-3 (H87), and DENV-4 (8144669)] were propagated in the C6/36 mosquito cells and harvested after cytopathic effects were observed. The virus stock titer was determined using focus-forming assay (FFA), as described by Zandi et al. (2011).

The three peptides used in this study were derived from the domains I, II, and III of E protein of DENV, corresponding to aa 25–42 (LEHGSCVTTMAKDKPTL), 98–109 (DRGWGNGCGLFG), and 339–354 (GQGKAHNGRLITANP), denoted as Pep1, Pep2, and Pep3, respectively (Table 1). The peptides were commercially synthesized using solid-phase peptide synthesis at GenScript (USA). The identities of the peptides were confirmed using LC–MS, and the peptide purity was > 96%. The amino and carboxyl terminus as well as cysteine residues of the peptides were not protected.

2.2. Cell proliferation assay

Vero and C6/36 cells were treated with the three peptides to determine their toxic effects using the MTT [3-(4,5-dimethylthiazol-2-yl)-

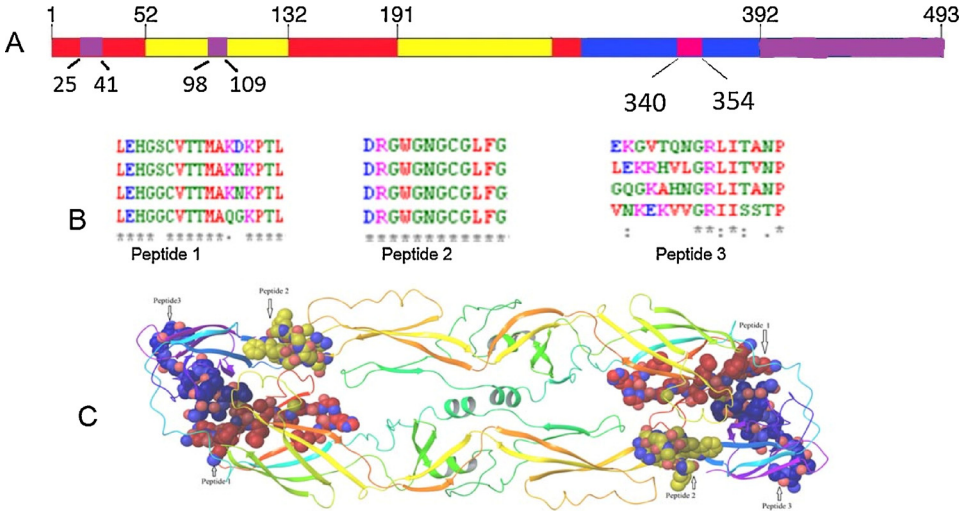

Fig. 1. Envelope glycoprotein of DENV-3 serotype. A. Schematic representation of Envelope glycoprotein containing domain I, domain II, domain III and stem region. The locations of three peptides are filled in purple (Peptide 1 & Peptide 2) and in **pink** (peptide 3). B. The amino acid sequences of the peptides and their conservation in the four serotypes of Dengue virus. C. Ribbon diagram of E protein (Modis et al., 2005; PDB-ID: 1UZG) the position of three peptides are marked as Peptide 1, Peptide 2 and Peptide 3 and represented in CPK model in E protein.

Table 1
Synthetic peptides used in this study.

Peptide no	Amino acid sequence	Position	Structural domain	Length	Molecular Weight (Da)
Pep 1	LEHGSCVTTMAKDKPTL	25 - 42	Domain I	17	1831.13
Pep 2	DRGWGNGCGLFG	98 - 109	Domain II	12	1238.34
Pep 3	GQGKAHNGRLITANP	340 - 354	Domain III	15	1636.84

2,5-diphenyltetrazolium bromide)] assay based on the ability of viable cells to reduce MTT via cellular oxidoreductases, thereby yielding a crystalline blue formazan product (Zandi et al., 2011). The experiments were performed in triplicates, and cell viability (%) was calculated as follows :[(Absorbance$_{sample}$ − Absorbance$_{Blank}$)/ Absorbance$_{Control}$ − Absorbance$_{Blank}$)/ × 100)], where A_{sample} and $A_{Control}$ are the absorbances of the treated and control cells, respectively.

2.3. Focus-forming unit reduction assay

The inhibitory activities of the peptides were measured on the basis of the number of viral foci formed by DENV in the treated and untreated wells. The experiment was performed following the protocol of Zandi et al.(2012). The cells were seeded in microplates and infected with DENV. The infected cells were then overlaid with 2% CMC overlayer medium containing MEM and FCS, followed by incubation for 3 days. To visualize the focus-forming units (FFUs; indicator of viral titer), the cell culture medium was discarded, and cells were gently washed thrice with phosphate-buffered saline (PBS). The cells were then fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 1% Triton X-100 in PBS for 10 min, and washed with PBS thrice. After washing, the cells were incubated with 100 µl of 4G2, an antiflavivirus antibody, at 4°C for overnight. The cells were then washed thrice with PBS and incubated with horseradish peroxidase-conjugated anti-mouse IgG at a final concentration of 1:1000 for 1 h at 37°C. Finally, the substrate 3′-diaminobenzidine (DAB; 1 µg/ml) was added to each well for staining and incubated for 5 min at 37°C.The cells were then washed with PBS to stop the reaction and observed under a microscope. Each red spot that appeared in the wells was manually counted. Wells with untreated infected cells were used as the negative control. The inhibitory activities of peptides were measured by determining the number of viral foci formed by DENV in the treated and untreated wells. Focus reduction (RF%) was calculated by comparing the treated and untreated wells as follows: RF(%) = (C − -T) × 100/C, where C is the mean of the focus forming units (FFUs) in the negative control (mock) and T is the mean of the FFUs formed in treated wells. All experiments were performed in triplicates.

