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# Computational identification of self-inhibitory peptides from envelope proteins

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**ABSTRACT**

**Fusion process is known to be the initial step of viral infection and hence targeting the entry process is a promising strategy to design antiviral therapy. The self-inhibitory peptides derived from the enveloped (E) proteins function to inhibit the protein–protein interactions in the membrane fusion step mediated by the viral E protein. Thus, they have the potential to be developed into effective antiviral therapy. Herein, we have developed a Monte Carlo-based computational method with the aim to identify and optimize potential peptide hits from the E proteins. The stability of the peptides, which indicates their potential to bind *in situ* to the E proteins, was evaluated by two different scoring functions, dipolar distance-scaled, finite, ideal-gas reference state and residue-specific all-atom probability discriminatory function. The method was applied to α-helical Class I HIV-1 gp41, β-sheet Class II Dengue virus (DENV) type 2 E proteins, as well as Class III Herpes Simplex virus-1 (HSV-1) glycoprotein, a E protein with a mixture of α-helix and β-sheet structural fold. The peptide hits identified are in line with the druggable regions where the self-inhibitory peptide inhibitors for the three classes of viral fusion proteins were derived. Several novel peptides were identified from either the hydrophobic regions or the functionally important regions on Class II DENV-2 E protein and Class III HSV-1 gB. They have potential to disrupt the protein–protein interaction in the fusion process and may serve as starting points for the development of novel inhibitors for viral E proteins.**

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**Key words: peptide inhibitor; envelope protein; dengue virus; HIV-1 gp41; HSV-1 gB; optimization.**

## INTRODUCTION

As fusion process is the initial step of viral infection, targeting the entry process represents a promising strategy to design antiviral therapy.[1] The entry step involves the fusion of the viral and the cellular receptor membranes mediated by viral envelope (E) proteins. There are mainly three types of envelop proteins[2]: Class I E proteins are mainly α-helical structures which are exemplified by influenza virus hemagglutinin and retrovirus Human Immunodeficiency Virus 1 (HIV-1) gp41; Class II E proteins are mainly in β-sheet organization comprising the Flaviviridae family that includes a number of important human flavivirus pathogens such as Dengue virus (DENV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV), West Nile virus (WNV), hepatitis C virus (HCV), and Togaviridae family represented by the alphavirus Semliki Forest virus; Class III E proteins are generally a mixture of α-helix and β-sheet and comprised of the vesicular stomatitis virus, Herpes Simplex virus-1 (HSV-1), and Human cytomegalovirus (HCMV). Although the exact fusion mechanism remains elusive, it was suggested that three Classes of viral fusion proteins adopt similar hairpin conformation, and therefore, may share a similar mechanism of membrane fusion.[3]

Dengue fever is mosquito-borne disease caused by one of the four serotypes of DENV. There are about 50–100 million infection cases of dengue fever each year in more than 80 countries all over the world, and the infection results in about 500,000 cases of dengue hemorrhagic

Additional Supporting Information may be found in the online version of this article.

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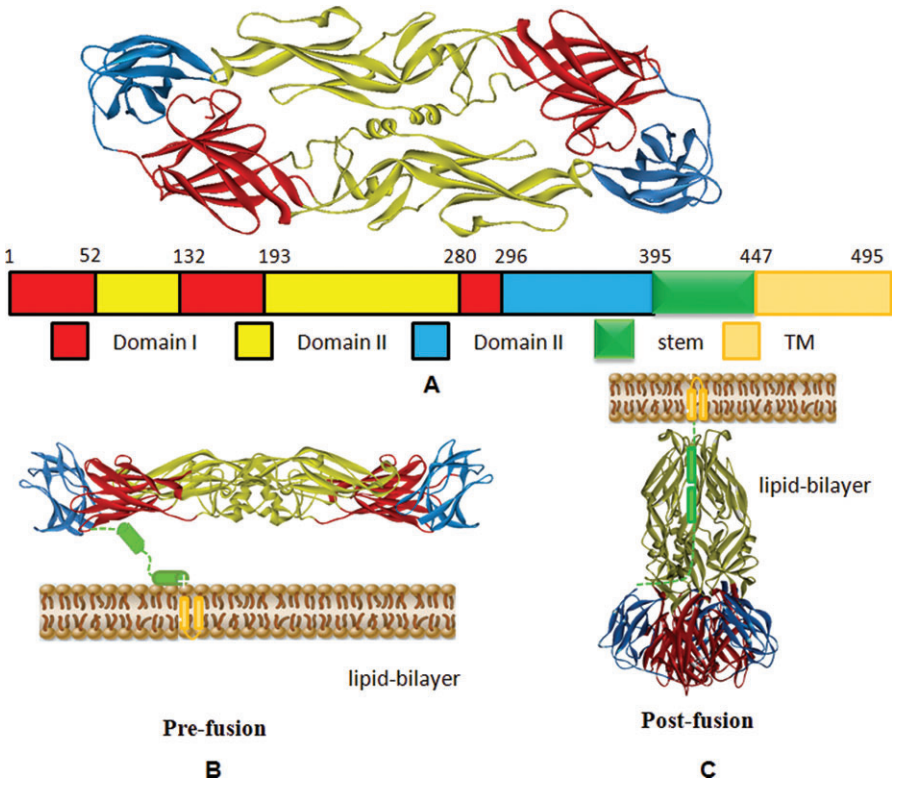



**Figure 1**

The structural construct of the DENV-2 E protein. (**A**) Schematic representation of E protein Domain I (red), Domain II (yellow), Domain III (cyan), Stem (green), and Anchor (orange). (**B**) Schematic representation of prefusion E protein homodimeric structure on membrane. (**C**) Schematic representation of postfusion E protein homotrimeric structure on membrane. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

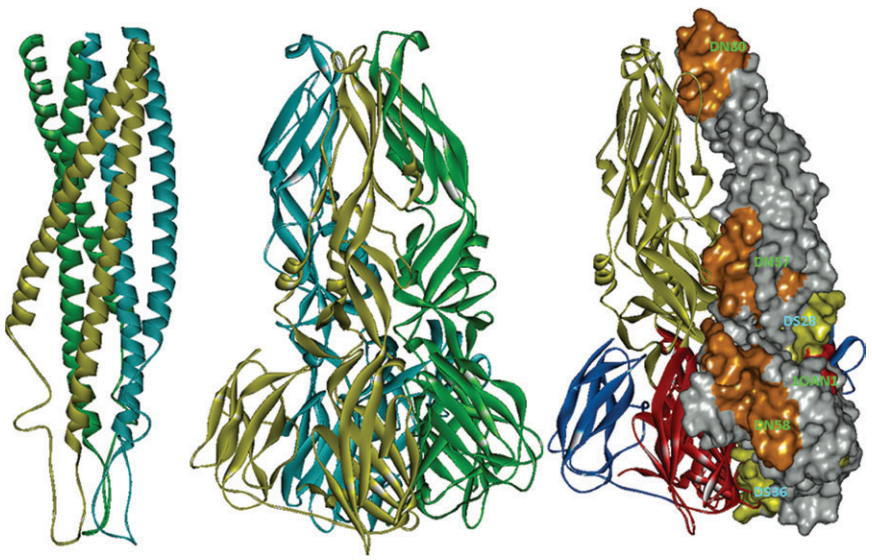



**Figure 2**

Postfusion structures of the E proteins. (**A**) Trimeric HIV-1 gp41 glycoprotein. (**B**) Trimeric DENV-2 E protein. (**C**) The inhibitory peptide regions identified on DENV-2 E protein. The hydrophobic inhibitory regions, DN80, DN81/DN57, and DN58 are shown in orange; the inhibitive peptide 1OAN1 is shown in red and the predicted inhibitory peptide regions DS28 and DS36 are shown in yellow. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

