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Review

Peptides as Therapeutic Agents for Dengue Virus

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Abstract

Dengue is an important global threat caused by dengue virus (DENV) that records an estimated 390 million infections annually. Despite the availability of CYD-TDV as a commercial vaccine, its long-term efficacy against all four dengue virus serotypes remains unsatisfactory. There is therefore an urgent need for the development of antiviral drugs for the treatment of dengue. Peptide was once a neglected choice of medical treatment but it has lately regained interest from the pharmaceutical industry following pioneering advancements in technology. In this review, the design of peptide drugs, antiviral activities and mechanisms of peptides and peptidomimetics (modified peptides) action against dengue virus are discussed. The development of peptides as inhibitors for viral entry, replication and translation is also described, with a focus on the three main targets, namely, the host cell receptors, viral structural proteins and viral non-structural proteins. The antiviral peptides designed based on these approaches may lead to the discovery of novel anti-DENV therapeutics that can treat dengue patients.

Key words: Dengue virus, Drug discovery, Peptides, Antiviral therapeutics.

Introduction

Dengue is a mosquito-borne disease caused by the infection of dengue virus (DENV). It has been estimated that 390 million dengue infections occur annually, of which 96 million manifest clinically [1]. Before 1970, only nine countries experienced dengue epidemics. Currently, dengue is endemic in more than 100 countries, primarily in tropical and sub-tropical countries [2]. There are four dengue virus serotypes, DENV-1-4, which are genetically and antigenically distinct, although each serotype elicits a similar range of disease manifestations during infection [3]. In humans, dengue infection causes a spectrum of illnesses ranging from asymptomatic, fever, rash, joint pain and other mild symptoms to life-threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [4]. Infection with one DENV serotype induces lifelong immunity against the homologous serotype but not against the other three heterologous serotypes. In fact, studies have shown that secondary infection with a different DENV serotype is an important risk factor in causing more severe complications, such as DHF and DSS, due to a phenomenon designated as antibody dependent enhancement (ADE) or the original antigenic sin [5-7].

One of the strategies that has been undertaken to halt DENV infection is by vector control. *Aedes aegypti* and *Aedes albopictus* are the primary transmission vectors for DENV [8]. Strategies such as fogging and the release of genetically modified mosquitoes which could lead to the production of fewer progenies [9] have failed to lessen the mosquito population, as witnessed by the emergence of new dengue cases in places that were dengue-free or had less dengue cases in the past [10-12]. While active research on vaccine development for dengue has been ongoing for the past few decades, the development of vaccines has been held back by several challenges. The major constraints for dengue vaccine development include the lack of good animal models, the complexity of developing a vaccine against all four antigenically distinct DENV serotypes, as well as the need to achieve balanced tetravalent responses that could

exhibit significant immunity against all four viruses without the adverse effects of ADE or original antigenic sin [13]. The first dengue vaccine, Dengvaxia®, (CYD-TDV, chimeric yellow fever virus-tetravalent dengue vaccine) developed by Sanofi Pasteur was licensed in December 2015 in Mexico. It is a live-attenuated tetravalent vaccine comprising structural proteins (pre-membrane and envelope proteins) of DENV based on the yellow fever 17D virus backbone [14, 15]. The approved regimen involves three doses, given at the 0th, 6th and 12th months, for individuals between 9-45 years of age. Outcomes from phase III clinical trials showed that the vaccine successfully reduced dengue hospitalizations by 80%. However, its average efficacy against DENV was low, especially against DENV-1 at approximately 50% and against DENV-2 at 39%. Its average efficacy against DENV-3 and DENV-4, meanwhile, was slightly higher at 75% and 77%, respectively [16, 17]. Furthermore, previous clinical trials revealed that CYD-TDV vaccination caused elevated risks of hospitalization for children less than nine years of age [18]. The World Health Organization has therefore recommended the use of CYD-TDV vaccine only in countries where epidemiological data indicated a high burden of dengue [19].

The lack of efficient vector control strategies and the uncertainty of long-term protective efficacy of CYD-TDV vaccine against all four DENV serotypes call for an urgent need for dengue therapeutics, especially in endemic countries with poor resource setting. There are no antiviral drugs available and at present, supportive treatment with emphasis on fluid therapy and close clinical monitoring during the critical phase of illness are the only course of action for dengue disease. Many antiviral candidates have failed to reach clinical trials due to their poor selectivity and physiochemical or pharmacokinetic properties [20]. Although nucleoside analogs, such as NITD-008 and balapiravir, have entered preclinical animal safety study and clinical trials, they were terminated due to lack of potency [21]. Balapiravir, for instance, did not improve the clinical and virological parameters in patients in the phase II clinical trial, although it was shown to have good *in vitro* antiviral activities with EC_{50} values of 1.3–3.2 μM in DENV infection assays using primary human macrophages [21]. Treatment of DENV-infected mice with another nucleoside analog NITD-008, on the other hand, completely prevented mice death, but severe adverse events were observed in rats and dogs after two weeks of oral dosing [20, 22]. Likewise, other anti-DENV candidates, including chloroquine, prednisolone, celgosivir and lovastatin, have gone through clinical trials but failed to meet the defined primary end points, whereby neither significant viremia nor evidence of beneficial effects on clinical manifestations was observed [23-26]. At present, two candidates, namely ivermectin and ketotifen, are undergoing clinical trials (trial number NCT02045069 and NCT02673840, respectively). However, their long-term clinical efficacies remain to be determined. In contrast to small molecules, peptides are generally known to have high selectivity and possess relatively safe characteristics which make them attractive pharmacological candidates [27]. Due to their attractive pharmacological profiles, this review will highlight the current status and the rational drug design of antiviral peptides and peptidomimetics as therapeutics for dengue.

Dengue Virus (DENV)

DENV is an enveloped, positive, single-stranded (ss) RNA virus classified under the genus *Flavivirus* of the *Flaviridae* family [28]. Other closely related viruses classified under the *Flavivirus* include yellow fever virus (YFV), west nile virus (WNV), japanese encephalitis virus (JEV) and zika virus. The dengue virion is a spherical particle, approximately 50 nm in diameter with envelope (E) and precursor-membrane (prM) / membrane (M) proteins located on its surface, while the capsid (C) proteins sit underneath the lipid bilayer, encapsulating the viral genome [29]. The DENV genome (~11 kb) constitutes a single open reading frame (ORF), encoding three structural proteins (C, prM/M and E proteins) followed by seven non-structural (NS) proteins (NS1, NS2A and 2B, NS3, NS4A and 4B, NS5) (Figure 1) [30]. The translated polyprotein is then cleaved by cellular signal peptidases and virally encoded protease (NS2B and NS3) to generate individual proteins. The structural proteins form the viral particle while the non-structural proteins participate in replication and invasion of the immune system [30]. To design peptides with therapeutic potential against dengue virus, it is necessary to understand the viral replication cycle.

DENV infection in humans starts with a DENV-infected mosquito bite. DENV can replicate in a wide spectrum of cells, including liver, spleen, lymph node, kidney and other organs [31, 32], but monocytes, macrophages and dendritic cells (DC) have been shown to be the major targets for DENV [33, 34]. The life cycle of dengue virus is initiated by the virus attachment through the interaction between viral surface proteins and attachment/receptor molecules on the surface of the target cell (Figure 2). Receptor recognition is believed to be mediated by the domain III of E protein to enable the virus to enter into host cells by receptor-mediated, clathrin-dependent

endocytosis (primary method) [35, 36]. However, studies have also shown that viral entry could occur by the direct fusion of the virus and host cells [37-39]. After internalization, dissociation of the E homodimers takes place as a result of the acidic environment in the endosome. Subsequently, domain II of the E protein will project outwardly and the hydrophobic fusion loop in domain II will insert itself into the endosomal membrane [40, 41]. This will then trigger domain III to fold back and force the virus particle and endosomal membrane to move towards each other and fuse together [42, 43]. The fusion of the virus with vesicular membranes would then release the nucleocapsid into cytoplasm, resulting in genome uncoating [44]. Subsequently, the viral RNA genome is released. The viral RNA is translated into a single polyprotein and processed co- and post-translationally by cellular and virus-derived proteases into three structural proteins and seven NS proteins (Figure 1). Upon protein translation, the NS proteins initiate viral genome replication at the intracellular membranes, resulting in the production of more viral RNA strands [45]. Then, the newly synthesized RNA is packed by C proteins to form the nucleocapsid [46]. The prM and E proteins, on the other hand, form heterodimers that oriented into the lumen of ER and are believed to induce a curved surface lattice which guides virion budding [47]. Hence, the virus assembles and buds from the ER before migrating to the trans-Golgi for maturation process. The slightly acidic pH of the trans-Golgi network prompts the dissociation of prM/E heterodimers to form 90 dimers with prM capping the fusion peptide located at the domain II of the E protein [48]. This is followed by the cleavage of the prM at Arg-X-(Lys/Arg)-Arg by cellular endoprotease (furin), (where X is any amino acid) to produce membrane-associated M and "pr" peptide [49, 50]. Both prM and the "pr" will act as chaperones to stabilize the E protein during the secretory pathway by preventing premature membrane fusion. At the end, the "pr" peptide will dissociate upon the release of the progeny by exocytosis [45].

