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Synthetic peptide optimization improves the inhibition of dengue NS2B-NS3 protease and dengue replication *in vitro*

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Summary. – Dengue virus (DENV) infection is one of the most widely-spread flavivirus infections with no effective antiviral drugs available. Peptide inhibitors have been considered as one of the best drug candidates due to their high specificity, selectivity in their interactions and minimum side effects. In this study, we employed computational studies using YASARA, HADDOCK server and PyMOL software to generate short and linear peptides based on a reference peptide, CP5-46A, to block DENV NS2B-NS3 protease. The inhibition potencies of the peptides were evaluated using in-house DENV2 serine protease and fluorogenic peptide substrates. *In vitro* analyses were performed to determine the peptides cytotoxicity and the inhibitory effects against DENV2 replication in WRL-68 cells. Our computational analyses revealed that the docking energy of AYA3, a 16 amino acid (aa) (-81.2 ± 10.6 kcal/mol) and AYA9, a 15 aa peptide (-83.8 ± 6.8 kcal/mol) to DENV NS2B-NS3 protease were much lower than the reference peptide (46 aa; -70.9 ± 7.8 kcal/mol) and the standard protease inhibitor, aprotinin (58 aa; -48.2 ± 10.6 kcal/mol). Both peptides showed significant inhibition against DENV2 NS2B-NS3 protease activity with IC_{50} values of 24 μM and 23 μM, respectively. AYA3 and AYA9 peptides also demonstrated approximately 68% and 83% of viral plaque reduction without significantly affecting cell viability at 50 μM concentration. In short, we generated short linear peptides with lower cytotoxic effect and substantial antiviral activities against DENV2. Further studies are required to investigate the inhibitory effects of these peptides *in vivo*.

Keywords: peptide inhibitors; dengue virus; NS2B-NS3 protease; plaque reduction

Introduction

Dengue virus (DENV) infection is one of the major health problems worldwide. DENV infection is endemic in more than 100 countries in the tropical and subtropical regions (WHO, 2018). The World Health Organization (WHO) estimated that DENV infects over 390 million people and cause 20,000 deaths annually (WHO, 2018). Clinical symptoms caused by DENV infection ranges from mild fever to severe dengue hemorrhagic fever and dengue shock syndrome.

DENV is a member of the *Flaviviridae* family that possesses a positive sense single-stranded RNA genome. The virus uses the host's ribosomes to translate its genomic RNA to a full-length precursor polyprotein. Host cell furin and viral NS2B-NS3 serine protease (NS2B-NS3) cleaves the viral polyprotein into structural and non-structural proteins (Stadler *et al.,* 1997). The activity of NS3 protease domain depends on its interaction with NS2B cofactor (47 aa) to form NS2B-NS3pro complex (Yusof *et al.*, 2000). Disruption of viral NS2B-NS3pro function impairs viral replication and therefore is an ideal target for antiviral drug development (Geiss *et al.,* 2009).

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Abbreviations: DENV = dengue virus; NS2B-NS3pro = NS2B-NS3 protease complex; WHO = World Health Organization

Peptide inhibitors are considered as the best drug candidates for designing and developing new therapies (Vlieghe *et al.*, 2010). These peptides possess high specificity and selectivity which maximize their potencies and reduce the possible side effects (Di, 2015). However, the drawback of using peptide inhibitors as antiviral drug is the high cost of manufacturing. Here, we optimized and assessed the antiviral activities of several short and linear peptides in an effort to substantially reduce the production cost. Our short peptides showed high inhibitory effect against DENV2 protease compared to the reference peptide, CP5-46A, and the standard protease inhibitor, aprotinin. Our peptides also showed substantial antiviral effect *in vitro* without significantly affecting cell viability.

Materials and Methods

Computational work. Fifteen short peptides (AYA1-AYA15) were modelled and geometrically optimized (steepest descent) by Hyperchem 8.0.10 based on a template peptide (Kügler *et al.,* 2012). The interaction between the designed peptides and DENV2 NS2B-NS3pro was identified by protein-protein docking study. Protein Data Bank (PDB) file of NS2B-NS3pro (in-house amino acid sequences) was built using homology modelling by YASARA (Krieger *et al.,* 2002). Docking simulations were performed in an information-driven flexible docking approach using an available online HADDOCK server (http://haddock.science.uu.nl/services/HADDOCK/haddockserver-easy.html) (van Zundert *et al.,* 2016). Final docking output files were analyzed for hydrogen bonds using PyMOL software version 1.3 (PyMOL™ 2010 Schrodinger, LLC).