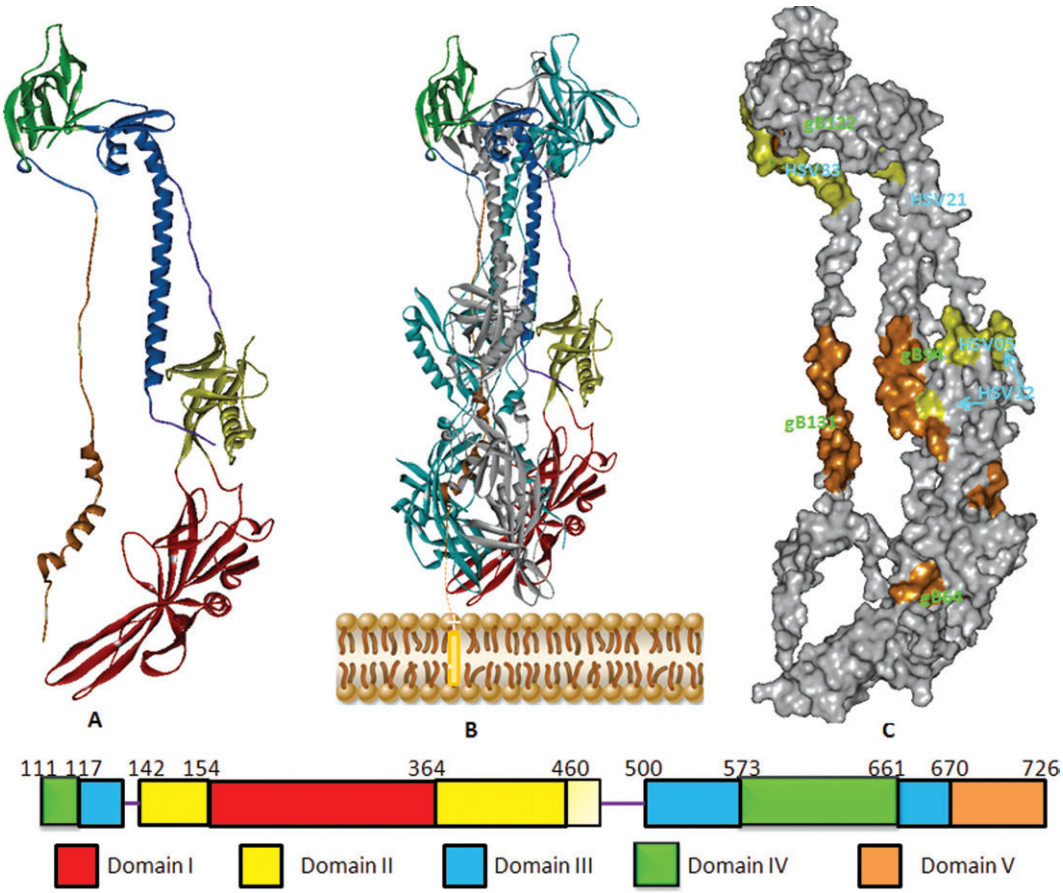



**Figure 3**

Postfusion structures of the Class III HSV-1 glycoprotein B. (**A**) Domain I-V shown in red, yellow, blue, green, and orange, respectively. (**B**) Schematic representation of the postfusion HSV-1 gB on membrane. (**C**) The inhibitory peptide regions identified on HSV-1 gB. The active peptide regions, gB64, gB94, gB122, and gB131 are shown in orange, and the predicted inhibitory peptide regions HSV05, HSV12, HSV21, and HSV33 are shown in yellow.

fever (DHF) that requires hospitalization[4] and about 25,000 cases of death caused by the more severe form of DHF, Dengue Shock Syndrome.[5] Although vaccines are currently available for JEV, TBEV, and YFV, there is no vaccine for DENV and WNV.

The infection by DENV is initiated by fusion of the viral and cellular membranes, a process mediated by DENV E protein. Dengue E protein includes three structural domains (DI, DII, and DIII) and a stem segment which links the stably folded soluble E (sE) fragment with the C-terminal transmembrane anchor (Fig. 1). DI, the N-terminal portion of the protein, is located in the middle of the Dengue E protein. The loop region at the tip of the extended DII is involved in fusion of the viral and the target membrane.[6] Electron microscopy has shown that the E protein undergoes a significant structural rearrangement to the reduced pH of an endosome when DENV enters a host cell,[7] which brings about the fusion between DENV and host-cell membranes (Fig. 1). Domain III has been shown to become putative receptor-binding domain where virus is attached to the host cell.[8] Furthermore, antibody neutralization epitopes were reported to be located on the DIII of the E protein.[9–12]

The essential step in the HIV-1 infection is the fusion between the viral and the host-cell membranes mediated by the interaction between heptad repeat 1 and heptad repeat 2 of HIV-1 envelop glycoprotein and the formation of a stable six-helix bundle structure [Fig. 2(A)]. The conformational change during the formation of the six-helix bundle drives the membrane fusion, and agents that prevent six-helix formation would block HIV entry.[13–15]

Class III Fusion proteins display a combination of α-helical and β-structural folds. It was revealed from the crystal structure of HSV-1 glycoprotein B (gB)[16] that its central α-helical coiled-coil domain III resembles Class I proteins, whereas two extended β-hairpins in domain I of HSV-1 gB with hydrophobic tips inserting into the cell membrane, resemble the fusion loop in the Class II Proteins (Fig. 3).

Peptides derived from the protein–protein interface have been shown to be able to mimic the modes of binding of its original domain to its specific partner protein. Thus, they may serve as promising leads for drug development.[17] Peptides which mimic the regions of HIV-1 gp41 have been demonstrated as effective inhibitors for the HIV entry process. Enfuvirtide (T20), a peptide that mimics the helix of the post fusion six-helix bundle HIV-1 gp41 protein and effectively inhibits conformational change during fusion and thus prevents viral infection, is the first FDA-approved HIV-1 fusion inhibitor.[18–20] The success of developing peptide inhibitors derived from HIV-1 gp41 into clinical use has triggered interests for the design of inhibitors to interrupt protein–protein interactions in the fusion process of Class II and Class III envelope proteins. Recently, a peptide derived from HCV was shown to inhibit the postbinding step in HCV entry.[21] Several hydrophobic peptides derived from DENV and WNV E proteins were found to have potent inhibitory activities.[22] These dengue peptide inhibitors were further optimized by a computational approach and two optimized peptides in domain II showed improved inhibitory activities. In addition, a novel peptide inhibitor designed on the basis of the DENV-2 E protein exhibits potent inhibition activity toward the DENV.[23] Recently, it was reported that peptides derived from Class III HSV-1 gB and HCMV gB showed inhibitory activities toward the viruses.[24–26]

Here, we developed a Monte Carlo-based optimization method to identify the druggable regions in E proteins, and to optimize peptide hits as potential inhibitors to disrupt the protein–protein interactions in membrane fusion mediated by the E proteins.

## MATERIALS AND METHODS

### Computational identification of hydrophobic region as potential peptide inhibitors

A physico-chemical algorithm, the Wimley–White interfacial hydrophobicity scale (WWIHS),[27] was used to identify the regions on the DENV-2 E protein and HSV-1 gB that would preferably interact with the hydrophobic surface within the E proteins and therefore have the potential to be developed into inhibitors to prevent viral entry and viral infectivity.

### Optimization of potential peptide inhibitors

A 20-amino acid sliding window was moved from the N- to the C-terminus of the Dengue E protein (PDB code: 1OAN, chain A)[28] in an increment of 10 amino acids. A window of 20 amino acids was settled to slide the sequence of the E protein sequence as it is approximately the length of potential inhibitory peptides.[22] Thirty-eight 20-mer peptides were obtained by sliding along the crystal structure of DENV-2 E protein, and were then used as templates for amino acid mutations. For each of the peptides, the position for amino acid mutation was selected randomly and the selected amino acid was replaced by one of the other 19 naturally occurring amino acids. The amino acid replacement was performed using a backbone-dependent side-chain rotamer library and a linear repulsive steric energy term implemented in SCWRL (version 4.0).[29]

The resulting peptide was subjected to 200 steps minimization using the AMBER ff99SB force field[30] in the AMBER package (version 11).[31] The stability of the proteins following amino acid mutations was evaluated by two scoring functions, the residue-specific all-atom probability discriminatory function (RAPDF)[32] and dDFIRE (dipolar distance-scaled, finite, ideal-gas reference state), a knowledge-based statistical energy function.[33]

The RAPDF is based on the conditional probability function that represents preference of atomic distance. The score is given by the following logarithm of conditional probability:

$$S(\{d_{ab}^{ij}\}) = -\sum_{ij} \mathrm{In}\frac{\mathrm{P}(d_{ab}^{ij}|C)}{\mathrm{P}(d_{ab}^{ij})}\alpha - \mathrm{InP}(C|\{d_{ab}^{ij}\}) \qquad (1)$$

where

$P(C)$: the probability that any structure picked at random is a member of the "correct" set.

$P(d_{ab}^{ij}|C)$: the probability of observing a distance $d$ between two atoms $i$ and $j$ of types $a$ and $b$ in a correct structure.

$\mathrm{P}(d_{ab}^{ij})$: the probability of observing such a distance in any structure, correct or incorrect.

$\mathrm{P}(C|d_{ab}^{ij})$: the probability the structure is a member of the "correct" set, given it contains the distances $\{d_{ab}^{ij}\}$.