Development of Peptides as Therapeutics

Peptides are biologically active molecules comprising the combination of at least two amino acids through a peptide bond. In contrast to large proteins, they are smaller in size and are considered to contain less than 100 amino acid residues. Peptides are known to have attractive pharmacological profiles due to their highly selective and relatively safe characteristics [27]. They readily exist in the human body and exert diverse biological roles, predominantly as signalling and regulatory molecules in a variety of physiological processes [51]. In the past, peptides were held back in the drug development pipelines due to their instability, whereby they could be easily degraded by at least 569 proteases in the human body [52]. Nevertheless, a number of technological breakthroughs and advancements have reversed the situation. This has resulted in the spark of interest in peptide drug development. Current technologies have allowed the modification of peptides to create artificial variants with improved stability and overcome pharmacodynamic weaknesses. For instance, advances in automated-liquid handling devices, synthetic peptide synthesis, mass spectrometry and *in silico* drug design have allowed high-throughput drug screening. In addition, advances in peptide manipulations such as synthesis of D-amino acids, cyclic peptides, incorporation of chemicals and nanocarriers have further increased the bioavailability of peptides [53]. Currently, ample examples of efficacious and safe peptide drugs are available in the market [54-57]. Great success has been achieved for the peptide drug Fuzeon™ (enfuvirtide), a synthetic peptide that blocks viral fusion by binding to the gp41 (polypeptide chain) of the human immunodeficiency virus (HIV) type-1 envelope protein [55]. It is the only antiviral peptide which has been commercialised. Other antimicrobial peptide candidates, such as MU1140, Arenicin, IMX924, Novexatin and Lytixar, are being evaluated in the preclinical and clinical trials [58, 59]. Myrcludex B, an anti-Hepatitis B and Hepatitis D

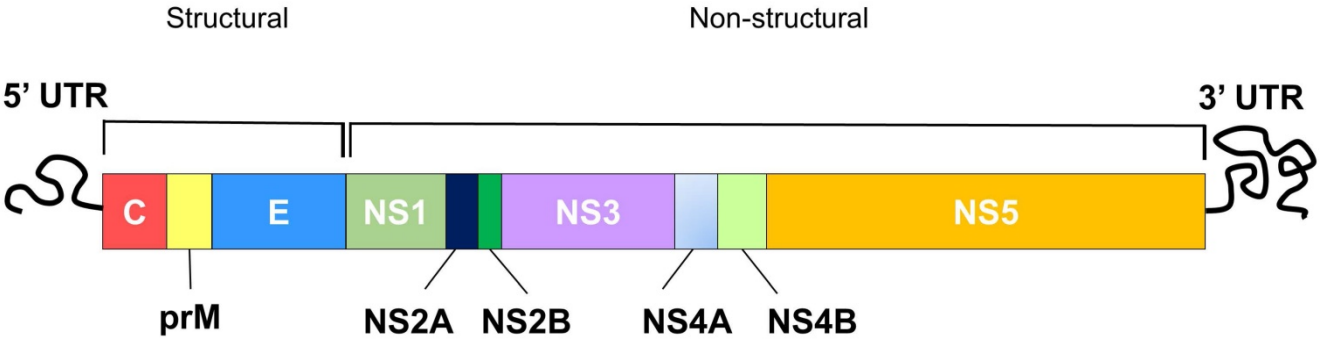

Figure 1. Schematic diagram of the DENV genome showing structural and non-structural polyproteins that are encoded by the DENV genome.

peptide targeting sodium taurocholate co-transporting polypeptide (NTCP) of liver cells, is also being studied in a phase II clinical trial [60].

At present, the value of global peptide therapeutics market is predicted to increase from US$21.3 billion (2015) to US$46.6 billion in the year 2024 [61]. There are at least 60 therapeutic peptides that have been approved by the US Food and Drug Administration (FDA) and approximately 140 peptide therapeutics are being evaluated in clinical trials [62]. In 2011, 25 of the US-approved peptide drugs accounted for the global sale of over US$14.7 billion, while Victoza®, Zoladex®, Sandostatin®, Lupron® and Copaxone® each had global sales of over US$1,000 million [63]. Some other examples of therapeutic peptides include glucagon-like peptide-1 (GLP-1) and analogues [57], deletion peptides of insulin [56] and a deletion peptide of the heat shock protein 60 [54] that have been used widely in the treatment of diabetes. This has demonstrated the potential and importance of peptides as pharmacological agents. Additionally, as the number of new entities approved by the FDA rapidly decreases over the years [64] and the number of publicities about the side effects of popular small molecules increases (such as the cancer chemotherapeutic or COX-2 inhibitors) [65-67], the pharmaceutical industry is now reviving their interest in peptides as potential drug candidates. With good pharmacology properties and new technologies to mitigate the weakness of peptides, the number of therapeutic peptide candidates will continue to grow.

Mode of Action for Therapeutic Peptides

Antiviral peptides that either interact with the virus particles or target at critical viral replication steps of the life cycle can potentially be used as treatment or prophylaxis for dengue. Several approaches have been explored thus far to inhibit dengue virus infection, including targeting the host cell receptors or attachment factors, viral structural proteins and non-structural (NS) proteins. Drugs that were designed against these three main targets employ different mechanisms of action to stop virus infection. By targeting the host cellular receptors or attachment factors, it will prevent the attachment and binding of viral proteins with the host cell, hence stopping the subsequent entry of DENV. Drug candidates directing at the viral structural proteins [capsid (C), pre-membrane (prM/M) and envelope (E)], on the other hand, might be able to interfere with the binding of viruses to host cells, thereby inhibiting the viral attachment/fusion and viral entry. Lastly, as non-structural proteins are essential components of replication machinery, designing drug candidates against NS proteins will interfere with the viral replication cycle to effectively ameliorate dengue.

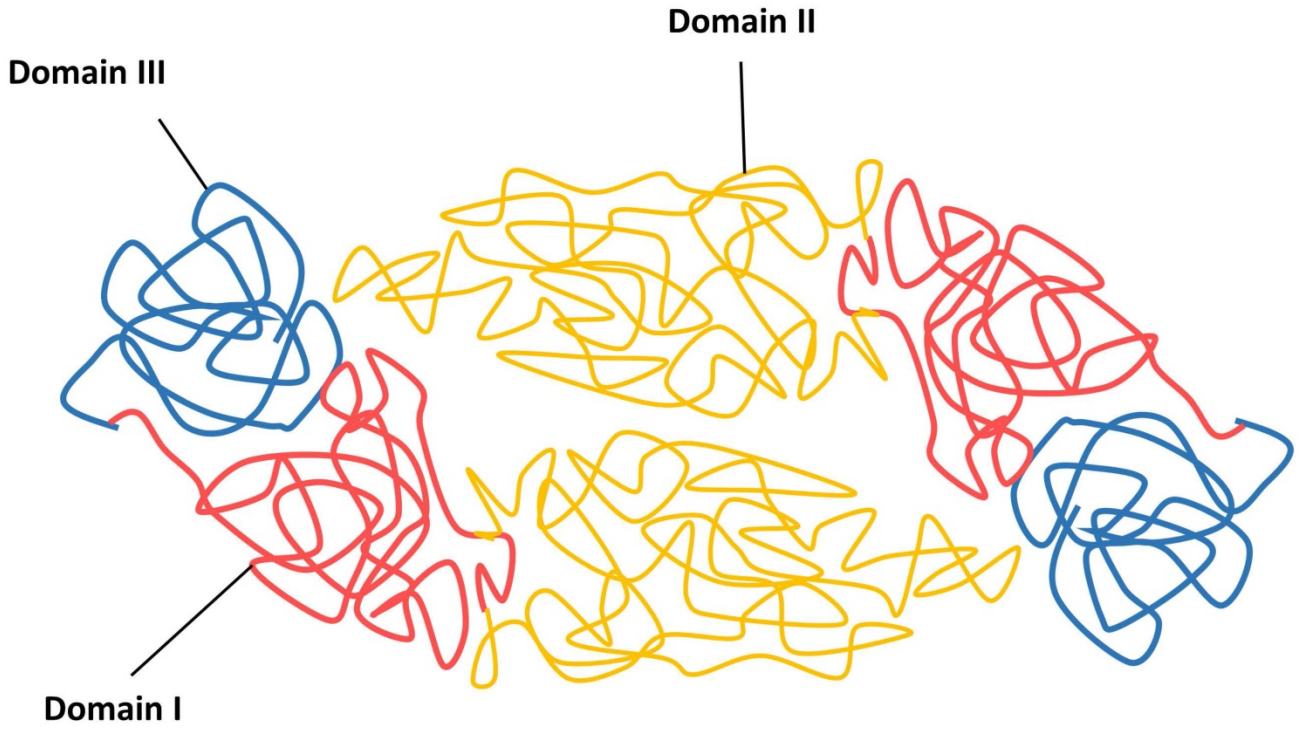

Figure 2. Schematic diagram of DENV replication cycle and summary of antiviral peptides. The antiviral peptides are classified according to their mechanism of actions, which include entry inhibitors, fusion inhibitors, translation inhibitors and replication inhibitors.