Production of recombinant dengue NS2B-NS3 protease in Escherichia coli. cDNA fragment of NS2B-NS3pro was generated using the NS2BF (5'-ATACTGAGGATCCGCCGATTTGGAACTG-3') and NS3R (5'-ATTGATCAAGCTTAAGCTTCAATTTTCT-3'), which was then used as a template to amplify the NS2B (aa 49–95) and NS3pro (aa 1–180) by PCR reactions. To produce a single chain NS2B-(G_4-T-G_4)-NS3pro, the NS2B fragments were amplified separately by PCR using the primer pairs NS2BF and NS2BlikR (5'-ATACTGAGGATCCGCCGATTTGGAACTG-3', 5-ACCTACTAGGTACCTCCTCCACCCAGTGTCTGTTCTTC-3') while NS3pro was amplified using NS3likF and NS3R primers (5'-ATCTATAGGTACCGGCGGTGGAGGTGCTGGAGTATTGTGG-3', 5'-AGCATAAGCTTAAGCTTCAATTTTCT-3'). G_4-T-G_4 linker sequence and *Kpn*I restriction sites were added to NS2BlikR and NS3likF primers. The PCR products were subsequently digested with *Bam*HI and *Kpn*I, and the purified fragments were cloned into a pQE30 plasmid downstream of the 6xHis tag (all restriction sites are underlined).

Enzyme purification and confirmation. E. coli XL1-Blue strain (Promega, USA) was transformed with pQE30-NS2B (G4-T-G4) NS3pro. The recombinant *E. coli* was inoculated in Luria-Bertani liquid medium (1% tryptone, 1% NaCl, 0.5% yeast extract w/v, pH 7.0) supplemented with 100 mg/l ampicillin and cultured overnight at 37°C. In general, 10 ml of overnight-grown culture was added to 1000 ml of culture medium and incubated with shaking at 37°C until the optical density at 600 nm reached 0.5. Isopropylthio-β-D-galactoside (IPTG) was then added to the culture medium at a final concentration of 0.5 mM to induce protein expression, and further incubation was performed for another 5 h at 37°C in a shaking incubator. Bacterial cells were harvested by centrifugation at 6,000 rpm for 15 min at 4°C. The protein was purified using His GraviTrap™ Flow pre-charged Ni Sepharose™ 6 Fast column (Amersham Biosciences, USA) according to the manufacturer's instructions. In brief, the column was equilibrated with phosphate buffer (20 mM sodium phosphate buffer and 500 mM NaCl, pH 8.0). The sample was loaded into the column and the column was washed with binding buffer (phosphate buffer containing 20 mM imidazole, pH 8.0). The recombinant protein was eluted with elution buffer (phosphate buffer containing 150 mM imidazole, pH 8.0). The final product was verified by SDS-PAGE and western blot. Purified recombinant NS2B-NS3 protease (20 µg) was separated in polyacrylamide gel and transferred onto nitrocellulose membranes. The membrane was blocked with blocking buffer overnight, and then incubated with anti-His tag antibody for 1 h. After washing three times, the membranes were incubated with anti-mouse IgG conjugated to horseradish peroxidase (Dako, Denmark) at 1:1000 for 2 h. Chromogenic substrate was added for colour development.

NS2B-NS3 protease assay. This assay was carried out as we previously described (Rothan *et al.* 2013). In brief, the endpoint reaction mixtures with a total volume of 200 µl was prepared, consisting of 40 µM fluorogenic peptide substrate (Pyr-RTKR-amc), 1 µM of recombinant NS2B-NS3pro, with or without the peptides of varying concentrations, buffered at pH 8.5 with 200 mM Tris-HCl. The peptides were previously prepared in distilled water and assayed at five different concentrations, ranging from 12.5–200 µM. The reaction mixtures were incubated at 37°C for 10 min. Subsequently, the substrate was added to each reaction mixture and further incubated for another 30 min. Measurements were performed in triplicates using Varian Eclipse Cary fluorescence spectrophotometer. Substrate cleavage was detected with 360/440 ex/em and normalized against the control. The readings were then used to plot DENV serine protease inhibition and IC_{50} values of peptide inhibitors was determined using non-linear regression models in GraphPad Prism 5.0 software.