In the dDFIRE-based statistical energy function, the atom–atom potential of mean force between two polar atoms $p$ and $q$, $\bar{u}^{\mathrm{dDFIRE}}(r_{pq}, \theta_p, \theta_q, \theta_{pq})$ is given by

$$\bar{u}^{\mathrm{dDFIRE}}(r_{pq}, \theta_p, \theta_q, \theta_{pq}) = \bar{u}^{\mathrm{dDFIRE}}(r_{pq}) + \bar{u}(\theta_p|r_{pq}) + \bar{u}(\theta_q|r_{pq}) + \bar{u}(\theta_{pq}|r_{pq}) \qquad (2)$$

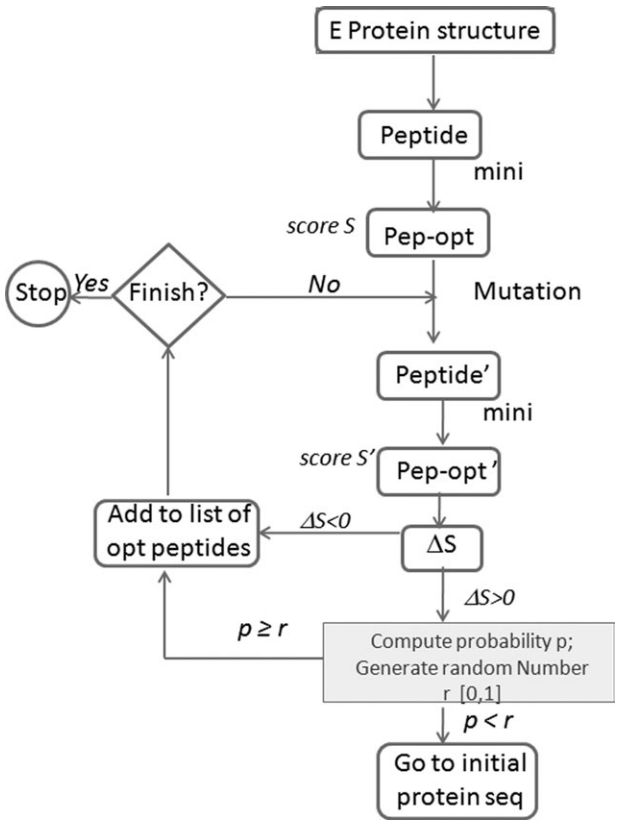
where

$$\bar{u}(\theta_p|r_{pq}) = -RT\mathrm{In}[P_{pq}^{\mathrm{obs}}(\theta_p|r)/P_{pq}^{\mathrm{obs}}(\theta_p|r_{\mathrm{out}})]$$

$$\bar{u}(\theta_q|r_{pq}) = -RT\mathrm{In}[P_{pq}^{\mathrm{obs}}(\theta_q|r)/P_{pq}^{\mathrm{obs}}(\theta_q|r_{\mathrm{out}})]$$

$$\bar{u}(\theta_{pq}|r_{pq}) = -RT\mathrm{In}[P_{pq}^{\mathrm{obs}}(\theta_{pq}|r)/P_{pq}^{\mathrm{obs}}(\theta_{pq}|r_{\mathrm{out}})]$$

Here, $P_{pq}^{\mathrm{obs}}(\theta_p|r), P_{pq}^{\mathrm{obs}}(\theta_q|r)$ *and* $P_{pq}^{\mathrm{obs}}(\theta_{pq}|r)$ are conditional probabilities.




**Scheme 1**

Identification and optimization of self-inhibitory peptides from Envelope proteins by Monte Carlo method. The peptide stability scores were calculated using RAPDF or dDFIRE scoring functions.

Considering the computational demand required, for each amino acid position that was randomly selected in the peptides, the amino acid substitutions were performed 10 times randomly, namely, each amino acid was replaced by 10 different amino acids out of all the 19 possible amino acids, and the most stable peptides were saved for subsequent substitution. The entire selection procedure was based on the Metropolis Monte Carlo criterion so that the search was biased toward the most stable peptide with the global low energy (Scheme 1).[34] The substitution iteration was performed 20,000 times for each peptide to retain the peptides with the best stability scores.

Similar procedure was applied to optimize the peptides that were retrieved from the HIV-1 gp41 E protein. A 30-amino acid sliding window was moved from the N- to the C-terminus of the HIV-1 gp41 E protein model structure that was built based on the NMR structure of HIV gp41 (PDB code: 1IF3),[35] in an increment of 10 amino acids. Ten 30-mer peptides were obtained by sliding the structure of gp41 E protein and used as templates for subsequent amino acid mutations.

The recently reported self-derived peptide inhibitors for HSV-1 gB have the length of 15 amino acids,[24] and therefore a 15-amino acid sliding window was moved along the C-terminus half of the HSV-1 gB ectodomain (PDB code: 2GUM, Chain B).[16] The missing loops 331–336 and 462–490 were built using Modeller (version 9.9. UCSF, USA).

## RESULTS AND DISCUSSION

### Identification of inhibitory peptides for HIV-1 gp41

#### *Evaluating the stability of the substituted protein using dDFIRE scoring function*

The known HIV-1 peptide inhibitors[36,37] (Table I) can be approximately divided into two groups: the first group includes 5-Helix, N36, and DP107, which are mainly located around the amino acid sequence 5–51; the other group of peptide inhibitors consists of C34, HIV-31, T20, and T-1249, which contain the similar amino acid sequence of 97–126. These known peptide inhibitors span the α-helical structure of the HIV-1 gp41 structure.

To identify potential peptide inhibitors, a 30-residue sliding window was moved from the N-terminus to the C-terminus of the HIV-1 gp41 protein structure in an increment of 10 amino acids. Ten 30-mer peptides were obtained and denoted sequentially as HIV01 (1–30), HIV02 (11–40), HIV03 (21–50), and so on up until HIV10 (91–120).

For each 30-mer peptide, the position of each amino acid was randomly selected and substituted by the other 19 native amino acids. A Monte Carlo optimization method was used to identify the potential peptide inhibitors based on the stability of the mutated 30-residue peptides. The regions with the best scores indicate the locations on the proteins where potential peptide inhibitors may bind *in situ*. The stability was first evaluated using dDFIRE, a statistical energy function that treats each polar atom as a dipole with a direction. dDFIRE has been shown to be effective in *ab initio* protein folding.[33] Although the HIV-1 gp41 structure is a model built using the NMR structure of SIV gp41 as template, the

**Table I**

Fusion Peptide Inhibitors for HIV-1 gp41

| Name | Location | Potency (in vitro) |
|---|---|---|
| 5-Helix | 5–38 | nM |
| N36 | 7–42 | nM – μM |
| DP107 | 14–51 | nM – μM |
| C34 | 89–122 | nM |
| HIV-31 | 99–125 | nM |
| T20 | 99–126 | nM |
| T-1249 | 97–126 | nM |

The inhibitor potency is given as the approximate 50% inhibitory concentration in nM or μM ranges in standard *in vitro* infectivity assays.[36]
The Locations are the position of the inhibitors in the HIV-1 gp41 E protein.