Entry inhibitors: Targeting host cells

One of the attractive approaches to inhibiting virus infection is by blocking the cellular receptors or attachment factors, or mimicking the cellular receptors, hence preventing the virus from attaching and entering host cells. This will form the first barrier to block viral infection. Studies have shown that this is a feasible approach to halting viral infections [68-70]. Pugach *et al.* (2008) and Lieberman-Blum *et al.* (2008) demonstrated that a small molecule, CCR5 inhibitor, Maraviroc, successfully inhibited human immunodeficiency virus type 1 (HIV-1) infection by binding to the CCR5 co-receptor of host cells [68, 69]. On the other hand, Myrcludex B, a lipomyristolated peptide containing 47 homologous amino acid residues of hepatitis B virus pre-S1 protein, was able to bind to the NTCP of host cells and resulted in the restriction of virion uptake in the host cells [70]. The identified DENV receptors or attachment factors on mammalian cells were reviewed by Perera-Lecoin *et al.* (2014) and Cruz-Oliveira *et al.* (2015) [71, 72]. Some of the important attachment factors or receptors are the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) [34], heparan sulfate [73], mannose receptor [74], HSP90/HSP70 [75], laminin receptor [76], and the TIM and TAM proteins [77]. To date, several small molecules were identified as receptor antagonists or mimics for DENV. For instance, CC-chemokine receptor 5 (CCR5) antagonists, Met-R and UK484900 (a Maraviroc analogue) prevented CCR5 activation and reduced DENV load [78], while heparin sulfate mimetics, such as PI-88 [79], fucoidan [80] and CF-238 [81], were shown to block viral entry. Interestingly, to the best of our knowledge, no peptide inhibitors were found to block DENV infection by binding to cellular attachment factors or receptors. This represents a big research gap that should prompt researchers to investigate.

The DC-SIGN is a type II transmembrane protein that falls into the category of C-type lectin with an extracellular domain that can bind specifically to carbohydrates [82]. DC-SIGN has been shown to be an essential cellular factor required for the infection of ebola virus [83, 84], HIV-1 [85, 86] and human cytomegalovirus (CMV) [87] into dendritic cells. Studies have also shown that dendritic cells that abundantly express DC-SIGN are highly susceptible to DENV infection [33, 88, 89]. Tassaneetrithep *et al.* (2003) further validated the importance of DC-SIGN as a DENV receptor [34], whereby the transfection of DC-SIGN into THP-1 cells resulted in DENV infection while dendritic cells blocked with anti-DC-SIGN prevented DENV infection [34]. These results suggest that DC-SIGN is a feasible target for designing therapies that prevent DENV infection. Furthermore, dendritic cells were activated after capturing antigen and resulted in the stimulation of naïve T cells to produce cytokines and chemokines [90]. Blocking the binding of DENV to DC-SIGN can prevent DENV infection, as well as the initiation of immune response which can lead to severe dengue characterized by the cytokine storm. Based on the literature, limited DC-SIGN inhibitors are found to stop DENV infection. In a study, Alen *et al.* (2011) evaluated the inhibitory properties of various carbohydrate-binding agents (CBAs) which are mannose-specific plant lectins by using the Raji/DC-SIGN+ cell line. Results showed that *Hippeastrum hybrid* (HHA), *Galanthus nivalis* (GNA) and *Urtica dioica* (UDA) were able to bind to the envelope of DENV, hence preventing the subsequent viral attachment [91]. Similarly, pradimicin-s (PRM-S), a small-size non-peptidic CBA, was shown to exert antiviral activity against DENV by binding to the DENV envelope in monocyte-derived dendritic cells [91].

Another important known DENV receptor is the glycosaminoglycans (GAG). Among the GAG family, heparin sulfate (HS) is the most ubiquitous member of the family and is the putative receptor for DENV [92-94]. Studies have shown that HS acted as the first interactive attachment factor to facilitate DENV binding to the second receptor [92, 95]. It was demonstrated that DENV-HS interacted via positively charged E(III) residues, especially Lys291 and Lys 295 binding to the negatively charged HS [73, 96]. Many heparan mimetics were identified to block DENV infection [79, 80, 97]. Lee *et al.* (2006) showed that a heparin sulfate mimetic, phosphomannopentaose sulfate (PI-88), significantly increased the survival rate of DENV-infected mice [79]. In another study, a sulphated polysaccharide, fucoidan, which was extracted from the marine alga *Cladosiphon*, was able to inhibit DENV-2 infection by binding to the DENV envelope protein [80]. Interestingly, Talarico *et al.* (2005) showed that iota-carrageenan and dl-galactan hybrid C2S-3 (sulphated polysaccharides isolated from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*) inhibited DENV infection in a virus serotype and host cell dependent manner [97]. Many other heparin mimetics, including CF-238 [81], sulphated galactomannan [98], curdlan sulfate [99], sulphated galactan [98], sulphated K5 [100] and chondroitin sulfate [101], were found to inhibit DENV infection but no antiviral peptide was identified to either bind to cellular receptor or act as a receptor mimetic to block DENV entry thus far. Likewise, to the best of our knowledge, no antiviral peptide was found to inhibit DENV infection by targeting other

receptors, including mannose receptor [74], HSP 90/70 [75], laminin receptor [76], and the TIM and TAM proteins [77]. Furthermore, inhibitors targeting host cellular receptor(s) are anticipated to be less prone to develop resistance as compared to those targeting viruses. Therefore, this may serve as an interesting research gap to be explored.

Although studies demonstrated that DENV mainly enters host cells via receptor initiated-clathrin mediated endocytosis [102-104], viral entry via clathrin-independent endocytic route has also been observed [104]. In addition, evidence of direct entry via fusion with plasmatic membrane leading to direct penetration into cytoplasm without undergoing endocytosis has also been described [105, 106]. Furthermore, evidence showed that DENV is able to infect a variety of cell types, including those isolated from humans [107, 108], monkeys [92, 93], hamsters [95, 109] and mosquitoes [110, 111] via different receptors. Therefore, the DENV entry pathway is greatly dependent on the cell type and viral strain. Due to the variability in viral entry routes and broad tissue tropism, targeting the viral structural proteins is easier than the vastly different cellular receptors, as DENV possesses the capability to utilize a wide range of cellular receptors and pathways to enter host cells. Viral structural proteins, especially the E protein, is therefore a popular target for antiviral inhibitors to interfere with the virus-host interactions and stop subsequent viral entry.

Entry Inhibitors: Targeting Envelope (E) proteins

The viral infection cycle starts with the interaction of viral structural proteins, mediated mainly by the E protein with the host cell receptors or attachment factors to facilitate the entry of virus. The DENV E protein is 53 kDa in size and composed of three distinct domains, namely the domain I E(I), flanked by the dimerization domain E(II) containing the fusion peptide and an immunoglobulin-like domain E(III) that protrudes from the virion surface, followed by a membrane proximal stem and a transmembrane anchor (Figure 3) [45, 112].