Cytotoxicity assay. WRL-68 cells were seeded at $1x10^4$ cells per well in triplicates in 96-well plates and cultured at optimal conditions (37°C, 5% CO_2 in humidified incubator). AYA3 and AYA9 peptides were diluted serially (0, 12.5, 25, 50, 100, 200 µM) in DMEM media supplemented with 2% FBS. Each dilution was tested in triplicate. Two control wells were included in each experiment: culture medium without peptides and culture medium with different concentrations of the designed peptides in the absence of cells to subtract the background value of the peptides in the culture medium. Cell viability was analyzed at 48 h using Non-radioactive

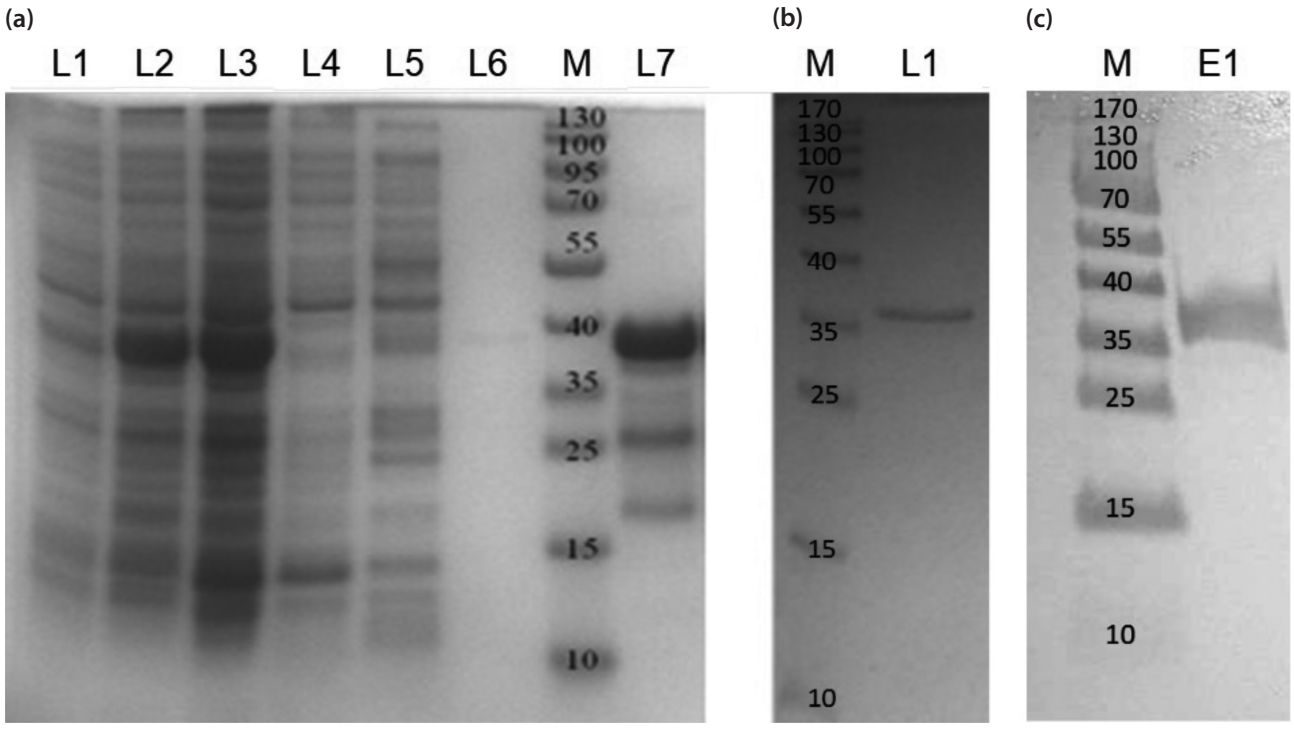

Fig. 1

Expression and purification of recombinant dengue NS2B-NS3pro

(a) Recombinant NS2B-NS3pro was produced as a single chain in *E. coli* (XL1-Blue) and purified using Ni^{+2}-NTA affinity chromatography column. L1 protein profile before IPTG induction; L2 after IPTG induction; L3 total protein after cells lysis; L4 unbound protein; L5-L6, washing steps; M marker; L7 eluted protein. **(b)** SDS-PAGE analysis of purified protein, L1, purified protease. **(c)** Immunoblot analysis using His-tag antibody. E1: elution fraction.

cell proliferation assay (Promega, USA) according to the manufacturer's instructions.