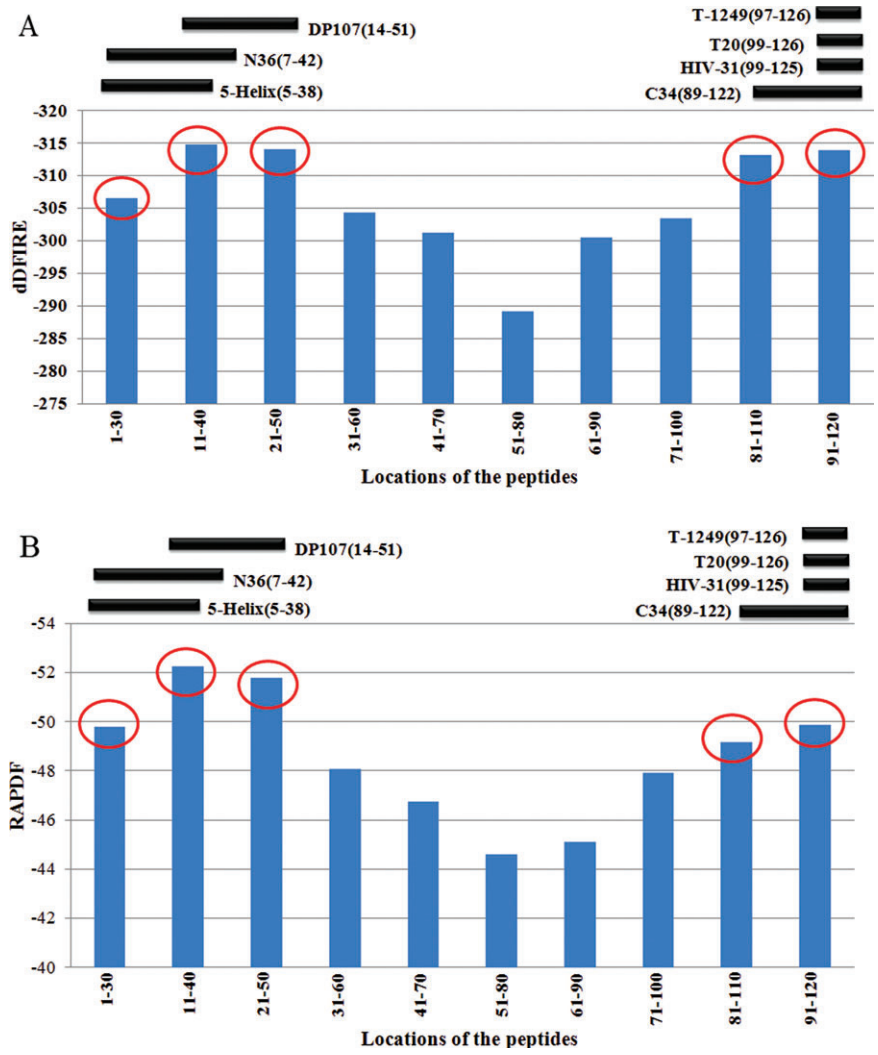



**Figure 4**

(**A**) The regions of the predicted potential peptide inhibitors for HIV-1 gp41. The *x*-axis is the location of the peptides derived from HIV-1 gp41 and the *y*-axis represents dDFIRE scores. (**B**) The regions of the predicted potential peptide inhibitors for HIV-1 gp41. The *x*-axis is the location of the peptides derived from HIV-1 gp41 and the *y*-axis represents RAPDF scores. The regions that correspond to the known peptide inhibitors of DENV are circled in red. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

structure is sufficiently robust because HIV-1 gp41 and SIV gp41 share high similarity in functions, biochemistry, and structures, as well as high sequence identity (46%) around the loop region.[38]

Two clusters of regions with the best (lowest) dDFIRE scores were identified [Fig. 4(A)]. The first cluster included three amino acid sequences, HIV01 (1–30), HIV02 (11–40), and HIV03 (21–50). The amino acids in the first cluster have better dDFIRE scores than the neighboring regions of amino acid sequence and are comprised in the self-inhibitory peptide inhibitors, 5-Helix, N36, and DP107. The HIV01 peptide has a slightly higher score than the rest three amino acid sequences in the first cluster, presumably because only part of the HIV01 is contained in the active inhibitor 5-Helix. The other cluster of regions comprised four amino acids, HIV09 (81–110) and HIV10 (91–120) which have lower dDFIRE scores than the neighboring regions. Two amino acids regions, HIV09 and HIV10, are contained in all the four active inhibitors (C34, HIV-31, T20, and T-1249). HIV08 (71–100) is only partially comprised in the C34 peptide inhibitor, whereas residues 71–82 in HIV08 span the loop region. As none of the HIV-1 gp41 inhibitors has been derived from the loop region, HIV08 is associated with a relatively higher dDFIRE score compared with HIV09 and HIV10. Generally, these inhibitory regions identified by the Monte Carlo method combined with dDFIRE scoring function are in good agreement with the known self-derived HIV-1 inhibitors that span mainly the α-helical region

**Table II**
Five Hydrophobic Regions Identified on DENV-2 E Protein using WWIHS Hydrophobicity Scale and the Optimized Peptides Obtained using RAPDF Biased Monte Carlo Method

| Name | Sequence | Location | $IC_{50}(\mu m)$ |
|---|---|---|---|
| DN80wt | MVDRGWGNGCGLFGKGGIV | 96–114 **DII** | – –[22] |
| **DN80opt** | WFCCFFVIYCWCFCCCCCQ | | |
| DN81wt | AWLVHTQWFLDLPLPWLPG | | – –[22] |
| **DN81opt** | **WIFIRYEFFRSFKFLWRGN** | 205–223 **DII** | |
| DN81opt[23] | RQMRAWGQDYQHGGMGYSC | | 36 ± 6[23] |
| DN57wt | AWLVHTQWFLDLPLPWLPGADTQGSNWI | | – –[22] |
| **DN57opt** | **WYFIRKEFFERIRFLPQRNPHRRDDEWD** | 205–232 **DII** | |
| DN57opt[23] | RWMVWRHWFHRLRLPYNPGKNKQNQQWP | | 8 ± 1[23] |
| DN58wt | GDSYIIIGVEPGQLKENWFKKGSSIGQMF | 374–402 DIII | |
| **DN58opt** | **TWWCFYFCRRHHPFWFFYRHN** | | |
| DN59wt | MAILGDTAWDFGSLGGVFTSIGKALHQVFGAIY | 412–444 stem | $10^{22}$ |

Tested peptides that did not reach 50% inhibition activity are marked with dashed lines. The optimized peptide sequences from this study are highlighted in bold. Part of the DN58 sequence, and the whole of DN59 sequence are labelled in grey as their coordinates are not known.

of the gp41 glycoprotein. We also calculated the stability of the 10 30-mer amino acid sequences using an updated DFIRE energy function, DFIRE2.0[39] where dipolar interactions are neglected. Similar result was observed (Supporting Information Fig. S1). For the rest of the E proteins, only dDFIRE function was employed.

#### *Evaluating the stability of the substituted protein using RAPDF scoring function*

In addition to dDFIRE, we also implemented RAPDF scoring function in the Monte Carlo-based optimization method to select the most stable peptides. RAPDF measures the binding pseudoenergy and was previously used to predict structural stability of proteins and potential of *in situ* binding to the partner regions. It correlated well with the experimentally determined inhibition activities of viral-target cell membrane fusion.[40] In this study, RAPDF has also been shown to identify the three potent inhibitory peptides, HIV01, HIV02, and HIV03 that corresponded to the first group of inhibitors [Fig. 4(B)]. Moreover, the HIV9 and HIV10 peptides were also identified with low RAPDF scores, indicating they have the potential to bind to the opposite helix in HIV-1 gp41 protein. Therefore, a Monte Carlo-based selection method combined with dDFIRE scoring function is as effective as that combined with RAPDF to identify potent peptide inhibitors for the Class I α-helical E proteins.

### Identification of inhibitory peptides for DENV-2 E protein

#### *Identification of hydrophobic regions on DENV-2 E*

A physicochemical algorithm, WWIHS, was used to identify the hydrophobic regions where potential peptide inhibitors may bind. The sequence of DENV-2 E protein was scanned and five hydrophobic regions, DN80 (amino acids 96–114), DN81 (205–223), DN57 (205–232), DN59 (412–444), and DN58 (374–402) were identified (the denotations used in the previous literature[22] are utilized, Table II). Among them, DN80 located at the tip of the extended domain II was suggested to be involved in the viral fusion with the endosomal membrane and the release of the genome into the host cytosol.[8] Recently, a novel epitope for antibody neutralization $^{98}$DRXW$^{101}$ was identified in the fusion loop region.[41] DN57 and DN81 are in close proximity to the kl hairpin 270–279, which was shown to be a potential binding pocket of inhibitors, and significant conformational change around this region was observed during the fusion process.[28] DN59 is located in the highly conserved stem region of the DENV-2 E protein and several promising self-inhibitory peptide inhibitors have recently been identified around this region.[42,43] DN58 peptide spans from domain III to the stem region of the DENV-2 E protein. Domain III of E protein undergoes the most significant displacement from prefusion homodimeric to postfusion homotrimeric structures.[6] It has been demonstrated that exogenous domain III is a dominant-negative inhibitor of flavivirus membrane fusion and infection.[44] Hence, we propose that peptides which mimic this region may prevent the conformational change and therefore inhibit the membrane fusion in the viral entry step. Although the structure of the amino acids 395–402 located in the stem region has not yet been resolved, the 3D co-ordinates of the DN58 sequence 374–394 located in Domain III have been known from the crystal structure of DENV-2 E protein[28] and could be used in the peptide optimization.