2.4. Inhibitory activities of peptides

Vero and LLC-MK2 cells were seeded in the microplates and incubated overnight. Two hundred FFUs of DENV-1 (Hawaii), DENV-2 (NGC), DENV-3 (H87), and DENV-4 (8144669) were incubated with or without the peptides Pep1, Pep2, and Pep3 at different concentrations (10, 20, 30, 40, 50, and 100µM) in MEM for 1 h at 37°C. Post incubation, the peptide-treated viruses were allowed to infect confluent monolayers of Vero and C6/36 cells and incubated for 2 h at 37°C. The infected cells were overlaid with 2% CMC overlay medium and incubated for 3 days. Postincubation, the antiviral activities of the peptides were determined using FFA.

2.5. Prophylactic analysis with peptides

To evaluate the mode of action of the peptides in terms of whether they can bind to the receptors of host cells or to the E protein of DENV, Vero and LLC-MK2 cells were treated with peptides at different time points of infection. For this, Vero cells were treated with 40µM of the peptides for 5 h prior to the infection. The cells were then washed twice with PBS. The cells were then infected with 200 FFUs of DENV-1 (Hawaii), DENV-2 (NGC), DENV-3 (H87), and DENV-4 (8144669) in the respective microplates. The infected cells were subsequently overlaid with 2% CMC overlay medium and incubated for 3 days. To visualize the FFUs, the above-mentioned procedure was followed, and the FFUs were counted.

2.6. Anti-adsorption activity

Anti-adsorption activities were assessed to examine the roles of the peptides against the adsorption of DENV in animal cells. Here, Vero and LLC-MK2 cells were treated with peptide-pretreated DENV-1 (Hawaii), DENV-2 (NGC), DENV-3 (H87), and DENV-4 (8144669) and incubated for 1 h. The cells were then washed with sterile PBS and overlaid with 2% CMC containing MEM and FCS. After incubation for 3 days, FFA was performed as described above, the FFUs were counted, and the RF% was calculated.

2.7. Post-adsorption antiviral activity

Post-adsorption antiviral activities were assessed to evaluate the antiviral activities of the peptides after the entry of DENV. For this, Vero and LLC-MK2 cells were infected with 200 FFUs of DENV [DENV-1 (Hawaii), DENV-2 (NGC), DENV-3 (H87), and DENV-4 (8144669)] in the respective microplates for 1 h at 37°C. Postincubation, the cells were washed with PBS to eliminate the unabsorbed viruses, 40 µM of all three peptides was added to the respective wells, and the wells were overlaid with 2% CMC containing MEM and FCS. After incubation for 3 days, FFA was performed as described above, the FFUs were counted, and the RF% was calculated to determine the anti-dengue activities of the peptides.

2.8. Statistical analyses

All statistical analyses were performed using GraphPad Prism 7.0 (2005; GraphPad Software Inc., San Diego, CA, USA). The cytotoxic concentration (CC_{50}) and inhibitory concentration of FFA (IC_{50}) were determined using GraphPad Prism. $P < 0.05$ was considered statistically significant. Error bars are expressed as mean ± standard deviation.

3. Results

3.1. Cytotoxicity of peptides

Prior to the analysis of antiviral activity, the toxic effects of the peptides in the Vero and C6/36 cells were examined using the MTT assay. None of the three peptides (Pep1, Pep2, and Pep3) affected cell viability to < 80% even at a concentration as high as 200 µM (Fig. 2A). Cytotoxicity assays of Pep1 and Pep2 in Vero cells indicated that > 90% cells were viable at a concentration of 50 µM. In the C6/36 cells, none of the three peptides showed any cytotoxic effect (Fig. 2B).