Treatment of DENV-infected cells with antiviral peptides. WRL-68 cells were infected with DENV (MOI of 1) for 2 h with gentle shaking every 15 min for optimal virus to cell attachment. Then, fresh complete DMEM medium containing different concentration (0, 12.5, 25, 50, 100, 200 μM) of each peptide was added to the cells and incubated for 48 h. Afterwards, cellular supernatants were collected and stored at -80°C for viral load quantification.

Virus quantification by plaque formation assay. Vero cells were seeded in 6-well plates. A 10-fold serial dilution of 200 μl culture supernatant of DENV-infected cells was added to fresh serum-free DMEM. The cells were incubated for 2 h at 37°C with gentle shaking every 15 min. Supernatants of uninfected cells were added to the negative control wells (mock control cells). After washing with phosphate-buffered saline (PBS), 2 ml of 0.5% agarose overlay diluted in DMEM maintenance medium was added to each well. Viral plaques were stained with crystal violet dye after a 3-day incubation period. Virus titers were calculated according to the following formula: titer (PFU/ml) = number of plaques / inoculum volume × dilution factor.

Results

Recombinant NS2B-NS3pro production in E. coli

In this study, we produced DENV2 NS2B-NS3 protease as a single chain recombinant protein in *E. coli* in a soluble form tagged with 6x histidine. IPTG treatment induced a strong expression of a >35kDa peptide (Fig. 1a). The final eluted fraction showed a clear >35kDa band (Fig. 1a). The eluted fraction was further purified, yielding a single band on SDS-PAGE (Fig. 1b). To verify the protease, we performed western blot using his-tag antibody and detected a single band with a similar size to the band in the SDS-PAGE analysis (Fig. 1c).

Short peptide optimization and computational interaction analysis

We designed and optimized several (AYA1-AYA15) short peptides which were derived from CP5-46A peptide that was previously reported to demonstrate antiviral activity (Kügler *et al.* 2012). The aim of our computational study was to create shorter and linear peptides with high bind-