### Evaluating the stability of the substituted protein using dDFIRE scoring function

A 20-residue sliding window was moved from the N-terminus to the C-terminus of the DENV-2 E protein in an increment of 10 amino acids. Thirty-eight 20-mer peptides were generated and used as templates for amino

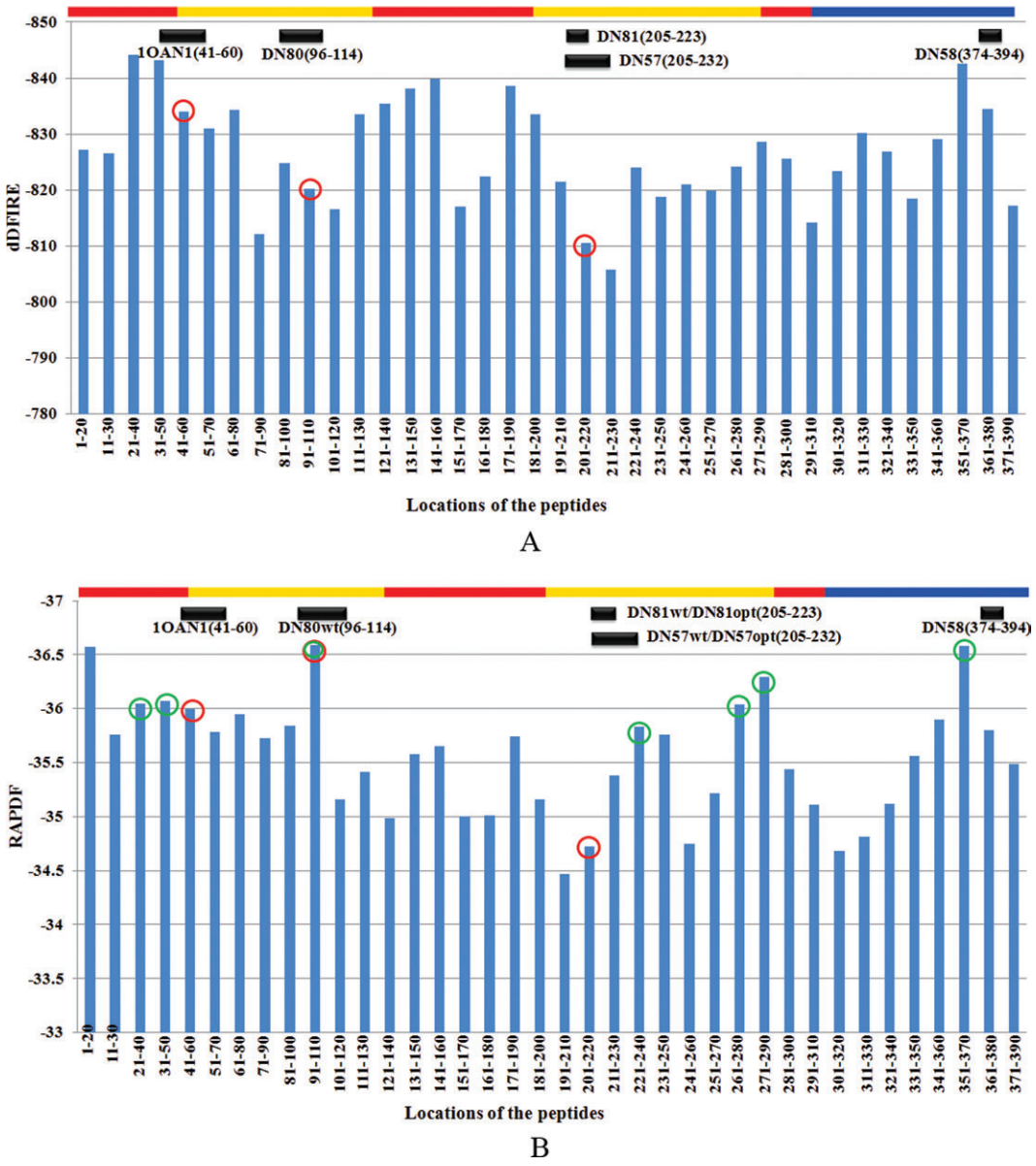



**Figure 5**

(**A**) The regions of the predicted potential peptide inhibitors for DENV-2 E. The *x*-axis is the location of the peptides derived from DENV-2 E and the *y*-axis represents dDFIRE scores. (**B**) The regions of the predicted potential peptide inhibitors for DENV-2 E. The *x*-axis is the location of the peptides derived from DENV-2 E and the *y*-axis represents RAPDF scores. The regions that correspond to the known peptide inhibitors of DENV are circled in red, and the novel regions that were predicted to have potential inhibitory activities are circled in green. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

acid substitution. They were denoted sequentially as DS01 (amino acids 1–20), DS02 (11–30), DS03 (21–40), and so on up until DS38 (371–390).

Both the four hydrophobic regions (DN57, DN80, DN81, and DN58) and the 38 20-mer peptides were subjected to amino acid substitutions. Each of the amino acid in the peptides was randomly substituted by one of the other 19 native amino acids, and the stability of the resulting peptides was evaluated using dDFIRE and RAPDF scoring functions. The entire Monte Carlo-based optimization protocol was similar to that applied to the HIV-1 gp41 E protein.

The score of DS05 (amino acids 41–60) was used as the reference to identify potential peptide inhibitors for DENV-2 E protein as this peptide region corresponds to the active DENV-2 E protein inhibitor 1OAN1.[23] Four clusters of inhibitory regions with dDFIRE scores comparable to that of 1OAN1 were identified (Fig. 5). The first cluster consists of three amino acids, DS03 (21–40), DS04 (31–50), and DS05. Except for DS05 which spans

**Table III**
The Optimized Peptides Derived from the DENV-2 E Protein using RAPDF Biased Monte Carlo Method

| Name | Sequence | Location |
|---|---|---|
| DS03wt | VDIVLEHGSCVTTMAKNKPT | 21–40 |
| DS03opt | FPFDFHHDRYYHFHWKRYQH | |
| DS04wt | VTTMAKNKPTLDFELIKTEA | 31–50 |
| DS04opt | IWWRPRDWPTFIFYFREWRW | |
| DS10wt | VCKHSMVDRGWGNGCGLFGK | 91–110 |
| DS10opt | IVIIYFCCCCLYCCCWCYCC | |
| DS27wt | HTALTGATEIQMSSGNLLFT | 261–280 |
| DS27opt | KEYFRRFFHCHNHQREWHWH | |
| DS28wt | QMSSGNLLFTGHLKCRLRMD | 271–290 |
| DS28opt | KEKRREWEWRFRWEFRLYFE | |
| DS36wt | LITVNPIVTEKDSPVNIEAE | 351–370 |
| DS36opt | RHWEQFYFRRRERKFWLFFW | |

the domain I and domain II, the low-score peptides are located in domain I of the E protein. Both of the second cluster populated around DS13 (121–140), DS14 (131–150), and DS15 (141–160), and the third cluster centred on DS18 (171–190) are located in domain I. The fourth cluster, focused on DS36 (351–370), is located in domain III, which was suggested to be involved in the receptor binding.

## Evaluating the stability of the substituted protein using RAPDF scoring function

We also used RADPF to assess the stability of the peptides and their potential *in situ* binding affinities. Six scattered clusters were disclosed by the Monte Carlo-based selection, implemented with RAPDF scoring function. The first cluster, comprised DS03, DS04, and DS05, was similar to those identified by dDFIRE, although the difference in the RAPDF scores of the three peptides is less obvious compared to those observed in dDFIRE. The low RAPDF score associated with DS05 is in good agreement with the observed inhibitory activity of 1OAN1 (41–60), which covers the same region as DS05. The optimized peptide DS10 (91–110) was observed to exhibit an exceptional low RAPDF score. This peptide, mainly comprised Cys and aromatic amino acids, is located in the domain II (Table III). The location of DS10 dovetails with DN80wt, a peptide inhibitor derived from DENV-2 E protein.[22] DS21 (201–220) corresponds to the inhibitory peptides DN81/DN57, two self-inhibitory peptides derived from the hydrophobic regions of DENV-2 E protein. Low-stability scores associated with DS21 were not observed either with the dDFIRE or with the RAPDF scoring function. However, the adjoining cluster centred on DS23 (221–240) was observed to have comparable RAPDF score to that of 1OAN1. The good score associated with DS23 may be accounted by its partial coverage of the location of the inhibitory peptides DN81/DN57.

Among the four hydrophobic peptides tested in the previous studies, only the stem region DN59 inhibited DENV infection, and all other three hydrophobic regions, DN57, DN81 on domain II, and DN80 at the tip of the domain II did not reach 50% of inhibition in the micromolar range.[22] Recently, DN57, DN81, and DN80 were optimized using a computational approach that utilized ENCAD force field to minimize the mutated protein structures.[23] Although the optimized DN57 and DN81 exhibited improved inhibitory activities, the optimized DN80 showed no activity. Unlike the previously reported computational approach that used ENCAD force field, our method implemented AMBER force field to minimize the protein structures and identified an optimized DN80 region (as well as corresponding DS10) that is associated with exceptional good RAPDF score [Fig. 5(B)], indicating the optimized DN80 may possess promising inhibitory activity.