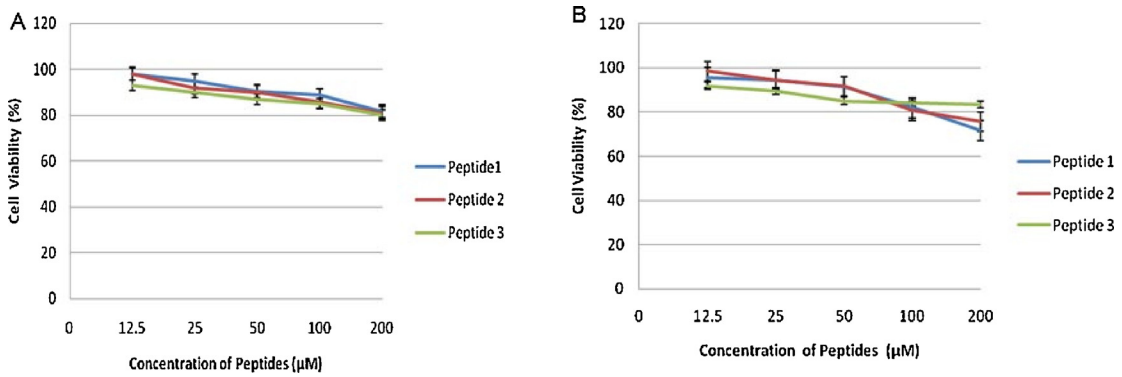

Fig. 2. Cytotoxicity of peptides towards Vero cells (A) and C6/36 cells (B). The cytotoxic effect of peptides was evaluated at 12.5 μM to 200 μM concentrations using MTT assay. The experiments were performed in triplicate. Values are expressed in mean ± SE.

3.2. Effect of peptides on viral infection

The viral infectivity was measured by determining the difference in the number of foci formed between control and treated cells, as the peptides bind to E protein, which eventually blocks the entry of DENV. The numbers of FFUs obtained from control and treated cells were converted into percentages of focus reduction (RF%). The percentage reduction in FFUs increased with increasing concentration of peptides. High concentrations of peptides reduced the number of foci by more than 80%. There were no significant differences in the percentage reductions of foci at concentrations of 50 and 100 μM in all of the DENV serotypes. Fig. 3A indicates the antiviral activities of Pep1, Pep2, and Pep3 against DENV-1. Pep1 reduced the infectivity of DENV-1 by 58% ± 2.55% at 50 μM, whereas 50 μM of Pep2 and Pep3 showed reductions in foci by 88% ± 3.89% and 83% ± 3.72%, respectively. In DENV-1, 50% reduction of foci was achieved at 10, 10, and 33 μM concentrations of the peptides Pep1, Pep2, and Pep3, respectively. There was no statistically significant difference in the percentage of focus reduction among the three peptides on DENV-1. The reductions in FFUs in Vero cells treated with Pep1, Pep2, and Pep3 was observed (Fig. 4B–D) compared to untreated plates (Fig. 4A). Similarly, DENV-2 was inhibited by the three peptides and showed 50% foci reductions at a concentration of 10 μM (Fig. 3B; Fig. 4F–H). A reduction in 60% FFUs was achieved against DENV-3 by all three peptides at a concentration of 20 μM (Fig. 3C; 5B–D). For DENV-4, all three peptides at a concentration of 20 μM were found to be effective, reducing the infectivity by 55% (Fig. 3D; Fig. 5F–H).

3.3. Preincubation of peptides with cells does not effectively prevent viral infection

It was not clear whether the peptides reduced the infectivity by binding to host receptor proteins or the E protein. Therefore, a prophylactic assay was carried out to elucidate whether the peptides have prophylactic effects on mammalian cell lines. This was studied by treating Vero cells and LLC-MK2 cells with peptides for about 5 h prior to viral infection. Less than 50% inhibition was observed for all of the serotypes in Vero cells (Fig. 6A) and LLC-MK2 cells (Fig. 6B), which suggested that the peptides do not interact with the receptors of mammalian cell lines to block viral entry.