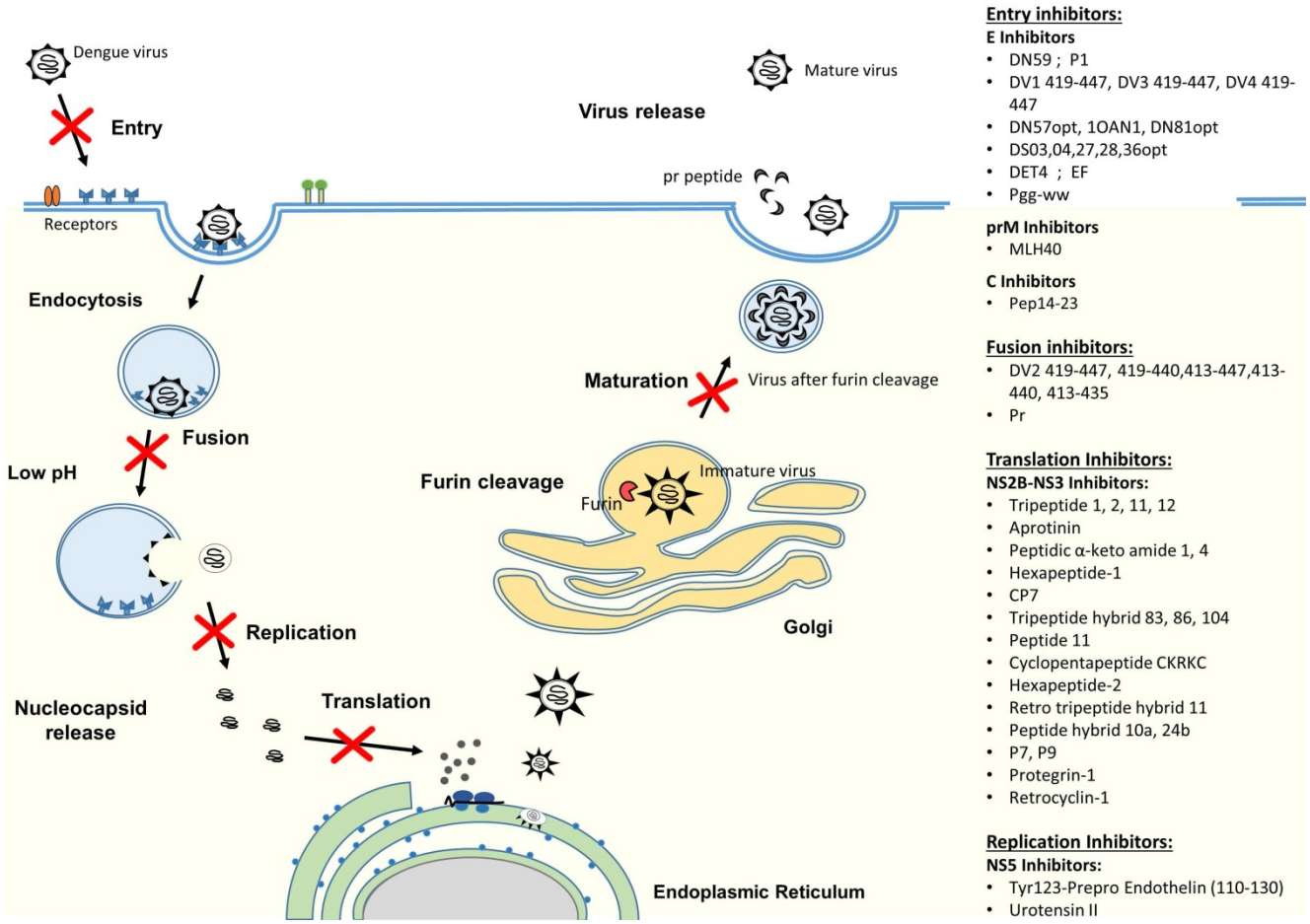

Figure 3. Schematic diagram of DENV envelope (E) proteins in their dimeric forms.

The function of E(I) has not been fully characterized, although it has been shown to be involved in the structural rearrangement of the E protein during internalization [112]. The E(II) contains a region known as fusion peptide, which is responsible for the viral fusion activity, and the E domain II also contains serotype-conserved epitopes, and contributes to the E protein dimerization [113, 114]. Previous studies have shown the E(III) is responsible for receptor recognition, which is essential for viral attachment to facilitate viral entry into host cells by receptor-mediated, clathrin-dependent endocytosis (primary method) [73, 102, 103]. Additionally, E(III) also harbours the serotype-specific neutralizing epitopes [115, 116]. Because of the involvement of receptor recognition and attachment, as well as its vital role in mediating viral and cellular membrane fusion to release viral genomic RNA for viral replication, the E glycoprotein is the most important protein facilitating viral entry. Hence, this makes the E protein a good antiviral target to stop viral entry.

The DENV E structural proteins have been well determined using nuclear magnetic resonance spectroscopy, X-ray crystallography and cryo-electron microscopy [112, 117, 118]. Recent advancements in the understanding of the high-resolution E structure have allowed researchers to utilize the information in combination with *in silico* molecular drug designing methods to search for potential antiviral candidates. Several research groups have utilized different strategies including *in silico* drug design to screen for novel antiviral peptides against the E protein (Table 1). By using Wimley-White interfacial hydrophobicity scale (WWIHS) in combination with known structural data of the E protein, Hrobowski *et al.* (2005) were the first group to identify a novel peptide DN59, corresponding to the stem region of E, which showed >99% DENV-2 inhibition at <25 μM [119]. The D59 peptide was suggested to function through a sequence of specific mechanisms as a scrambled peptide failed to inhibit DENV infection. It was hypothesized that the DN59 peptide might interfere with the intramolecular interaction, disrupt structural rearrangements of fusion proteins or interact with target cell surface components to exert its inhibitory effects [119]. The mechanism of action for peptide DN59 was further evaluated by a later study (2012) where it was shown that the peptide D59 inhibited DENV infectivity by interacting directly with virus particles, causing the formation of holes at the five-fold vertices in the virus particles [120]. This led to the release of viral genomic RNA and exposure of the viral RNA to exogenous RNase [120].

Likewise, Schmidt *et al.* (2010) also hypothesized and proved that the stem peptides could inhibit dengue virus infection [121]. In the study, a set of overlapping peptides based on the DV2 stem region (from amino acid residues 396-447) were synthesized and tested for their binding affinities with soluble form of DV2 E (sE, covering only the first 395 residues of E) via fluorescence polarization. Among the set of overlapping peptides, a peptide ($DV2^{419-447}$) was found to bind selectively to the post-fusion of sE with the concentration of half-maximal change in fluorescence polarization (FP IC_{50}) of 0.125 μM and K_d at approximately 150 nM, while the scrambled peptide $DV2^{419-447}$ neither bound to pre-fusion nor post-fusion conformers of sE. Schmidt *et al*. (2010) proposed that the peptide $DV2^{419-447}$ inhibited DENV infection through a two-step mechanism during late-stage fusion intermediate, whereby the peptide first binds non-specifically to the viral membrane, followed by specific interaction with the E protein when E proteins undergo conformational rearrangement at low pH. Interestingly, they observed that the reduction of the $DV2^{419-447}$ hydrophobicity (by changing the 441-447 amino acid residues) greatly reduced the inhibitory property of the peptide, but not its binding affinity against dengue virus E proteins. This suggested the importance of peptide hydrophobicity to non-specific host membrane interaction before high binding affinity to the DENV E protein.

The importance of hydrophobicity was further supported by Hrobowski *et al.* (2005), whereby the novel antiviral peptide was successfully identified using the Wimley-White interfacial hydrophobicity scale (WWIHS) screening method. This screening method calculated a sliding hydrophobicity score to determine the segments of the protein with a propensity to interact with membrane interfaces [119]. By using a similar strategy, another study has identified five hydrophobic regions located on the DENV-2 E protein [122]. Amino acids derived from these regions were screened via WWIHS and further optimized using RAPDF biased Monte Carlo method. This resulted in the identification of several novel peptides, namely DS03/DS04, DS27/DS28 and DS36, which could potentially interrupt protein-protein interactions during DENV fusion. Likewise, many antiviral peptides were identified against other viruses such as type-1 herpes simplex virus (HSV-1) [122], severe acute respiratory syndrome coronavirus (SARS) [123], human cytomegalovirus (HCMV) [124] and rift valley fever virus (RVFV) [125] via the same approach. This again has validated the importance of hydrophobicity property for antiviral peptides.

In addition, it was observed that many of the antiviral peptides known to inhibit entry of enveloped

viruses to cells have hydrophobic and/or amphipathic properties to facilitate the interaction with cellular lipid membrane interfaces [126, 127]. Besides using WWIHS, the interfacial helical hydrophobic moment (iHHM) is another physio-chemical determining strategy which can be used to augment the peptide-membrane interfaces. This approach quantified the segregation of hydrophobic and hydrophilic faces of a peptide folded into an α-helix structure [128]. Higher iHHM value indicates stronger membrane interaction with peptides [129, 130]. Additionally, Badani *et al.* (2014) also suggested hydrophobicity and amphipathicity to be critical properties for peptides in their interaction with cellular membrane and thereby inhibiting viral entry [126]. Therefore, for future drug design and development, researchers could consider incorporating the hydrophobic and amphipathic properties into antiviral peptides to further enhance antiviral efficacies.