Six amino acids, $N_{37}$, $Q_{211}$, $D_{215}$, $P_{217}$, $H_{244}$, and $K_{246}$ that are conserved in the DENV-2 E species and exposed in the prefusion dimer or postfusion trimer of DENV-2 E protein were identified by computational analysis.[45] These sites are suggested to comprise the possible binding sites and would become potential target for the development of diagnostics and vaccines to DENV. Three highly conserved sequence regions, $V_{24}$–$D_{42}$, $V_{97}$–$S_{102}$, and $V_{208}$–$W_{220}$ where the binding sites were derived, are contained in the peptides that displayed the highest RAPDF scores (DS05, DS10, and DS23) in our study. This suggests that these peptides may bind at the hot-spot regions along the protein–protein interface and are therefore particularly important for the development of potential DENV inhibitors.

While only 1OAN1 region was identified by the Monte Carlo optimization method implemented with the dDFIRE scoring function, all of the three known DENV-2 peptides inhibitory regions, namely 1OAN1 and the hydrophobic region peptides (DN80, DN81/DN57) were identified by the RAPDF-based peptide stability evaluation in the Monte Carlo method. This indicates that RAPDF to be more suitable for the identification of inhibitory regions for β-sheet Class II E proteins.

We examined the minimized conformation of the optimized peptides and found the backbone conformations of the optimized peptides remaining to be similar to those of the wild-type peptides (Fig. 6). This indicates that the opti-

**Table IV**
Sequences and Activities of Peptide Inhibitors for HSV-1 gB[24]

| Name | Sequence | Location | Domain | $EC_{50}$(μm) |
|---|---|---|---|---|
| gB64(HSV02) | TTPKFTVAWDWVPKR | 346–360 | I | 85 |
| gB64-opt | VPERFLLFRWHHHKQ | | | |
| gB82(HSV11) | VGQPQYYQANGGFLI | 436–450 | II | ND |
| gB82-opt | PEHWVWAFERKYFFW | | | |
| gB94(HSV17) | KTTSSIEFARLQFTY | 496–510 | III | 6.5 |
| gB94-opt | QDDEERKEKYEERKS | | | |
| gB122(HSV31) | GHRRYFTFGGGYVYF | 636–650 | IV | 15 |
| gB122-opt[a] | WWWWWWWYWWYFWFW | | | |
| gB131 | HEVVPLEVYTRHEIK | 681–695 | V | 18 |

The Locations are the position of the inhibitors in the HSV-1 gB. Names in the bracket are those that were used in our computational analysis.

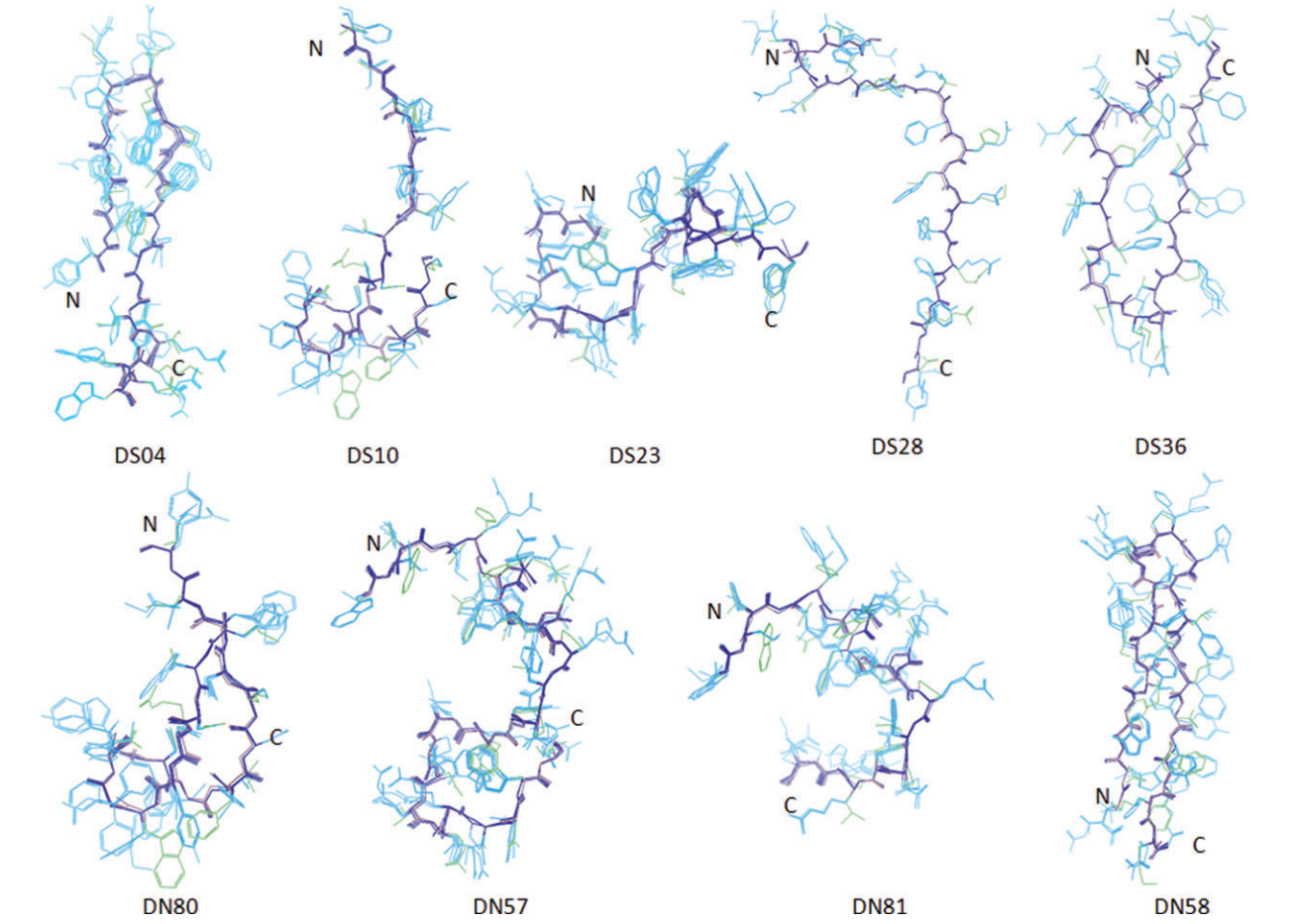



**Figure 6**

The top 10 optimized peptides and the corresponding wild-type peptides derived from the DENV-2 E protein. DS04, DS10, DS23, DS28, and DS36 were identified by scanning the DENV-2 structure; DN80, DN57/DN81, and DN58 are the hydrophobic regions. DS10 and DN80 are partially overlapped and DS23 and DN57/DN81 are partially overlapped and hence they are arranged in the same way. The backbones of the optimized peptides are shown in blue and the side chains of the optimized peptides are shown in cyan; the backbones of the wild-type peptide are shown in purple and the side chains of the wild-type peptides are shown in green. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mized peptides may function similarly to their respective ancestors to bind *in situ* to the counterpart of the Dengue E protein. Furthermore, the optimization of the side chain may result in improved binding affinities as well as specificity.

### Identification of novel peptides with potential DENV inhibitory activities

In addition to the peptide regions which coincide with the locations of the known DENV inhibitors, we here identified additional peptides with better RAPDF scores than that of the DENV peptide inhibitor 1OAN1 (Fig. 5). It is likely that these peptides also have potent inhibitory activities to prevent viral fusion.

The cluster centred around peptide DS36 (351–370) in domain III identified using dDFIRE scoring function also exhibit low RAPDF scores, indicating that this region has the potential to bind *in situ* to the E protein. The third novel cluster regions with good RAPDF scores were observed around DS27 (261–280) and DS28 (271–290). The crystal structure of the DENV-2 E protein revealed a hydrophobic pocket around the hinge region (kl hairpin) comprising the amino acids 270–279.[28] The flexibility of the hinge region allowed the protein to possess different angles between domain I and domain II during the fusion process. Regulators which can stabilize the flexibility of the hinge region will interrupt the fusion and could therefore be developed into potential inhibitors for DENV. Both DS27 and DS28 peptides covered the hinge region, and hence they have potential to exhibit inhibitory activities toward DENV.

The four hydrophobic peptides which were identified by hydrophobicity scale, namely DN80, DN57, DN81, and DN58 were also optimized. The DN58 peptide

**Table V**
Seven Hydrophobic Regions Identified on HSV-1 gB using WWIHS Hydrophobicity Scale

| Name | Sequence | Location | Domain |
|---|---|---|---|
| HSV-a | VTVSQVWFGHRYSQFMGIF | 168–186 | I |
| HSV-b | FVLATGDFVYMSPFYGYRE | 287–305 | I |
| HSV-c | PTTRNLLTTPKFTVAWDWVPKRPSVCTMTKW | 339–369 | I&II |
| HSV-d | YGGSFRFSSDAISTTFTTN | 380–398 | II |
| HSV-e | YYQANGGFLIAYQPLLSNT | 441–459 | II |
| HSV-f | PCTVGHRRYFTFGGGYVYF | 634–650 | IV |
| HSV-g | YAYSHQLSRADITTVSTFI | 653–671 | III, IV&V |

belonging to the cluster centered around the potential inhibitory peptide DS36 also exhibited good RAPDF score. The DN81 peptide is contained in the sequence of DN57. Notably, we found that the optimized DN81 and DN57 peptides share the common sequence (Table II). Thus, although the Monte Carlo selection method is based on random amino acid substitution, the selection tends to coverage of the most stable peptides depending on their structural stability.