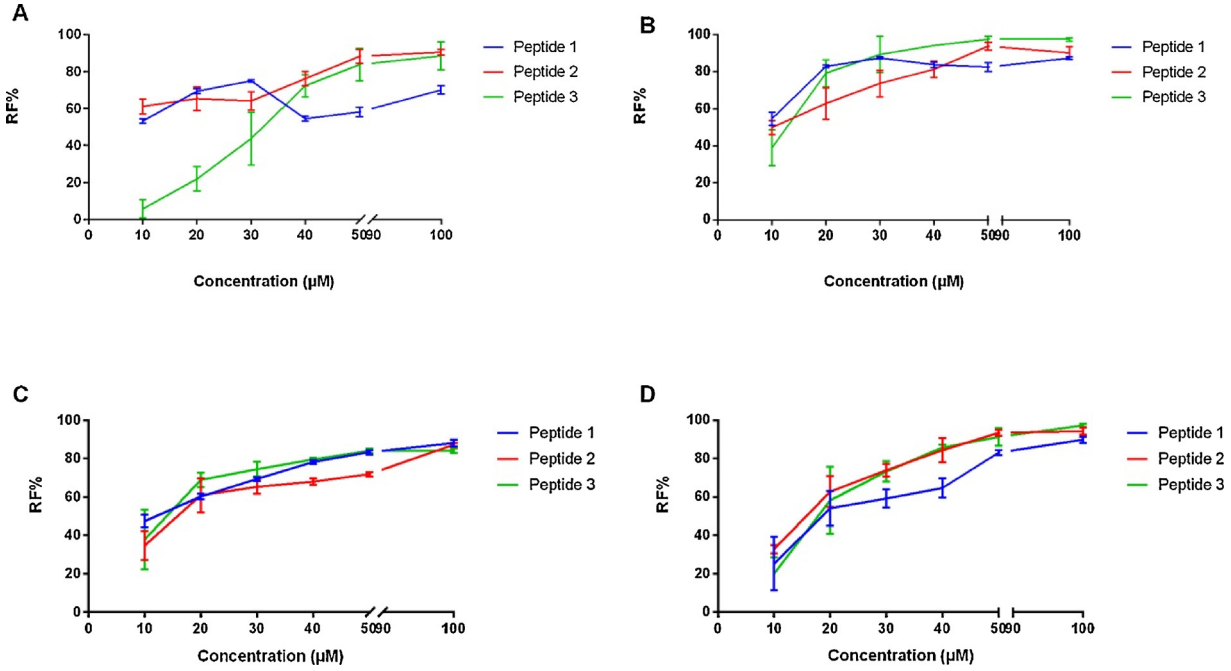

Fig. 3. Determination of peptide inhibitory dose against the four serotypes of DENV. A. DENV-1, B. DENV-2, C. DENV-3, D. DENV-4 All measurements were made in triplicates and the values expressed with mean +/- SD.

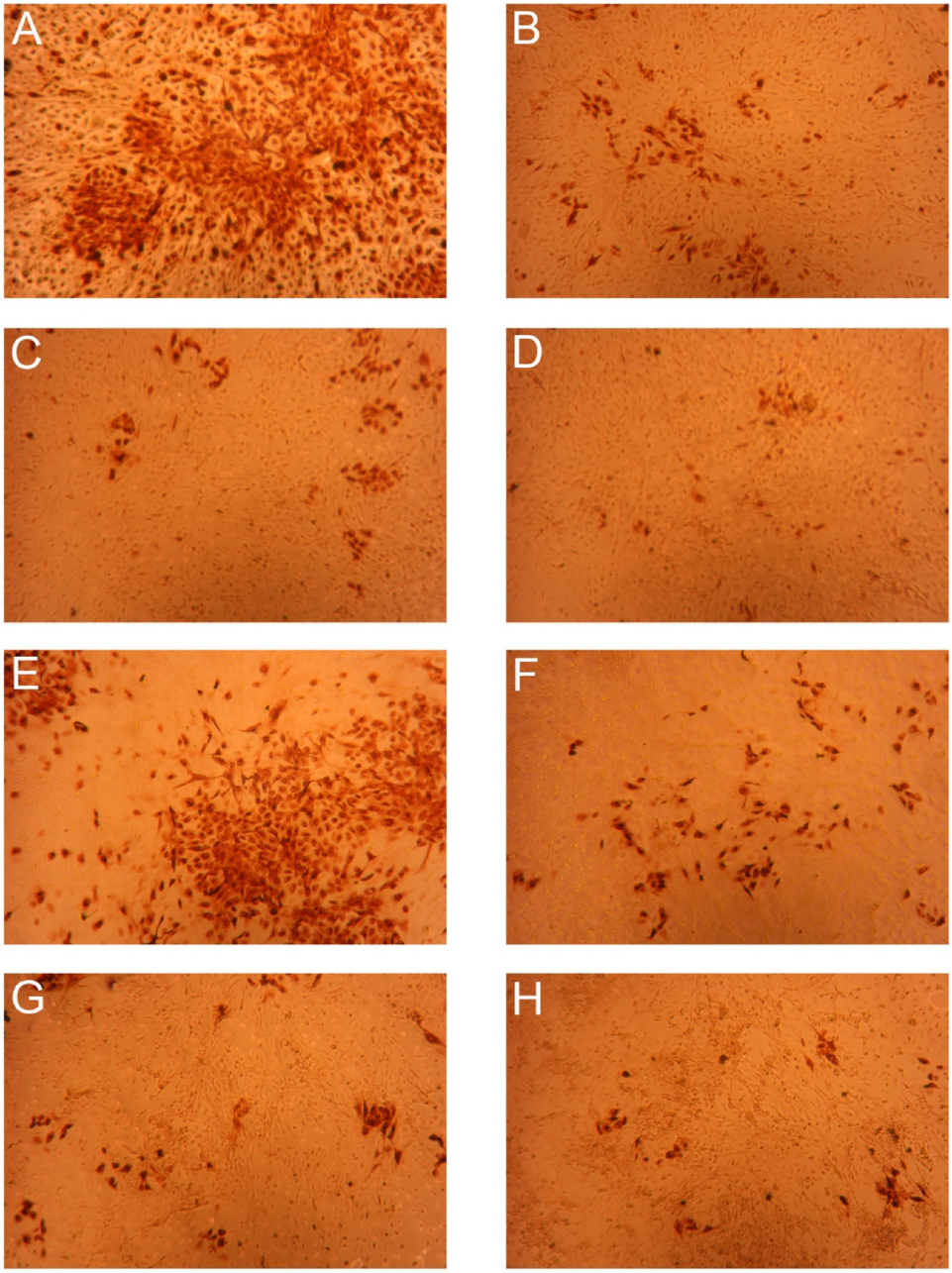

Fig. 4. Focus forming units in Vero cells infected with peptides treated and untreated DENV-1 and DENV-2. Vero cells infected with DENV-1 without peptides (Control) (**A**), infected with DENV-1treated with 40 μM Peptide 1 (**B**), Peptide 2 (**C**) and Peptide 3 (**D**). Vero cells infected with DENV-2 without peptides (Control) (**E**), infected with DENV-2 treated with 40 μM of Peptide 1 (**F**), Peptide 2 (**G**) and Peptide 3 (**H**). The virus are stained **brown** using DAB staining.