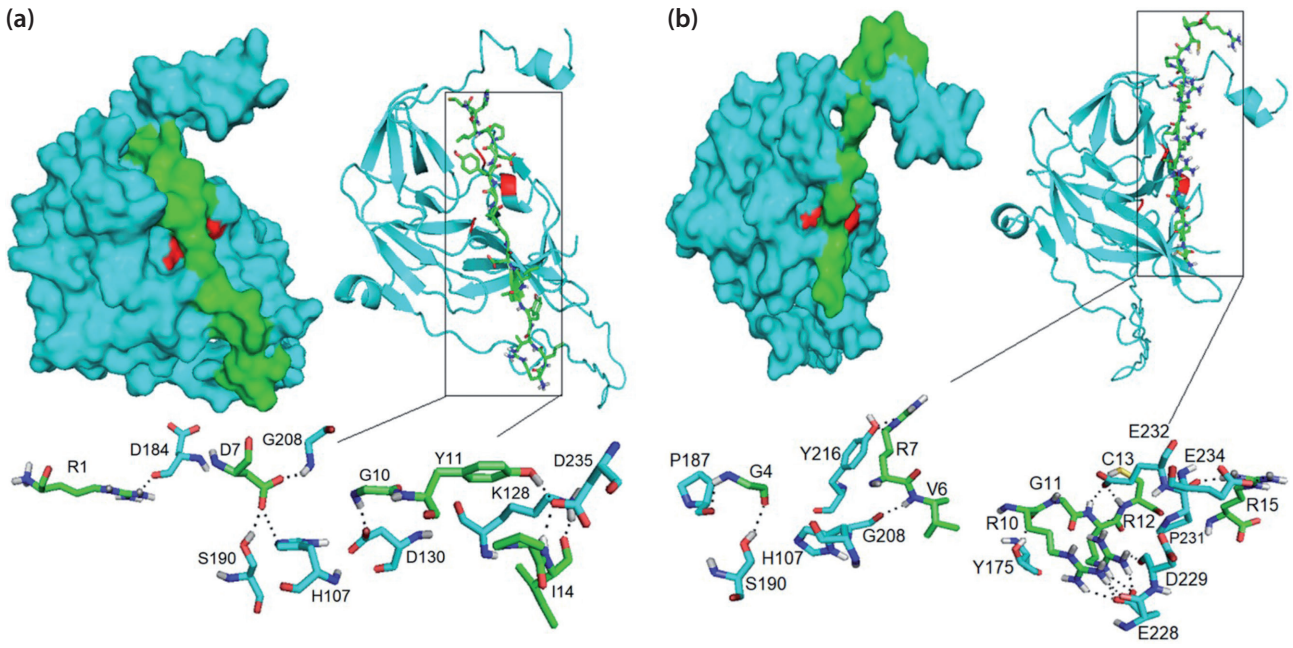

Fig. 2

Docking studies of the AYA3 and AYA9 peptide to DENV2 protease

Space-filling, ribbon, stick diagrams of AYA3 **(a)** and AYA9 **(b)** interaction with DENV NS2B-NS3 protease. The ribbon diagrams show peptide (green) binding to the protease (cyan) in close proximity to the active sites (red) of the protease. Stick diagrams show putative hydrogen bonds (dotted lines) between the peptide (green) with the protease residues (cyan).

Table 1. The parameters of AYA peptides docking energy towards dengue NS2B-NS3pro

Peptide name	Docking energy (kcal/mol)	Amino acids	Peptide sequence
CP5-46A	-70.9 ± 7.8	21	GELGRLVYLLDGPGYDPIHCD
Aprotinin	-48.2 ± 10.6	58	RPDFCLEPPYTGPCKARIIRY-FYNAKAGLCQ TFVYGGCRA KRNNFKSAEDCMRTCGGA
AYA 1	-70.9 ± 8.5	13	GRLVYLLDGPGYD
AYA 2	-74.9 ± 3.9	7	RLVYLLD
AYA 3	-81.2 ± 10.6	16	RLVYLLDGPGYDPIHC
AYA 4	-44.0 ± 2.2	10	LDGPGYDPIH
AYA 5	-42.6 ± 2.8	6	RLVYLD
AYA 6	-54.9 ± 9.2	11	GRLDGPGYIHC
AYA 7	-51.5 ± 7.0	8	RLDPYIHC
AYA8	-60.5 ± 2.1	13	CYGGRLRVRRGRC
AYA 9	-83.8 ± 6.8	15	CYGGRLRVRRGRCPR
AYA 10	-58.8 ± 3.7	14	CYGRLRVRRRCGPR
AYA 11	-74.3 ± 1.9	10	CYGRLVRVRR
AYA 12	-78.7 ± 1.7	10	CYGGRLRVRR
AYA 13	-76.4 ± 11.1	9	CYGRLRVRR
AYA 14	-73.3 ± 2.8	9	GPGYDPIHC
AYA 15	-70.4 ± 7.9	9	GELRLRVRR

Table 2. Predicted peptide interactions with DENV protease

DENV protease	CP5-46 (21 aa)	AYA3 (16 aa)	AYA9 (15 aa)
Docking energy (kcal/mol)	-70.9 +/- 7.8	-81.2 ± 10.6	-83.8 +/- 6.8
Total H bonds	6	8	14
S190	1	1	1
D130	0	1	0
H107	0	1	0

ing affinity. Among the fifteen peptides, AYA3 and AYA9 peptides showed the lowest and better docking energy to DENV NS2B-NS3pro (-81.2 ± 10.6 kcal/mol and -83.8 ± 6.8 kcal/mol) than the control peptides, aprotinin and CP5-46A, indicating potential inhibitory effects against DENV genome replication (Table 1). For our full length recombinant DENV NS2B-NS3 protease, the catalytic triad refers to H107, D130, and S190 residues which correspond to the reported catalytic triad residues H51, D75 and S135 (Salaemae *et al.*, 2010). AYA3 and AYA9 were predicted to form eight and fourteen hydrogen bonds with DENV NS2B-NS3 protease (cyan), respectively, including several putative interactions with DENV protease active sites (red residues) as depicted in Fig. 2a and 2b. The number of hydrogen bonds and binding energy for the peptides are summarized in Table 2.