Recent membrane leakage experiments disclosed that both DN80 and DS28 corresponded to the hydrophobic

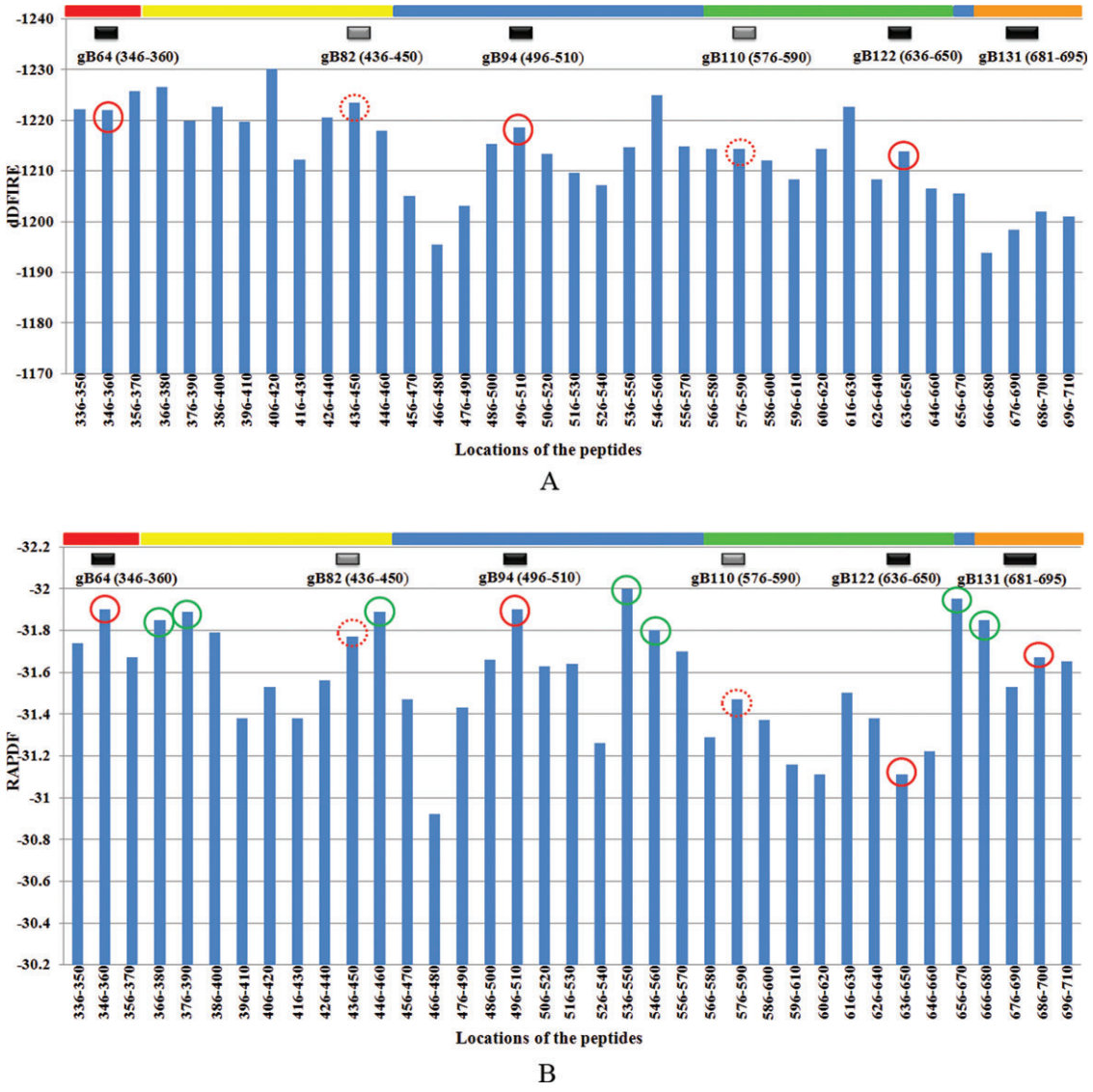



**Figure 7**
(**A**) The regions of the predicted potential peptide inhibitors for HSV-1 glycoprotein. The *x*-axis is the locations of the peptides derived from HSV-1 glycopotein and the *y*-axis represents dDFIRE scores. (**B**) The regions of the predicted potential peptide inhibitors for HSV-1 glycoprotein. The *x*-axis is the location of the peptides derived from HSV-1 glycoprotein and the *y*-axis represents RAPDF scores. The regions that correspond to the known peptide inhibitors of HSV-1 glycoprotein are circled in red, and the novel regions that were predicted to have potential inhibitory activities are circled in green. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

**Table VI**
The Optimized Peptides Derived from the HSV-1 gB using RAPDF Biased Monte Carlo Method

| Name | Sequence | Location |
|---|---|---|
| HSV04wt | MTKWQEVDEMLRSEY | 366–380 |
| HSV04opt | KREVFLDEFRVWYME | |
| HSV05wt | LRSEYGGSFRFSSDA | 376–390 |
| HSV05opt | RQRERDQEEFWHYRE | |
| HSV12wt | GGFLIAYQPLLSNTL | 446–460 |
| HSV12opt | KFFFIFWYWQFLPCA | |
| HSV21wt | LTLWNEARKLNPNAI | 536–550 |
| HSV21opt | ERWTQWFAEGICKFV | |
| HSV22wt | NPNAIASVTVGRRVS | 546–560 |
| HSV22opt | WFRIFREMYNPGHFI | |
| HSV33wt | SHQLSRADITTVSTF | 656–670 |
| HSV33opt | WHCRYELWLRESEDR | |
| HSV34wt | TVSTFIDLNITMLED | 666–680 |
| HSV34opt | KDRKDKTKRNKNKSN | |

membrane-rupture zones,[46] indicating that these membrane-active functional regions may play an important role in viral membrane fusion mediated by the DENV E protein.

Altogether, several novel peptides, DS03/DS04, DS27/DS28, and DS36, were identified to have the potential to interrupt protein–protein interactions in DENV fusion (Fig. 5). The stability of DS36 demonstrated by low RAPDF and dDFIRE scores indicates that it may possess inhibitory activity to DENV entry. Although DS36 is only partially exposed, its potential to be developed into DENV inhibitor cannot be ruled out, just like the potent DENV inhibitory activity observed in the peptide inhibitor 1OAN1 of which only the segment between domain I and domain II is exposed. These wild-type and optimized peptides may possess potent inhibitory activities and serve as starting points for the development of effective specific antiviral therapy.

## Identification of inhibitory peptides for HSV-1 gB

### *Identification of hydrophobic regions on HSV-1 gB*

The sequence of HSV-1 gB was scanned using WWIHS and seven hydrophobic regions were identified, HSV-a