3.4. Anti-adsorption activity

The anti-dengue activity of peptides was evaluated at different time points of viral infection, including during virus adsorption. The mechanism behind the reduction in infectivity was also not clear, in terms of whether it involved binding to the E protein or blocking the receptors of recipient cells. To confirm binding to the E protein, anti-adsorption activity was examined by treating Vero and LLC-MK2 cells with viruses in the presence and absence of peptides at a concentration of 50μM.

The cells were exposed to the virus and peptide mixture for 1 h and then washed with PBS. It was observed that the cells treated with the virus and peptide mixture developed fewer FFUs than the cells treated with viruses alone, for both Vero (Fig. 7A) and LLC-MK2 cells (Fig. 7B). The percentage FFUs reduction due to Pep1 for Vero cells was as high as 96.22 ± 1.03% and 94.84 ± 0.20% against DENV-1 and DENV-2, respectively. Pep2 showed the highest focus reduction of 95.01 ± 1.74% against DENV-2 and 94 ± 3.27% in DENV-1 for Vero cells. Similarly, Pep3 also affected the adsorption of DENV-1, DENV-2, and DENV-4 by reducing the focus formation by more than 93% (Fig. 7A). However, all three peptides showed only 60% reduction against DENV-3, contrasting with the effects for the other serotypes. As shown in Fig. 7B, similar effects were also found in LLC-MK2 cells. Pep1, Pep2, and Pep3 were shown to reduce the adsorption by a maximum of 80% in DENV-1, DENV-2, and DENV-3.

3.5. Incubation of cells with peptides after viral infection does not abolish this infection

The anti-dengue activities of the peptides was evaluated at the post-adsorption stage by infecting the Vero and LLC-MK2 cells with DENV