Previous studies have shown that the lateral loop located on E(III) played an important role in virus-host receptor(s) interaction [73, 131], hence making it an interesting target. A short sequence (380-IGVEPGQLKL-389) in the lateral loop on the DENV-2 E(III) was used as a target to screen for potential antiviral peptides using the BioMoDroid algorithm [132]. Four different peptides were designed and DET4 (one of the peptides) was found to inhibit 85% DENV-2 at 500 μM. TEM images indicated that DET4 inhibited DENV-2 entry by causing structural abnormalities and alteration of the conformational changes of E proteins. On the contrary, Panya *et al*. (2014) targeted on the n-octyl-β-D-glycoside (βOG) hydrophobic pocket located in the domain I domain II interface of DV E protein [133]. A previous study has shown that the shift of two β-hairpins located at the hydrophobic pocket was essential to cause correct conformational changes during virus fusion step [113]. The importance of the βOG hydrophobic pocket was further validated as several compounds targeting this hydrophobic pocket were able to stop DENV infection [134, 135]. By using AUTODOCK v4.2 and CDOCKER DISCOVERY STUDIO v2.1 software, Panya *et al.* (2014) found a di-peptide, EF, to be the most effective antiviral peptide as it inhibited DENV-2 infection with the IC_{50} value of 96 μM [133].

Studies have shown that the E stem region was well conserved among *Flaviviruses* [including WNV, tick-borne encephalitis virus (TBE), YFV and JEV] [121, 136]. Therefore, an antiviral peptide targeting against one DENV serotype might possess the possibility to inhibit other DENV serotypes and closely related *Flaviviruses*. To further examine this hypothesis, Schmidt *et al.* (2010) investigated the antiviral potential of stem peptides derived from DENV-2 and WNV against DENV 1, 2, 3 and 4 [136]. The amino acid residues from 419-447 of the genome of each of the four DENV serotypes were synthesized along with a solubility tag (RGKGR). Results showed that $DV2^{419-447}$ remained the strongest inhibitor against all four dengue serotypes, followed by $DV1^{419-447}$, $DV3^{419-447}$ and $DV4^{419-447}$. Nonetheless, when stem peptides (residues 419-447) from related flaviviruses were tested against DENV infection, none of these peptides inhibited any of the DENV serotypes. This might be due to the variation in the seven residues located at the C-terminal which could have affected the non-specific interaction with the viral membrane rather than poor affinity against E protein, as WNV had nearly identical binding affinities for trimeric DV2 sE [136]. To further validate the observation, mutagenesis was performed, confirming that residues 442-444 were important in conferring the antiviral activity of the stem peptide whereby increased hydrophobicity would increase inhibitory strength [136]. On the other hand, a similar situation was observed by Hrobowski *et al.* (2005), whereby the DN 59 peptide (peptide sequence corresponding to the pre-anchor stem of the E protein and highly conserved among flaviviruses) which was shown to inhibit DENV had also demonstrated cross-inhibitory activity against WNV (>99% inhibition with concentration of <25μM) [119].

Bai *et al.* (2007) found a peptide, P1, which was isolated from the murine brain cDNA phage display library by biopanning against the recombinant WNV E protein [137]. When P1 was tested against DENV-2, it inhibited ~99% DENV-2 at a concentration of 200 μM. Surface plasmon resonance (SPR) showed that P1 bound to the WNV E protein with a K_d of 6 μM. However, the specific binding site on E protein and the mode of action are unknown. Other peptides that blocked DENV infection by binding to the E proteins were DN57 opt and 1OAN1 [138]. In the study, a set of peptides were computationally designed based on the pre-entry dimeric E structure. Peptides DN57 and 1OAN1 specifically designed from the hinge of domain II and the first domain I/domain II connection, was shown to display IC_{50} of 8 and 7 μM, respectively [138]. Both peptides were shown to bind specifically (affinities ~1 μM) to the purified DENV-2 E protein. Images from cryoEM suggested that these peptides might have caused structural deformations to the DENV-2 surface, hence interfering the virus-host cell binding. Further study of peptide inhibitors 1OAN1 and DN59 has also revealed that both peptide inhibitors were able to inhibit the antibody dependent enhancement (ADE) effect *in*

vitro with an IC_{50} of 3 μM and 6 μM, respectively [139]. In a recent study, Chew *et al.* (2015) identified a novel peptide, peptide gg-ww, by biopanning a randomised phage display peptide library against the purified DENV-2 viral particles [140]. Approximately 96% inhibition was achieved at the concentration of 250 μM and the data indicated that peptide gg-ww inhibited DENV-2 entry. On the other hand, screening of commercial cyclic peptides through molecular docking resulted in the identification of a peptide, the brain natriuretic peptide fragment 7-32 (BNP7-32), which could bind to the E protein with a p*Ki* value of 32.7 and ΔG of -44.9 $kcalmol^{-1}$ [141]. Due to the fact that data were obtained via *in silico* design, further experiments were required to explore the inhibitory potential of the peptide against DENV.

Entry Inhibitors: Targeting prM/M and C proteins

Many studies have focused on the DENV E protein due to the nature of the virus structure, whereby the E proteins cover most of the surface area of the viral particle [112], and the vast and expanded knowledge on the E protein. Nonetheless, prM and C proteins are feasible targets to look into in the screening for antiviral peptides.

The prM protein (about 21 kDa) is the precursor of the M protein (approximately 8 kDa). The cleavage of the prM protein by the cellular protease (furin) would separate the prM protein into the "pr" peptide (1-91 residues), the ectodomain (92-130 residues) and the M protein (131-166 residues) [45, 142, 143]. The hydrophilic N-terminal region of the protein is responsible for coding the glycosylated "pr" segment of the prM protein. The prM is believed to protect the E protein from conformational changes during the maturation pathway in the acidic environment of the trans-golgi network. A previous study has shown that prM-containing-virus is more resistant to the low pH environment [144]. Upon release of the matured virions, "pr" will be separated, leaving the E and M proteins on the surface of the mature DENV. In a recent study, a peptide inhibitor (MLH40) mimicking the conserved ectodomain of the M protein was designed and it was shown to inhibit all four DENV serotypes with an IC_{50} of 24–31 μM [145]. Docking results indicated that MLH40 bound to the interior site of E homodimer, which is the same interacting site for the native M protein against the E protein. Additionally, the expression of the pr protein also successfully inhibited virus fusion at low pH and stopped viral infection [146]. Approximately 81–85% inhibition was achieved at 30 μM. Data indicated that the pr peptide interacted with a highly conserved histidine (H244) because the substitution of H244 to alanine had led to the disruption of the pr-E interaction [146]. This disruption could have resulted in a distorted E conformation, therefore interfering the pH-triggered fusion reaction in the endosome. Further optimization could be carried out using truncated pr peptides to identify the amino acid residues that are essential to block viral infection.

On the other hand, the DENV C protein (approximately 11 kDa) is composed of four α-helical regions arranged in antiparallel homodimers [147]. The structure of the C protein contains high net charge with an asymmetric distribution of basic residues which lie along the surface of the C protein to orchestrate RNA binding. In contrast, the opposite surface forms a hydrophobic region which may enable interactions with lipids [147, 148]. This configuration makes the C protein essential in virus assembly as it enables encapsulation of the ssRNA genome to form the nucleocapsid [45]. Despite a general understanding of the viral RNA assembly role, it is believed that the hydrophobic region of the C-terminal capsid protein contains a signal sequence for anchoring the protein into the endoplasmic reticulum (ER) membrane and partitioning the prM protein to the membrane [143, 149]. The C-terminal is then cleaved off by the viral NS2B-NS3 protease to form a mature protein during virus assembly [148]. Faustino *et al.* (2015) have designed a peptide inhibitor, pep14-23, based on the conserved region of DENV C protein. The pep14-23 was able to interfere with the interaction of the DENV C protein with the host intracellular lipid droplet, which was shown to be essential for viral particle formation [46, 150]. It was found that the binding forces between the C protein and lipid droplets were reduced from 33 pN to 19 pN with the addition of 100 μM pep14-23. Interestingly, despite the importance of the C protein for viral survival, limited antiviral peptide was designed against the C protein. This is a target worth looking into.