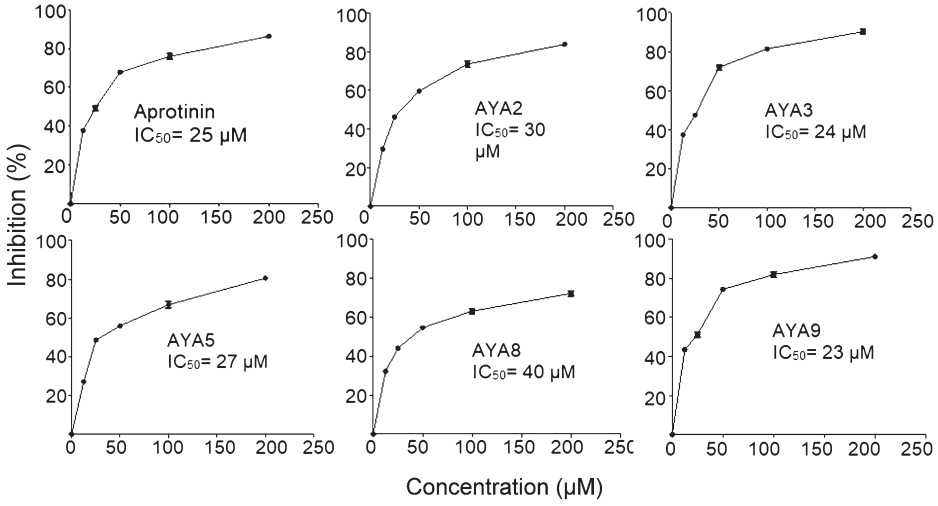

Fig. 3

Dengue NS2B-NS3pro inhibition assay

The designed peptides and aprotinin as a standard were evaluated in DENV NS2B-NS3pro assay. Each dot represents the average percentage of inhibition when normalized to the no-peptide/no-aprotinin controls for triplicate samples from three separate experiments with standard error of the mean (SEM) as the error bars.

Inhibitory potential of the AYA peptides against DENV2 NS2B-NS3pro

To verify our computational findings, we performed protease inhibition assay using our DENV NS2B-NS3pro. In this assay we used aprotinin, a 58 aa protease inhibitor, as the positive control. AYA3 and AYA9 peptides exhibited significant dose-dependent inhibition on dengue NS2B-NS3pro with IC_{50} values of 24 μM and 23 μM, respectively (Fig. 3). The inhibitory activities of these two peptides were comparable to aprotinin (IC_{50} 25 μM) with maximum inhibition of ≥90%. Other AYA peptides showed lower inhibition potential against DENV NS2B-NS3pro (AYA2 IC_{50}: 30 μM, AYA5 IC_{50}: 27 μM and AYA8 IC_{50}: 40 μM). The maximum DENV protease inhibition for these peptides at the highest concentration was also lower (around 80%).

AYA3 and AYA9 peptides inhibited DENV replication in human liver cells

AYA3 and AYA9, two peptides with the lowest IC_{50} values in DENV protease assay, were then tested for antiviral inhibition in human liver cells (WRL-68). In order to evaluate the efficacy of our peptide optimization method, the short peptides were compared to the reference peptide CP5-46A. We first evaluated the cytotoxic effects of these peptides. At lower concentrations, cell viability was similar among AYA3, AYA9 and CP5-46A treated groups. At 200 μM, viability for AYA3 and AYA9 treated group was >80% but viability for CP5-46A dropped to 62% (Fig. 4). Hence, the optimized peptides were less toxic to human cells when compared to the reference peptide. Next, we infected WRL-68 cells with DENV2 (MOI of 1) and treated the cells with increasing concentrations of the peptides for 48 h. The virus titer in the culture supernatants were evaluated by plaque formation assay. Supernatants from reference peptide (CP5-46A) treatment were used as the control in this experiment. Representative images showed clear reduction of the viral plaques starting from 50 μM concentration of AYA3 and

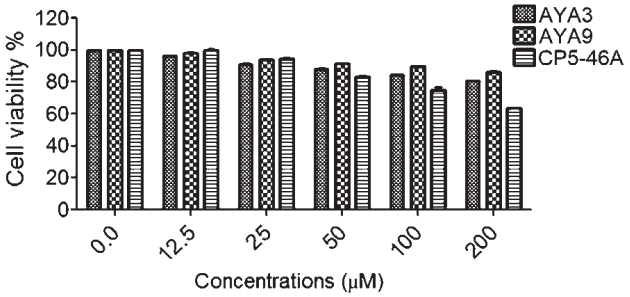

Fig. 4

The cytotoxic effect of AYA3, AYA9 and CP5-46A on viability of WRL-68 cells

The WRL-68 cells were seeded and treated with serial concentrations of the peptides (0, 12.5, 25, 50, 100 and 200 μM) for 48 h. Each bar represents the average percent viability when normalized to no-peptide sample with SEM as the error bar for triplicate samples from duplicate experiments.