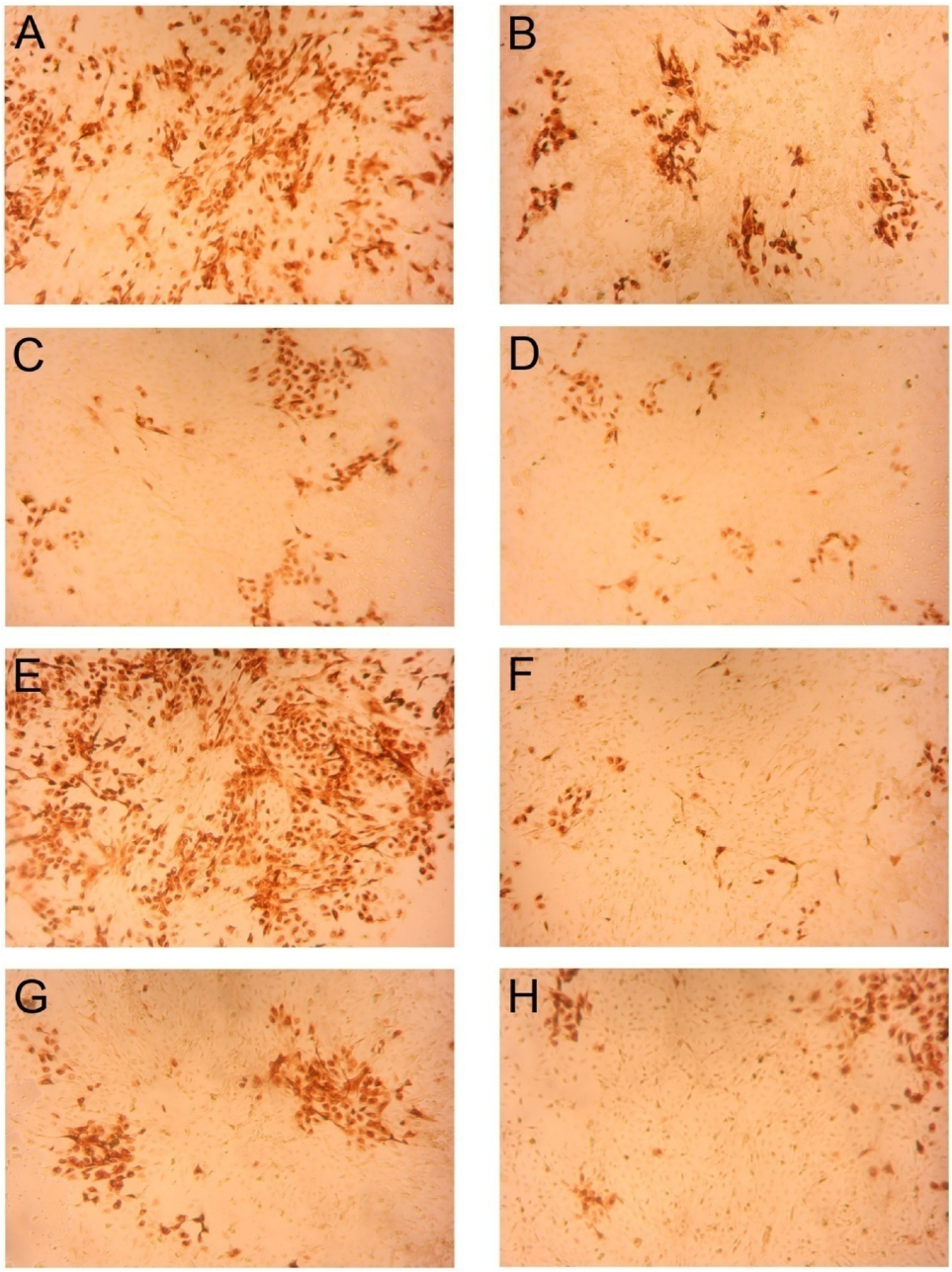

Fig. 5. Focus forming units in DENV-3 and DENV-4 infected Vero cells with or without peptides. Vero cells infected with DENV-3 without peptides (Control) (**A**), infected with DENV-3 treated with 40 μM Peptide 1 (**B**), Peptide 2 (**C**) and Peptide 3 (**D**). Vero cells infected with DENV-4 without peptides (Control) (E), infected with DENV-4treated with 40 of μM Peptide 1 (**F**), Peptide 2 (**G**) and Peptide 3 (**H**). The virus are stained **brown** using DAB staining.

for 1 h and then treating them with the peptides. Because the viral binding sites were not blocked by the peptides, viruses infected the host cells, and no significant difference was observed in viral infectivity between the control and treated wells for both Vero (Fig. 8A) and LLC-MK2 cells (Fig. 8B). Peptide treatment after virus adsorption did not show significant antiviral activity against the four serotypes of DENV or the restriction of viral spread to the neighboring cells.

4. Discussion

In patients who can potentially develop life-threatening DSS and DHF, the disease severity depends on the DENV titer. The identification of potentially effective drugs would be beneficial to patients in the viremic phase, because reducing the number of virion particles could be effective for improving the disease outcomes (Thisyakorn and Thisyakorn, 2014; Vannice et al.,2015). Apart from acting as a target for neutralizing antibodies, the E protein remains a promising target for developing antiviral agents.

The entry of viruses into host cells is a complex process. The E protein of DENV is a class II fusion protein, which facilitates viral entry into host cells via clathrin-mediated endocytosis and subsequent fusion of viral and cellular membranes (Acosta et al., 2008; Cruz-Oliveira et al., 2015; De La Guardia and Lleonart, 2014). Several studies have reported on anti-DENV inhibitors targeting the E protein (De La Guardia and Lleonart, 2014; Hrobowski et al., 2005; Panya et al., 2014; Poh et al., 2009; Schmidt et al.,2010a, 2010b, 2012; Zandi et al.,2011, 2012). Moreover, the E protein is a major target for docking small molecules, such as the stem domain (Lin et al., 2011), hydrophobic pocket (Modis et al., 2003), and receptor-binding domain III (De La Guardia and Lleonart, 2014), making it an ideal target for developing

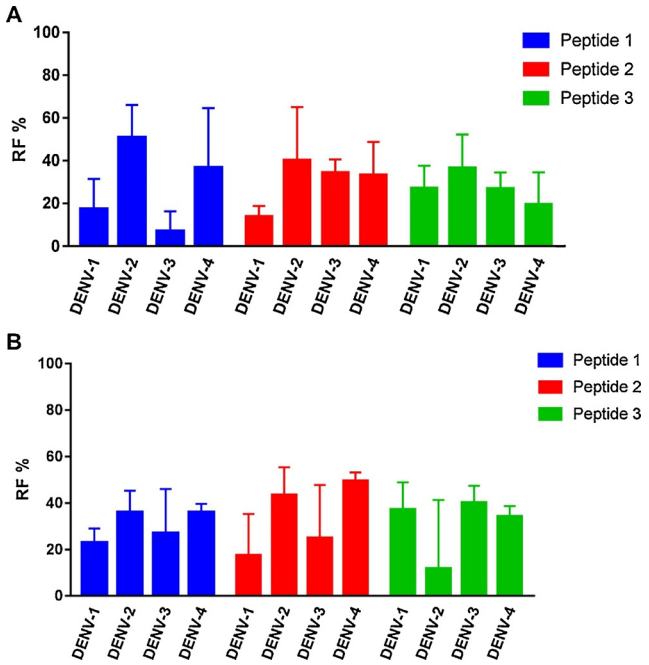

Fig. 6. Prophylactic activity of peptides on Vero (A) and LLC-MK2 cells (B). The prophylactic activity of the peptides on cells were analyzed by pretreating the cells with peptides prior viral infection. Later the cells were washed and infected with virus. Peptides introduced to cell lines prior to the viral infection reduced only 20%–50% focus forming units which suggested that the peptides have no prophylactic activitiy and reduced the infectivity of DENV by binding to E protein and not onto the receptors of host cell.