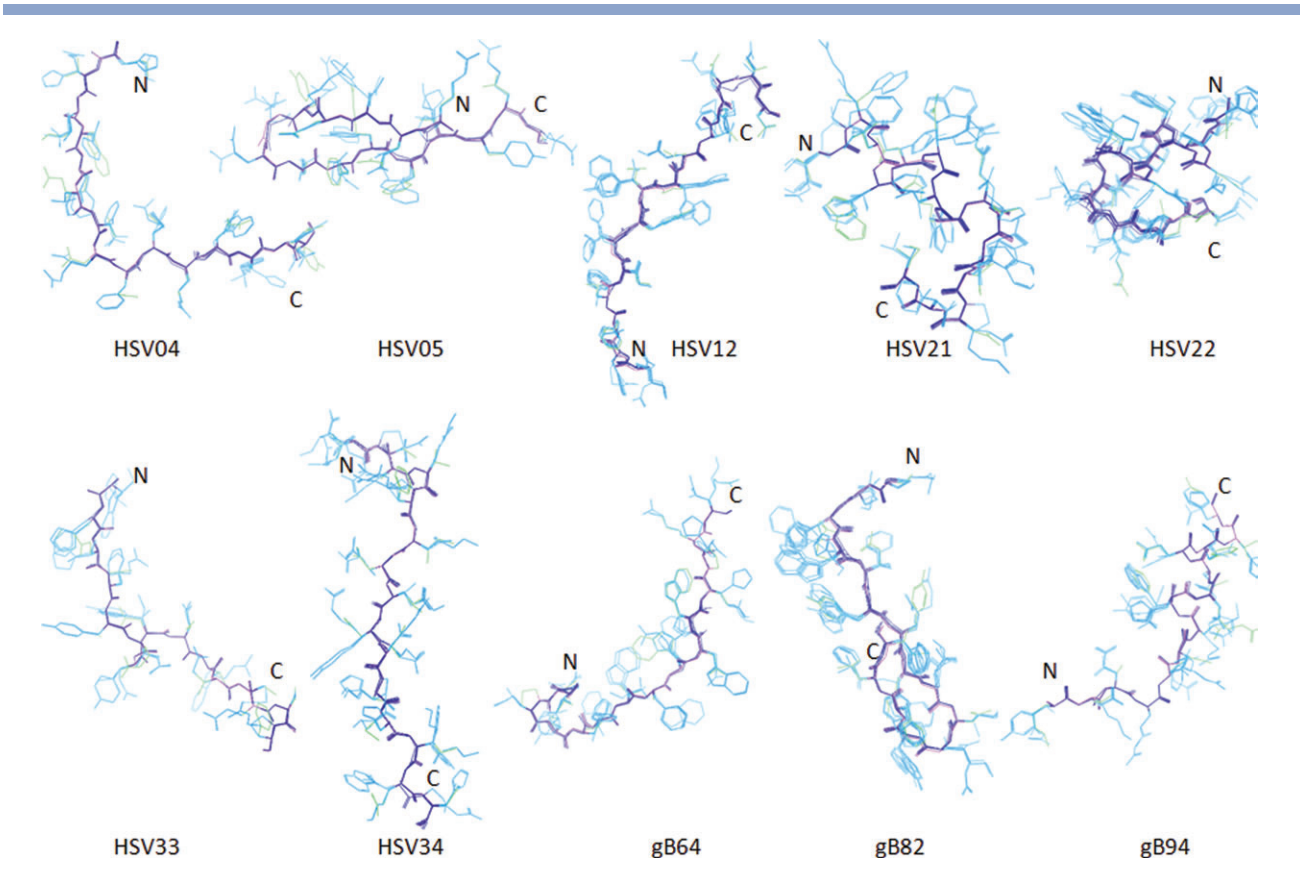



**Figure 8**
The top 10 optimized peptides and the corresponding wild-type peptides derived from the HSV-1 gB protein. HSV04, HSV05, HSV12, HSV21, HSV22, HSV33, and HSV34 were identified by scanning the HSV-1 gB structure; gB64, gB82, and gB94 are the active peptides. HSV12 and gB82 are partially overlapped and hence they are arranged in the same way. The backbones of the optimized peptides are shown in blue and the side chains of the optimized peptides are shown in cyan; the backbones of the wild-type peptide are shown in purple and the side chains of the wild-type peptides are shown in green. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(residues 168–186), HSV-b (287–305), HSV-c (339–369), HSV-d (380–398), HSV-e (441–459), HSV-f (634–650), and HSV-g (653–671) (Table V). HSV-a contains the tip of one extended β-hairpin in domain I, which is suggested to be involved in fusion of the viral and target membranes (Fig. 3). The β-hairpin of HSV-b on domain I interacts with the α-helix on domain V from another protomer. The partially exposed HSV-c contains a β-hairpin in domain I and extends to domain II. HSV-d and HSV-e are located on domain II and both interacting with the long coiled-coil in domain III on the same protomer. The HSV-f is located on domain IV interacting with domain III on another protomer. It was reported that a residue Y640 lying at the center of a shallow pocket corresponding to HSV-f is critical for inhibitory activity of a peptide derived from this region.[24] The exposed HSV-g spans from domain III to domain V and has no contact with the other two protomers.

### *Validating the functional regions on HSV-1 gB*

By high-throughput screening of library with 138 peptide sequences, seven, whose sequences clustered in the carboxy-terminal half of the ectodomain of HSV-1 gB demonstrated inhibition of β-galactosidase activity by 50% or more.[24] A 15-residue sliding window was moved from residues 336 to 710 in the C-terminus half in an increment of 10 amino acids. Thirty-seven 15-mer peptides were generated and used as templates for amino acid substitution. They were denoted sequentially as HSV01 (amino acids 336–350), HSV02 (346–360), HSV03 (356–370), and so on up until HSV37 (696–710).

All the 37 15-mer peptides were subjected to sequence optimization using the Monte Carlo protocol. The stability of the resulted protein after amino acid substitution was evaluated using dDFIRE and RAPDF (Fig. 7). The score of HSV17 (496–510), corresponding to an active peptide gB94[24] was used as reference to identify potential gB inhibitors.

All the first eight peptides from HSV01 until HSV08 have comparable dDFIRE scores to that of gB94. Among them, HSV02 (residues 346–360, corresponding to the region of an active peptide gB64) constitutes the hydrophobic region HSV-c that spans from HSV01 to HSV03; HSV06 corresponds to another hydrophobic region HSV-d. HSV11 (residues 436–450, corresponding to another active inhibitor gB82) that partially overlaps with the hydrophobic HSV-e, has comparable dDFIRE score to that of gB94. The scores of two active peptides gB110 (HSV25, residues 576–590) and gB122 (HSV31, residues 636–650) are slightly higher than gB94. gB122 corresponding to a region on domain IV, overlaps with the hydrophobic region HSV-f and may form a binding pocket for the entry receptor.[24]

RAPDF scoring function was then applied to the thirty-seven overlapping peptides. The optimized sequences of the peptides with low RAPDF scores are listed in Table VI and the structures of the optimized peptides are shown in Figure 8. Similar to what was observed by dDFIRE scoring function, low RAPDF score was observed for gB94 (HSV17). gB94 constitutes the central helical coiled-coil of the gB trimeric bundle. Peptides derived from this region may interact with the helix, and therefore disrupting trimer formation (Fig. 3). HSV21(residues 536–550) located opposite to the tip of the extended β-hairpin comprising the beginning of the helical coiled coil, is also associated with low scores evaluated by both dDFIRE and RAPDF scoring functions, indicating inhibitors derived from this region may also function to disrupt the trimer interaction. Further study on this region may provide information on the biological function of gB. The HSV02 located on domain I and HSV11 on domain II was identified to have comparable RAPDF scores to gB94, as evaluated by both dDFIRE and RAPDF scoring functions. HSV05, which is located on domain II, may interact with the extended domain V loops in the other two protomers by forming more favorable electrostatic interactions. Our hypothesis was confirmed by careful examining the optimized sequence and its potential binding interactions with the other two protomers. It was found that in HSV05-opt, mutations Y380R and D389R (Table VI) resulted in stronger ionic interactions with the negatively charged residues E679 from another protomer and with D680 from the third protomer, respectively (Supporting Information Fig. S2). In addition to the two hydrophobic regions HSV-c and HSV-d, another two hydrophobic regions HSV-e (corresponding to HSV12, residues 446–460) and HSV-g (HSV33, residues 656–670) were also identified to have low RAPDF scores. HSV36 (residues 686–700) located in domain V, whose sequence largely overlaps with the active peptide gB131 (residues 681–695) displays a low RADPF score. According to the crystal structure of gB,[16] the region has no contact with rest of the protomer and thus could exert its function by blocking the binding with the other two protomers. It is not surprising that RAPDF performs as well as dDFIRE scoring function to identify potential inhibitory peptides and functional important regions for the Class III fusion protein HSV-1 gB, as the active peptides could interact with either α-helix or extended loop (Fig. 3), and therefore disrupting trimer formation.

## CONCLUSIONS

We have developed a new Monte Carlo-based computational method to identify self-inhibitory peptides from E proteins to interrupt the virus fusion process. In the method, we implemented RAPDF and dDFIRE scoring functions to evaluate the stability of the mutated peptides to indicate their potential for *in situ* binding to the part-

ner proteins. The method was validated using α-helical Class I HIV-1 gp41, β-sheet Class II DENV-2 E and HSV-1 gB proteins as case studies. The locations of the known self-inhibitory peptides derived from the enveloped proteins are in good agreement of the inhibitory peptide regions identified. It is observed that the RAPDF-based Monte Carlo selection method is more suitable for β-sheet Class II E proteins, whereas both scoring functions display similar efficiency for α-helical Class I HIV-1 gp41 and Class III HSV-1 gB proteins. Implementing RAPDF scoring in the Monte Carlo method, we identified and designed several novel peptides derived from DENV-2 E or HSV-1 gB proteins with preferable stability scores. Their functional importance and potential as druggable regions are reinforced by hydrophobicity scan and conservation analysis. These peptides may inhibit the protein–protein interaction in the DENV or HSV fusion process and serve as starting points for the development of antiviral therapy to treat viral infection.

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