Replication and Translation Inhibitors: Targeting NS proteins

Viral proteases have been shown to serve as good inhibitory targets. For instance, protease inhibition was shown to be a successful strategy in treating HIV infection [151]. The HIV-1 protease cleaves the translated polypeptide chain into smaller functional proteins, thereby allowing the virus particle to mature [152]. By inhibiting the protease, the immature virus particles would not be able to transform into the mature virion, hence obstructing the viral replication. Several HIV-1 protease inhibitors were discovered and used clinically, such as saquinavir, ritonavir, indinavir, nelfinavir,

amprenavir (and its prodrug, fosamprenavir), lopinavir, atazanavir, and darunavir [153]. Similarly, the NS5, NS3 and NS2B (co-factor) proteins were known to play major roles in enzymatic activities for DENV infection, thus making them ideal antiviral targets [154, 155]. After DENV infection, translation of the viral genome will give rise to a polyprotein containing three structural and seven non-structural proteins. The polyprotein will be cleaved into individual proteins during virus maturation by the host proteases (signalase and furin) on the luminal side of the endoplasmic reticulum, as well as by the viral serine protease (NS2B-NS3 protease) on the cytoplasmic side to ensure the success of viral replication and maturation [49, 154, 156]. DENV NS3 contains a trypsin-like protease and it requires the NS2B cofactor to be active to cleave the DENV polyprotein at the Ser-His-Asp catalytic triad [157-160].

In a previous study, Schuller *et al.* (2011) synthesized a series of tripeptide aldehyde inhibitors whereby four of them had the IC_{50} values in the range between ~6.7 and 12.2 μM against the DENV-2 NS2B-NS3 protease [161]. Among the four tripeptide aldehyde inhibitors, tripeptide 1 (benzoyl-n-Lys-Arg-Arg-H) and tripeptide 2 (phenylacetyl-Lys-Arg-Arg-H) were reported to have the most potential as anti-DENV candidates with IC_{50} of 9.5 μM and 6.7 μM, respectively [162]. Further investigation of the tripeptide1 revealed that it bound covalently to the DENV-3 NS2B-NS3 protease and resulted in the formation of a closed conformation of the NS2B-NS3 protease in which the hydrophilic β-hairpin region of NS2B would wrap around the NS3 core [163]. Structural analysis of this protease-peptide complex further revealed a pocket located on the NS2B-NS3 protease which could act as a new antiviral target for drug development [163]. Another protease inhibitor, aprotinin, a large polypeptide [also known as bovine pancreatic trypsin inhibitor (BPTI)] was hypothesized to form multiple interactions with the NS2B-NS3 protease and gained its inhibitory activity from the steric hindrance of the active site [163]. In contrast to tripeptide 1 which required the interactions with NS2B, no direct binding was observed between aprotinin and NS2B [163].

In a recent study, several cyclic peptides were designed based on the binding loop of aprotinin which is highly similar to the sequence of the native NS2B-NS3 cleavage site and extends from the P3 to P4′ position at the active site of the NS2B-NS3 protease [164]. Results indicated that a peptide, CP7, was able to show good inhibitory property (*Ki* value of 2.9 μM) against the DENV-3 protease. Similar with the binding of aprotinin to the protease, strong hydrogen bonds contributed by the P1 and P2′ positions were observed but the inhibitory constant value was not as strong as aprotinin (*Ki* of 0.026 μM). This might be due to the flexibility of the cyclic peptide which resulted in the decreased affinity against the protease. Nevertheless, this study proved the feasibility of designing inhibitors against both prime and non-prime regions of the protease, and CP7 could act as an alternative candidate for developing a therapeutic against the NS2B-NS3 protease.

On the other hand, the N-benzoyl capped tetrapeptide sequence (Nle-Lys-Arg-Arg) was previously shown to be the favoured amino acid residues for the S1-S4 subsites of the NS2B-NS3 protease binding cavity [165, 166]. Yusof *et al.* (2000) showed that the Arg-Arg residues in the P1 and P2 positions located next to the cleavage site were responsible for the high binding affinity against the protease, while Li *et al.* (2005) found that Lys and Nle (norleucine) in the P3 and P4 positions were essential for high binding affinity [165, 166]. Interestingly, Nitsche *et al.* (2012) showed that the removal of an arginine resulted in better inhibitory activity [167].

Nitsche *et al.* (2012) showed that a retro-peptide based on the sequence R-Arg-Lys-Nle-NH_2 with an arylcyano-acrylamide group as N-terminal cap possessed the best inhibition activity at *Ki* value of 4.9 μM [167]. It is hypothesized that the arylcyanoacrylamide moiety mimic the first Arg in the P1 position while the Arg-Lys-Nle tripeptide bound to other protein pockets of NS2B-NS3 protease. Unfortunately, even though the drug candidate possessed good binding ability, it did not have significant antiviral activity in the cell culture against DENV. Therefore, Nitsche *et al.* (2013) further optimized the lead candidate via structure activity relationship assays in a subsequent study and successfully developed a thiazolidinedione-based peptide hybrid (hybrid 24b) containing a hydrophobic group with a better *Ki* value of 1.5 μM [168]. Nonetheless, drugs designed via structure-based activity faced the limitation due to the differences in protease structures derived from crystallization versus the *in vivo* protease structures, thereby the antiviral candidate which has high binding affinity against the crystallized protease structure might not have the same binding affinity against the protease *in vivo*. To overcome this challenge, Nitsche *et al.* (2013) further modified the N-terminal cap moieties and incorporated membrane-permeable peptide to increase the potential antiviral activities [168]. The peptide hybrid which possessed the best antiviral activity in the cell culture was found to be the rhodanine-based peptide hybrid 10a with an EC_{50} value of 16.7 μM and *Ki* value of 9.3 μM. From the

study, the Arg-Lys residues were found to be sufficient to create high target affinity with *Ki* values below 2 μM.

On the other hand, Behnam *et al.* (2015) utilized the Bz-Arg-Lys-Nle as the core sequence to further optimize the chemical structure to improve its antiviral potential against the NS2B-NS3 protease [169]. Tripeptide hybrid 83 was created by the combination of 4-CF_3-benzyl ether and the thiazole cap, while tripeptide hybrid 86 was created by the combination of 4-CF_3-benzyl ether and the thien-2-yl cap. Both of the peptide hybrids successfully showed improved *Ki* values of 12 nM and 19 nM, respectively. Thus far, these are the two peptide hybrids which possess the highest binding affinity against the NS2B-NS3 protease. Nonetheless, the derivative 83 which possessed the highest binding affinity had a lower EC_{50} value of 20 μM in reducing the virus titer as compared to the derivative 86 (EC_{50} value of 7 μM) while the derivative 104 and derivative 90 were found to have the most potent EC_{50} value of 3.42 μM and 4.06 μM, respectively. Theoretically, the inhibitor which has a higher binding affinity against a target would result in a higher antiviral property. But in contrast to the direct *in vitro* protein-peptide interaction assay, cellular antiviral assay in the cell culture is complicated by factors such as membrane permeability and metabolic stability. Data suggested that the lipophilicity of the tripeptide hybrids correlated well with the observed cellular antiviral activities which might be influenced by the fact that higher polarity (lower lipophilicity) could lead to weaker membrane permeability [169]. In this case, the tripeptide hybrid 104 was shown to be more lipophilic than the tripeptide hybrid 83. Moreover, data also indicated that the tripeptide hybrid 104 possessed a much higher half-life of 175 minutes compared to the tripeptide hybrid 83 (half-life of 45 minutes) in metabolic clearance via rat liver microsomes. These observations might offer the possible explanation of the higher antiviral activity of the tripeptide hybrid 104 in cellular assays as compared to the analogue of tripeptide hybrid 83 which possessed higher binding ability against DENV-2 protease *in vitro*.

In another study, 15 peptide inhibitors against the NS2B-NS3 protease were modified via molecular modelling method [170]. Results from the ADME/Tox calculation indicated that the peptide inhibitor 11 (Bz-Nle-Lys-Arg-Bip) possessed the best pharmacokinetic properties among all inhibitors due to its ability to be better absorbed into the intestine and to remain inactive in the central nervous system [170]. This results supported the use of Bz-Nle-Lys-Arg as the lead candidate for drug development against DENV by targeting the NS2B-NS3 protease. On the other hand, several hexapeptides derived from the NS2B-NS3 protease cleavage sites were synthesized and they were shown to inhibit DENV in a low micromolar range [171]. Among all candidates, the best hexapeptide inhibitor was derived from the NS2A/NS2B region with a *Ki* value of 12 μM. Likewise, several peptidic α-keto amide inhibitors mimicking the NS3/NS4 region were designed and two of the peptidic inhibitors were found to inhibit the NS3 protease with *Ki* values of 16 and 47 μM, respectively [172].