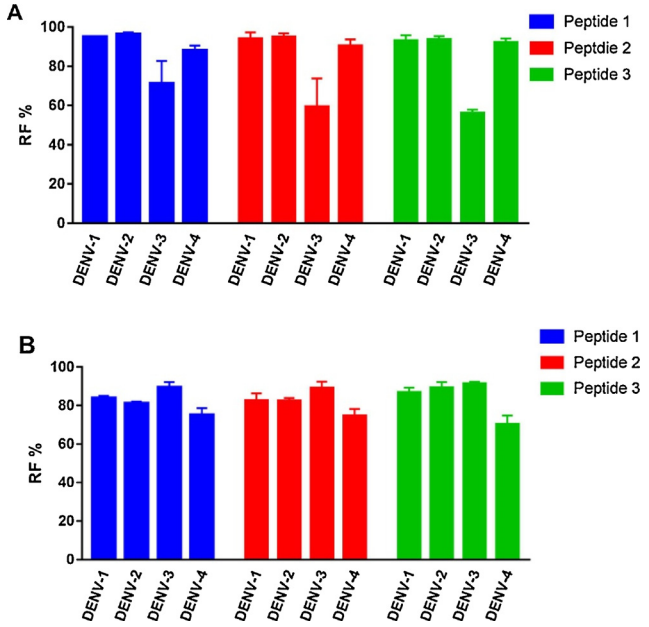

Fig. 7. Anti-adsorption activities of Peptides against DENV in mammalian cell lines. A. Vero cells B. LLC-MK2 cells. In order to understand the anti-adsorption activity of the peptides, the peptides were pre-treated with virus and later allowed to infect the cells. More than 80% reduction of foci formation units observed in this assay indicated that the peptides reduced the infectivity of DENV by binding to the surface of E protein and block the entry of DENV.

antiviral drugs. In the present study, three peptides derived from the E protein were selected because they were identified to contain a putative receptor-binding site and immunodominant epitopes. Moreover, the three peptides each comprised a part of the E protein domains. Approximately 90 E protein homodimers are presented on the surface of matured virion particles. This protein undergoes structural rearrangement in response to the reduced pH of the endosome, which leads to the insertion of fusion peptides into the target cell membrane, thereby forming a bridge between the virus and the host (Alen and Schols, 2012).

4.1. Peptide inhibition against dengue infection

Several *in vitro* studies have reported that peptides designed on the basis of the sequence of the E protein inhibit dengue infection (Costin et al., 2010;De La Guardia andLleonart 2014;Hrobowskiet al., 2005;Lalezari et al., 2003; Lok et al., 2012; Panya et al., 2014;Poh etal., 2009;Schmidt et al.,2010a, 2010b, 2012;Wang et al., 2009). For instance, peptides derived from various regions of the E protein, such as from the stem region aa 419–447 (Schmidt et al., 2010a, 2010b) and aa 692–724 (Hrobowski et al., 2005; Lok et al., 2012), domain II hinge regions aa 205–223 and 205–232 (Costin et al., 2010), and domain III region aa 380–389 (Alhoot et al., 2013), have demonstrated inhibitory effects against DENV. Envelope protein-derived peptide (DN59), corresponding to aa 692–724, demonstrated antiviral activity against DENV-2 in LLC-MK2 cells, exhibiting 99% viral inhibitory activity with an IC_{50} of 10μM (Hrobowski et al., 2005). Peptides targeted against the domain II hinge region also demonstrated an inhibitory activity of IC_{50} at a concentration of 8μM against DENV-2 (Costin et al., 2010). Moreover, a peptide (DET4; AGVKDGKLDF) targeting aa 390–389 of domain III demonstrated maximum inhibitory activity against DENV-2, with an IC_{50} of 35 μM (Alhoot et al., 2013). Here, we used peptides Pep1, Pep2, and Pep3 derived from aa 25–42 of domain I, aa 98–109 of domain II, and aa 339–354 of domain III, respectively, of the E protein of DENV (Table 1). Prior to testing the peptide inhibitory activity against DENV, the peptides were subjected to a cytotoxicity assay and were found to be nontoxic to Vero and C6/36 cells.

Upon testing the inhibitory activity of DENV, Pep2 showed the strongest inhibition against DENV-1, DENV-2, DENV-3, and DENV-4. Moreover, Pep2 is a sequence of fusion peptide located in domain II and is highly conserved among flaviviruses. It plays a significant role in the pH-dependent type II fusion process wherein fusion peptide becomes exposed and reoriented outward, thereby making it available for membrane fusion (Sultana et al., 2009). Pep1 is derived from domain I, which has an eight-stranded β-barrel with two insertion loops and two α-helices that act as a molecular hinge for the rearrangement. Pep3 mimics part of domain III, which is a receptor-binding region involved in membrane fusion. Compared with previously reported peptide inhibitors (Hrobowski et al., 2005; Lok et al., 2012; Schmidt et al., 2010a, 2010b), the peptide inhibitor used in the present study also showed similar inhibitory activity against all DENV serotypes. IC_{50} of Pep1, Pep2, and Pep3 are ranging from 10 to 33μM in four DENV serotypes (Fig. 4).