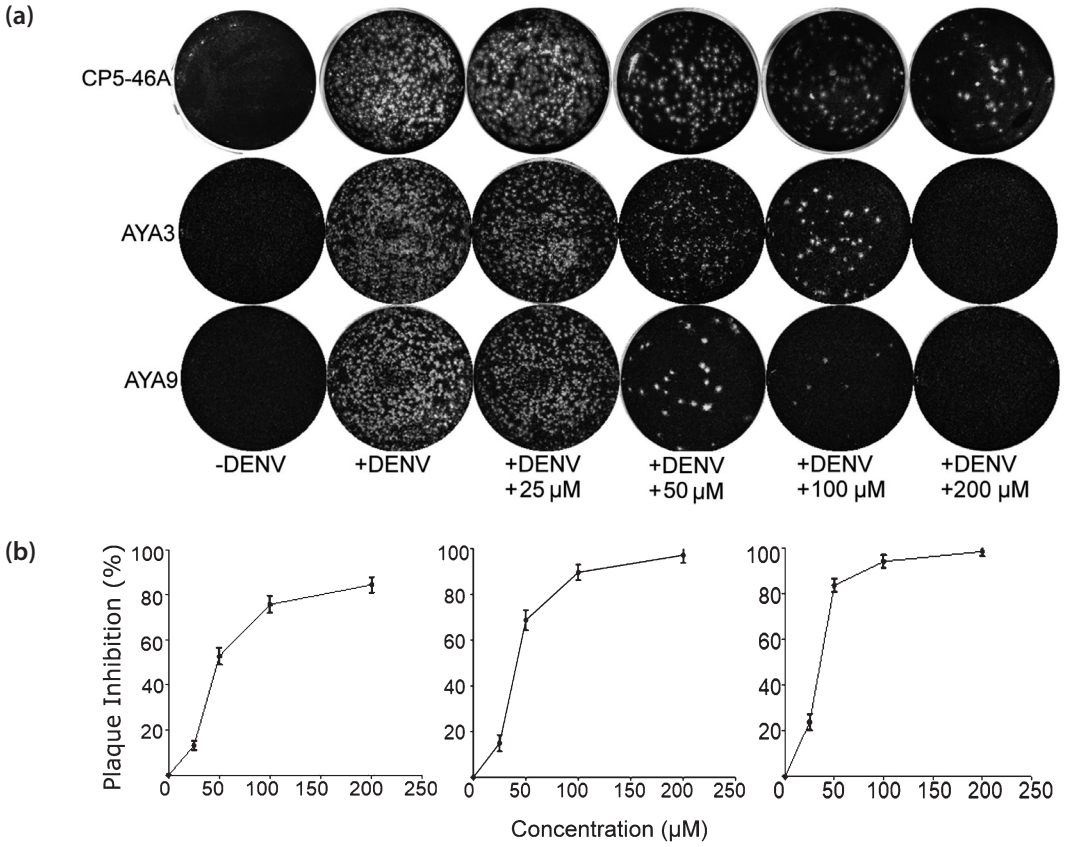

Fig. 5

The inhibitory effect of AYA3, AYA9 and CP5-46A against DENV replication

(a) Representative images of plaque reduction assay performed on Vero cells using supernatant of DENV-infected WRL-68 cells with or without peptide treatment. *In vitro* antiviral effects of CP5-46A peptide **(b),** AYA3 peptide **(c)** and AYA9 peptide **(d)** were quantified and plotted. Each point represents the average plaque reduction percentage when normalized to no-peptide samples (+DENV) ± standard deviation for triplicate samples in duplicate experiments.

AYA9 peptides when compared to the reference peptide CP5-46A (Fig. 5a). CP5-46A peptide showed approximately 55% of viral plaque reduction at 50 μM (Fig. 5b) whereas AYA3 and AYA9 showed approximately 68% and 83% of viral plaque reduction at the same concentration (Fig. 5c and 5d). Both AYA3 and AYA9 showed maximum inhibition of close to 100% at 200 μM whereas CP5-46A showed about 80% inhibition at the highest dose. These results showed significant improvement of the antiviral activity of our computer-based optimized peptides compared to the reference peptide.