Tambunan and Alamudi (2010) designed several cyclopentapeptides based on the substrate specificity for the NS2B-NS3 protease and a cyclopentapeptide, CKRKC, was found to be the best candidate with the estimated free binding energy of -8.39 kcal/mol and *Ki* of 0.707 μM [173]. Nonetheless, the cyclopentapeptide was designed *in silico*, further *in vitro* and cell-based experiments were required to further exploit the inhibitory potential of the peptide against DENV. Similarly, Velmurugan *et al.* (2014) found a hexapeptide with the highest binding energy of -80.4 kcal/mol against the NS2B-NS3 protease, but further investigation is required to verify its antiviral potential via *in vitro* studies [174]. In another study, a natural peptide library composed of conotoxins (a mixture of peptide neurotoxins produced by cone snails) was used to screen against the DENV NS2B-NS3 protease [175]. A 13-mer cyclic peptide inhibitor, MrIA (derived from *Conus marmoreus*), was shown to possess a *Ki* value of 9 μM. Mutagenesis study further revealed that the inhibitory activity was mainly mediated by a disulphide bond loop with a Lys residue at the active site. As the NS2B-NS3 protease was previously shown to have a preference for Ser at the P' position, further optimization was carried out. By changing the Leu to Ser, the resultant peptide 7 (P7) and peptide 9 (P9) were shown to have stronger inhibitory activities with improved *Ki* values of 1.4 μM and 2.2 μM, respectively. Nonetheless, stability assays showed that about half of the inhibitory activity of P7 was lost after 2.5h, while only ~25% inhibitory activity of P9 was lost after incubating with protease for 2.5h. Although P9 had a higher *Ki* value of 2.2 μM as compared to P7 (1.4 μM), the stability of P9 to withstand protease degradation and its efficient cell permeability in BHK-21 and Vero cells had shown that P9 is a better drug candidate. Rothan *et al.* (2012a and 2012b) identified two antiviral peptides, namely protegrin-1 and retrocyclin-1 against DENV NS2B-NS3 protease [176, 177]. It was previously shown that protegrin-1, a cationic cyclic peptide, possessed broad antimicrobial properties against different microorganisms [178]. In addition to having good binding affinity (*Ki* value of 5.85 μM)

against the DENV NS2B-NS3 protease, protegrin-1 was able to inhibit DENV replication in Rhesus monkey kidney (MK2) cells with an IC_{50} of 11.7 μM [176]. On the contrary, retrocyclin-1, a circular cationic peptide, was shown to inhibit the NS2B-NS3 protease at an IC_{50} of 21.4 μM [177]. Results indicated that retrocyclin-1 worked best when incubated with DENV-2 during simultaneous treatment, giving 85% reduction in viral replication after 75 hours. These findings suggest that both protegrin-1 and retrocyclin-1 are feasible candidates to be potential therapeutic drugs for dengue treatment.

NS5 is a large multifunctional protein which plays an important role in viral replication and modulation of the host immune response. It contains RNA-dependent RNA polymerase domain at the C-terminal end which is essential for viral replication and methyltransferase at the N-terminal region for RNA capping [155, 179]. These functions are essential for viral replication, thereby making NS5 a promising target for antiviral drug development. Inhibiting the polymerase or methyltransferase function can actively suppress virus growth and propagation in host cells [180]. Both NITD-008 and Balapiravir are examples of nucleoside inhibitors, whereby NITD-008 corresponds to an adenosine analogue while balapiravir is another nucleoside analogue originally developed for the treatment of hepatitis C virus. However, NITD-008 was terminated due to severe side effects while balapiravir failed due to its low efficiency in clinical trials [21, 22]. To search for novel antiviral peptide against the NS5 methyltranserase, Tambunan and colleagues (2014) screened over 300 commercial cyclic peptides and molecular docking results revealed two potential ligands, namely, tyr123-Prepro Endothelin (110-130) and urotensin II [181]. Both peptides were shown to bind to the NS5 methyltranserase and formed stable complexes. Docking results suggested that tyr123-Prepro Endothelin (110-130) was found to bind to the S-adenosyl-L-methionine (SAM) site of NS5 with a ΔG of -24.73 kkal/mol while urotensin II bound to the RNA-cap site of NS5 with a ΔG of -19.04 kkal/mol. Nevertheless, further *in vitro* verification is required to elucidate the antiviral potential of these inhibitors. Despite the importance of the NS5 protein, there are limited antiviral peptide screening studies targeting the NS5 protein. Perhaps this is due to the lack of a crystal structure that contains the full length of the NS5 protein. With the recent publication of the crystal structure of the NS5 protein [182], development of antiviral peptide targeting the NS5 protein can be expected.

Limitations of Antiviral Peptides

There are a number of limitations that hinder the use of peptides as therapeutic drugs. The main challenges are due to the poor stability and bioavailability of peptides. Unmodified peptides were shown to commonly degrade quickly in human serum, resulting in low *in vivo* activities [183, 184]. Nonetheless, various chemical modifications can be applied to manipulate the physicochemical properties of peptides, thereby increasing the stability and bioavailability of the peptides. These chemical modification approaches have been reviewed by Gentilucci *et al.* (2010) [53]. For instance, conjugation to polymers such as polyethylene glycol (PEG) increased the molecular weight of peptide, hence enhancing its peripheral stability to undergo hydrolysis in the brain [185]. A previous study also showed that the half-lives of unmodified peptides of less than 0.5 hour could be increased to approximately 1.5 hour after C-amidation and N-acetylation, while cyclization successfully increased the half-lives of the peptides to 6.5 hours [186]. On the other hand, the replacement of arginine residues with an amino acid derivate of arginine, α-amino-3-guanidino-propionic acid (Agp), could dramatically reduce the cleavage of peptide by trypsin [187]. The substitution of L-amino acids to D-amino acids of pandinin-2 increased its resistance to the degradation by bovine pancreatic trypsin and human elastase. The stability of the D-amino acid substituted pandinin-2 was increased to 4 hours as compared to L-pandinin-2 which was rapidly cleaved by these two enzymes [188].

Additionally, multiple strategies could also be incorporated into peptide drug development to enhance the antiviral properties of peptides. These include the mutagenesis assays to identify the vital amino acids which are responsible for the inhibitory effects and the addition of cell penetrating peptides to increase the cell permeability of peptides. For example, the IC_{50} of two antiviral peptides (DN57 opt and DN81 opt) were successfully reduced to 8 μM and 40 μM, respectively, after amino acid optimization was performed via residue-specific all-atom probability discriminatory function approach [119, 138]. Similarly, Schmidt and co-workers (2010a, 2010b) successfully enhanced the antiviral properties of the identified peptides after optimizations via mutagenesis assays and the addition of a solubility tag [121, 136]. On the other hand, due to the hydrophilicity and conformation properties of peptides, cellular uptake of peptides is constrained [189, 190]. Oral bioavailability of peptides is therefore limited by the membrane barrier. To overcome this challenge, cell-penetrating peptides (CPP) can be incorporated into the peptide sequence to act as a cargo delivery in carrying antiviral peptide across the permeability barrier and entering cells to exert its

inhibitory activity [191]. For instance, by conjugating the tat peptide (derived from the HIV-1 transcriptional activator protein) to galactosidase protein, it enabled the delivery of the fusion protein to all the tissues in mice while those without tat peptide-conjugated were restrained [192]. With the advancement of technology, the drawbacks of peptides serving as antiviral agents can therefore be overcome by various strategies. This, in turn, will aid the development and use of peptides as therapeutics.

Conclusions

The development of peptides as therapeutic drugs against viruses is a promising field for drug discovery. Peptides are known to be highly specific and selective against targets, have lower toxicity and low accumulation in tissues, all of which make them good candidates for drug development [193]. This phenomenon is in contrast to small molecule drugs, whereby off-target and toxicity are some of the biggest drawbacks for small molecule drugs [194, 195]. Although peptides possess several limitations, these could be overcome via chemical modifications.

Many peptides are shown to be effective as they demonstrated good *in silico* and *in vitro* binding affinity against viral targets (Table 1 and 2). However, many of these antiviral peptides against DENV have not been tested in cell-based assays and none of them has been evaluated in any *in vivo* study. Therefore, further studies should focus on elucidating the antiviral potential of these peptides in animal models, such as the AG129 mice which are highly susceptible to DENV infection as they lack both type I and type II IFN receptors [196-198]. Furthermore, an ideal DENV inhibitor will need to be effective against all four DENV serotypes. The inhibitory potential against all DENV serotypes have not been explored by many of the studies described in this review. With just a few exceptions, the mechanisms of inhibition of most of the peptides are not well defined. These are the areas where further studies should be performed to exploit the potential of these antiviral peptide candidates. Moreover, the commercially available vaccine (CYD-TDV) is unable to achieve the maximal protection against all four DENV serotypes, while the vector control strategy has failed to reduce the spread of DENV. Peptide inhibitors targeting host cells, viral structural proteins or non-structural proteins may help to overcome dengue infection. With effective antivirals, vaccination and vector controls such as fogging, dengue in endemic countries can be better controlled and the state of public health can be improved.