4.2. Mechanism of the peptide inhibitor

Our results showed that all three peptides have inhibitory activities against all dengue serotypes. To elucidate the mechanisms underlying these activities, experiments were performed as follows:1) preincubation of the peptides with the cells prior to viral infection, 2) preincubation of the peptides with the virus and then allowing the virus to infect the cells, and 3) postincubation of the peptides to virally infected cells.

The following observations were made: 1) preincubation of cells with peptides prior to viral infection resulted in focus reduction of < 20% (Fig. 6),2) preincubation of the virus with peptides prior to infection resulted in reduction in FFUs of > 80% compared with that in the control (Fig. 7),and 3) incubation of cells with the peptide after they had been virally infected did not reduce the DENV infection (Fig. 8). These findings indicate that rather than binding to a cellular receptor

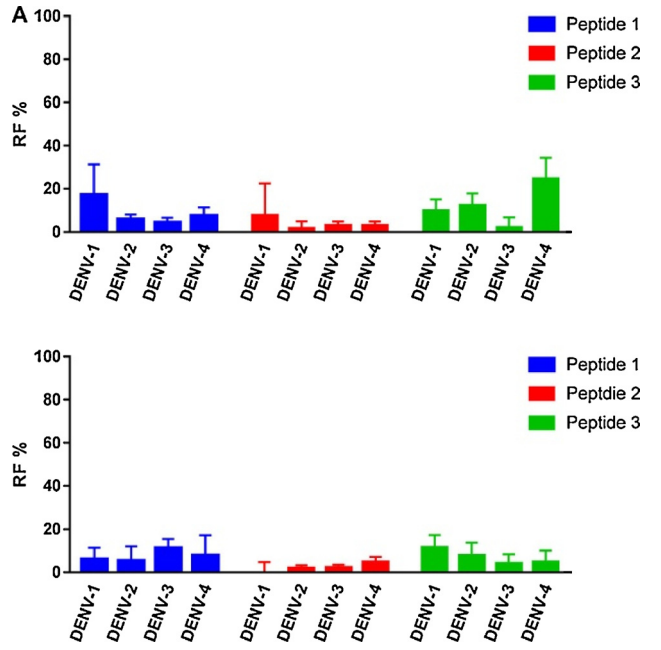

Fig. 8. Post-adsorption antiviral activities of Peptides against DENV. A. Vero cells B. LLC-MK2 cells. The post-adsorption antiviral activity of peptides was studied by infecting the cells with virus, after washing the infected cells were incubated along with peptides (for detailed information, see materials and methods). Our observation pointed out the peptides did not reduce the viral infectivity when it is introduced after the viral infection, suggesting that the peptides blocked the viral entry by binding to the E protein. When the host receptor binding sites of E protein are not blocked by peptides, DENV eventually enters into the host cells and increased the percentage of infectivity.

and blocking viral entry, the peptides bind to the virus and inhibit viral infection of the cells. The results of this study corroborate with those of previous reports on the effects of peptide inhibitors against DENV on the basis of the E protein (Costin et al., 2010; Lok et al., 2012; Schmidt et al., 2010a, 2010b).

It was reported that DN59 could interfere in the intramolecular interactions between the stem region and other parts of the class II viral fusion protein, thereby resulting in bilayer fusion (Hrobowski et al., 2005). It was subsequently found that the infectivity of DENV was reduced by DN59 peptide via destabilization of the viral membrane, which eventually releases the viral RNA genome from the particles (Lok et al., 2012). The peptides that mimic the stem region (aa419–447) of the DENV E protein particularly block viral fusion by binding to the trimeric, post-fusion sE conformer and not the prefusion dimer (Schmidt et al., 2010b). We also demonstrated that the peptides act as inhibitors by binding to the E protein, suppressing its conformational changes, and preventing its binding to the cellular receptor, thereby inhibiting viral entry.

5. Conclusions

The present study shows that Pep1, Pep2, and Pep3 exhibit anti-dengue activity toward four DENV serotypes. In particular, Pep2 showed the highest inhibitory activity toward all four DENV serotypes. These peptides inhibit viral entry by binding to the virus and not to the cellular receptors. In particular, this study demonstrated that the peptides inhibit viral infection by binding to the E protein and interfere with the conformational changes essential for viral entry. The experimental analyses proved that these peptides are potentially useful for antiviral treatment. Based on their structural and functional properties, these peptides can also be used to generate a pharmacophore model.

Conflict of interest

None.

Acknowledgments

This work was supported by the Bioinformatics Infrastructure Facility (BT/BI/25/017/2012), Bioinformatics Division, Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi. The authors would like to thank the Dean of the Faculty of Tropical Medicine, Mahidol University, Bangkok, for financial support. The authors would also like to thank Dr. S. Saira Banu, Associate Professor of English, for English correction and proofreading.

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