Discussion

DENV infection is a global health problem due to the increased frequency of DENV outbreaks and expanded DENV epidemic areas (Lim *et al.,* 2013). Although extensive efforts have been geared towards finding a cure for DENV, only one vaccine has been approved for DENV prevention (Pitisuttithum and Bouckenooghe, 2016). However, the application and efficacy of this vaccine are still very limited (WHO, 2018). Thus, an effective antiviral drug is urgently needed to combat DENV infection.

Synthetic therapeutic peptides are suitable candidates for anti-DENV drug development as these peptides can be tailored to target specific conserved regions of the virus which is crucial due to the diversity of DENV serotypes and the mutability of its genomic RNA (Hapuarachchi *et al.,* 2016). In this study, we employed computational studies to generate shorter linear peptides with high affinity against DENV NS2B-NS3 protease. The reference peptide, CP5-46A, was a 21 aa peptide with a slightly negative charge at physiological pH. Among the optimized peptides, AYA3 and AYA9 peptides showed the lowest docking energy towards DENV NS2B-NS3pro and AYA9 showed the best DENV inhibition post infection *in vitro*. AYA3 was a 16 aa peptide with a neutral charge whereas AYA9 was a positively charged 15 aa peptide at physiological pH. Net peptide charge could be

Table 3. CC_{50}, EC_{50} and selectivity index of the peptides

	CP5-46A	AYA3	AYA9
CC_{50} (μM)	>200	>200	>200
EC_{50} (μM)	49.7	41.8	32.2
Selectivity index (SI)	4.0	4.8	6.2

one of the reasons for the improved antiviral effects observed in AYA9-treated samples as cationic peptides were reported to exhibit increased cell surface binding and cellular uptake (Fei *et al.*, 2011). The positively-charged AYA9 also exhibited better solubility than the neutral-charged AYA3 which would improve peptide distribution in the aqueous environment in the *in vitro* culture. In addition to net charge, AYA9 peptide sequence lacks aspartic acid residue and aspartic acid-proline sequence when compared to AYA3 and CP5-46A, making it less prone to spontaneous cyclization and peptide hydrolysis (Piszkiewicz *et al.,* 1970; Capasso *et al.,* 1992). Due to these properties, AYA9 showed better antiviral activity with less effect on cell viability when compared to AYA3 and CP5-46A peptides. The selectivity index (SI) for AYA9 was higher (6.2) than the SI of AYA3 (4.8) and CP5-46A (4.0) (Table 3).

There are several ways to further improve the efficacy of the peptides. Residue optimization such as reversible lipidization, modification of the N- and C-termini and other strategies can be performed to increase peptide stability and prolong peptide half-life (Di, 2015). Peptide fusion is another method to improve the antiviral effects of the synthetic peptides. We recently published a synergistic improvement in peptide binding affinity towards Zika virus NS2B-NS3pro and significant Zika inhibition *in vitro* when two short antiviral peptides were fused to either side of MAP30 peptide (Abdulrahman *et al.,* 2019). Similar strategy may be used to improve the efficacy of the short peptides against DENV protease without compromising cell viability.

Although our peptides are shorter than aprotinin and the reference peptide, the cost of chemical synthesis of these short peptides might be still quite substantial considering DENV infection is mainly prevalent in developing countries. Alternative strategies are required to economically produce these synthetic peptides in bulk rapidly in case of a sudden outbreak. We previously produced our synthetic peptides as recombinant proteins (Abdulrahman *et al.,* 2017, 2019) and as inclusion bodies in *E. coli* (Rothan *et al.,* 2015). Both methods yielded functional antiviral peptides and could pave the way for a large-scale anti-DENV peptide production.

As previously mentioned, disruption of viral protease can reduce viral replication in the target cells (Geiss *et al.,* 2009). Here, the inhibition of DENV replication by interfering with the activity of DENV protease was postulated to be the main antiviral mechanism of our short peptide. Nonetheless, other potential mechanisms of antiviral effects such as viral inactivation and prevention of viral binding and viral entry into cells could not be ruled out. Further studies are required to determine the exact mechanisms of anti-DENV effects mediated by these peptides.

In conclusion, in this study, we developed AYA3 and AYA9 short peptides that exhibited considerable antiviral activity against DENV NS2B-NS3 protease and inhibited virus replication in human liver cells. These peptides possess short, simple linear structure and low toxicity properties when compared to the reference peptide.

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