Table 1. List of antiviral peptides against DENV structural proteins

Peptides	DENV serotypes	Sequences	Inhibitory activities	Target	References
DN59	All serotypes	MAILGDTAWDFGSLGGVFTSIGKALHQVFGAIY	IC_{50} less than 10μM for all serotypes	Envelope	[119,120]
P1	DENV-2	DTRACDVIALLCHLNT	200 μM (~99.3% inhibition)	Envelope	[137]
$DV2^{419-447}$	All serotypes	AWDFGSLGGVFTSIGKALHQVFGAIYGAA (solubility tag-RGKGR)	FP IC_{50} of 0.125μM (DENV-2)	Envelope	[121, 136]
$DV2^{419-440}$	All serotypes	AWDFGSLGGVFTSIGKALHQVF	FP IC_{50} of 0.25μM (DENV-2)	Envelope	[121]
$DV2^{413-447}$	All serotypes	AILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAA	FP IC_{50} of 0.275μM (DENV-2)	Envelope	[121]
$DV2^{413-440}$	All serotypes	AILGDTAWDFGSLGGVFTSIGKALHQVF	FP IC_{50} of 0.25μM (DENV-2)	Envelope	[121]
$DV2^{413-435}$	All serotypes	AILGDTAWDFGSLGGVFTSIGKA	FP IC_{50} of 2 μM (DENV-2)	Envelope	[121]
$DV1^{419-447}$	All serotypes	AWDFGSIGGVFTSVGKLVHQVFGTAYGVL (solubility tag-RGKGR)	IC 90: DV1- 1.5 μM; DV2- 2 μM DV3- >6 μM; DV4- >6 μM	Envelope	[136]
$DV3^{419-447}$	All serotypes	AWDFGSVGGVLNSLGKMVHQIFGSAYTAL (solubility tag-RGKGR)	IC 90: DV1- 0.1 μM; DV2- 2 μM DV3- 4 μM; DV4- 1.5 μM	Envelope	[136]
$DV4^{419-447}$	All serotypes	AWDFGSVGGLFTSLGKAVHQVFGSVYTTM (solubility tag-RGKGR)	IC 90: DV1- 5 μM; DV2- 6 μM DV3- >6 μM; DV4- 6 μM	Envelope	[136]
DN57opt	DENV-2	RWMVWRHWFHRLRLPYNPGKNKQNQQWP	8 μM	Envelope	[138]
1OAN1	DENV-2	FWFTLIKTQAKQPARYRRFC	7 μM	Envelope	[138]
DN81 opt	DENV-2	RQMRAWGQDYQHGGMGYSC	36 μM	Envelope	[138]
DS03opt	DENV-2	FPFDFHHDRYYHFHWKRYQH	na	Envelope	[122]
DS04opt	DENV-2	IWWRPRDWPTFIFYFREWRW	na	Envelope	[122]
DS27opt	DENV-2	KEYFRRFFHCHNHQREWHWH	na	Envelope	[122]
DS28opt	DENV-2	KEKRREWEWRFRWEFRLYFE	na	Envelope	[122]

DS36opt	DENV-2	RHWEQFYFRRRERKFWLFFW	na	Envelope	[122]
DET4	DENV-2	AGVKDGKLDF	35 μM	Envelope	[132]
EF	DENV-2	EF	96 μM	Envelope	[133]
Pgg-ww	DVEN-2	GGARDAGKAEWW	IC_{50} of ~77 -91 μM	Envelope	[140]
MLH40	DENV	SVALVPHVGMGLETRTETWMSSEGAWKHVQRIETWILRHPG	IC_{50} of 24-31μM	Pre-Membrane	[145]
pr	DENV-1 and DENV-2	pr protein	30 μM (81-85% inhibitions)	Pre-Membrane	[146]
Pep14-23	DENV	NMLKRARNRV	Binding forces were reduced to 19pN from 33pN with the addition of 100 μM pep14-23	Capsid	[150]

DENV: Dengue virus; na: not available

Table 2. List of antiviral peptides against DENV non-structural proteins

Peptides	DENV Serotypes	Sequences	Inhibitory activities	Target	References
Tripeptide 2	DENV-2	Phenylacetyl-K-R-R-H	IC_{50} of 6.7 μM	NS2B-NS3	[161]
Tripeptide 1	DENV-2	Benzoyl-n-K-R-R-H (n=norleucine)	IC_{50} of 9.5 μM	NS2B-NS3	[161-163]
Tripeptide 12	DENV-2	4-Aminophenylacetyl-K-R-R-H	IC_{50} of 11.2 μM	NS2B-NS3	[161]
Tripeptide 11	DVEN-2	4-Phenylphenylacetyl-K-K-R-H	IC_{50} of 12.2 μM	NS2B-NS3	[161]
Aprotinin	DENV-3	RPDFC LEPPY TGPCK ARIIR YFYNA KAGLC QTFVY GGCRA KRNNF KSAED CMRTC GGA	*Ki* of 0.026 μM	NS2B-NS3	[163]
Peptidic α-keto amide 4	DENV-2	Ac-FAAGRR-CHO	*Ki* of 16 μM	NS2B-NS3	[172]
Peptidic α-keto amide 1	DENV-2	Ac-FAAGRR-αketo-SL-$CONH_2$	*Ki* of 47 μM	NS2B-NS3	[172]
Hexapeptide-1	DENV	Ac-RTSKKR-$CONH_2$	*Ki* of 12 μM	NS2B-NS3	[171]
CP7	DENV-3	PCRARIYGGCA	*Ki* of 2.9 μM	NS2B-NS3	[164]
Tripeptide hybrid 83	DENV-2	Bz-Arg-Lys-L-Phg-NH_2 by the combination of 4-CF_3-benzyl ether and thiazole cap	*Ki* value of 12 nM; EC_{50} value of 20 μM	NS2B-NS3	[169]
Tripeptide hybrid 86	DENV-2	Bz-Arg-Lys-L-Phg-NH_2 by the combination of 4-CF_3-benzyl ether and thien-2-yl cap	*Ki* value of 19 nM; EC_{50} value of 7 μM	NS2B-NS3	[169]
Tripeptide hybrid 104	DENV-2	Bz-Arg-Lys-L-Phg-NH_2 by the combination of 3-OCH_3-benzyl ether and bithiophene cap	EC_{50} value of 3.42 μM	NS2B-NS3	[169]
Peptide inhibitor 11 (BDBM50175978)	DENV-2	Bz-Nle-Lys-Arg-Bip	*Ki* value of 1.16E+4 nM	NS2B-NS3	[162, 170]
Cyclopentapeptide CKRKC	DENV-2	CKRKC	*Ki* of 0.707 μM	NS2B-NS3	[173]
Hexapeptide-2	DENV-2	AIKKFS	Glide energy -80.4kcal/mol	NS2B-NS3	[174]
Retro tripeptide hybrid 11	DENV-2	R-Arg-Lys-Nle-NH_2 with an arylcyano-acrylamide group as N-terminal cap	*Ki* value of 4.9 μM	NS2B-NS3	[167]
peptide hybrid 10a	DENV-2	Rhodanine-based peptide hybrid bearing a cyclohexyl moiety at the heterocycle	EC_{50} value of 16.7 μM	NS2B-NS3	[168]
peptide hybrid 24b	DENV-2	thiazolidinedione-based peptide hybrid	*Ki* value of 1.5 μM; IC_{50} value of 2.9 μM	NS2B-NS3	[168]
P7	DENV-2	CGKRKSC	*Ki* value of 1.4 μM	NS2B-NS3	[175]
P9	DENV-2	CAGKRKSG	*Ki* value of 2.2 μM	NS2B-NS3	[175]
Protegrin-1	DENV-2	RGGRLCYCRRRFCVCVGR	IC_{50} of 11.7 μM	NS2B-NS3	[176]
Retrocyclin-1	DENV-2	GICRCICGRGICRCICGRIGGRVPGVGVPGVGHHHHHH	IC_{50} of 21.4 μM	NS2B-NS3	[177]
tyr123-Prepro Endothelin (110-130)	DENV-2	CQCASQKDKKWSYCQAGKEI	ΔG of -24.73 kkal/mol	NS5	[181]
Urotensin II	DENV-2	ETPDCFWKYCV	ΔG of -19.04 kkal/mol	NS5	[181]

DENV: Dengue virus; NS: non-structure

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Competing Interests

The authors have declared that no competing interest exists.

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