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Anti-dengue activity of synthetic peptides increases antiviral interferon-beta genes in LLC-MK2 cells

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ABSTRACT

Dengue virus (DV) causes dengue diseases in human via the Aedes mosquitoes. Fatal cases of dengue hemorrhagic fevers show a complication of acute kidney injury. Previous reports revealed that synthetic DV2(413-440), DV2(413-447), and DV2(419-447) peptides inhibit the dengue envelope proteins in *Aedes albopictus* C6/36 cells, but nothing is known about the effect of these peptides on mammalian cells in relation to adaptive and innate immunities. In this study, the anti-dengue activity of these synthetic peptides in Rhesus monkey kidney LLC-MK2 cells was investigated at the cellular and molecular levels. Moreover, the effects of these synthetic peptides on apoptotic caspase-10, pro-inflammatory interleukin-1beta, tumor necrosis factor-alpha, and antiviral interferon-beta genes in LLC-MK2 cells were also sought. The results revealed the maximum non-toxic doses of DV2 (413-440), DV2 (413-447), and DV2 (419-447) in LLC-MK2 cells, with values of 77.88± 0.52, 47.01±1.32, and 63.82±1.97 µM, respectively. At 25 µM concentration, synthetic DV2 (413-447) and DV2 (419-447) peptides showed 100% plaque inhibition in simultaneous treatment. By contrast, these peptides showed ≤58% plaque inhibition in pre- and post-treatment at 7 days post-incubation. These synthetic peptides also inhibited Dengue 2 virus, apoptotic caspase-10, pro-inflammatory tumor necrosis factor-alpha, and interleukin-1beta genes. On the other hand, these peptides upregulated the antiviral interferon-beta gene in innate immunity. This study is the first report to reveal the anti-dengue activity of synthetic DV2 (413-440), DV2 (413-447), and DV2 (419-447) peptides in adaptive and innate immunity.

Keywords: dengue; peptides; apoptosis; caspase-10; interleukin-1beta; tumor necrosis factor-alpha; interferon-beta

1. INTRODUCTION

Dengue virus (DV) serotypes 1, 2, 3, and 4 are the most prevalent arboviruses worldwide. *Aedes aegypti* and *Aedes albopictus* are the predominant vectors for DV transmission. DV infection (DVI) is found in over half of the global population, and 100 million symptomatic cases are reported annually (Uno and Ross, 2018). DV-infected patients present broad spectrum of the disease, ranging from subclinical (patients are unaware that they are infected) to severe conditions (hemorrhage and shock) (Pang et al., 2017). Severe dengue cases have been also associated with acute kidney injury (AKI) (Lee et al., 2009; Lizarraga and Nayer, 2014). AKI has been proposed to cause direct injury and present indirect (immune system, hemolysis, and rhabdomyolysis) and hypotensive mechanisms from shock. The case-fatality rate of 0.9%-60% was reported among patients with AKI (Diptyanusa et al., 2019).

High levels of soluble tumor necrosis factor-alpha (TNF-α) were demonstrated in the sera of patients with dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Bethell et al., 1998). TNF-308A allele is a risk factor for bleeding in patients with DHF (Fernandez-Mestre et al., 2004). A mouse model study showed that TNF-α deficiency significantly diminishes the hemorrhage development (Chen et al., 2007). DV and TNF-α induce the apoptosis of endothelial cells through the production of reactive oxygen (ROS) and nitrogen (RNS) species. This reaction activates tyrosine nitration to damage the endothelial cells and contribute to hemorrhage. Conversely, RNS and ROS inhibition significantly reduces hemorrhage development (Yen et al., 2008). A high level of interleukin-1 beta (IL-1β) was observed in monocytes of patients with DHF/DSS and confirmed in THP-1 (human monocyte-differentiated macrophages) and mouse bone marrow-derived macrophages infected with DV. IL-1β induces tissue injury and vascular leakage. This function was also tested in interferon (IFN)-α/β receptor 1 deficient C57BL/6 mice condition (Pan et al., 2019). The DV induces a high level of IL-1β through the activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome. Zika virus (ZIKV) infection induces AKI by activating the NLRP3 inflammasome and apoptosis through suppressing the Bcl-2 expression (Liu et al., 2019). DV2 increases the high pro-inflammatory cytokine levels in primary monocyte-derived DCs (moDCs), except IFN-α/β. By contrast, the addition of exogenous IFN-β in DV-infected moDCs fail to prime T cells, suggesting that DV plays a role in immune evasion by IFN-α/β inhibition (Rodriguez-Madoz et al., 2010).

Dengue RNA genome is a positive, single-stranded, and 11 kilobases in length, and it encodes a single polyprotein; three structural proteins (capsid-C, pre-membrane, and envelope E) located at the N-terminal and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are encoded at the C-terminal for virus propagation (Akey et al., 2014). Structural proteins play a role in viral entry, assembly, and budding. NS proteins are responsible for viral assembly, replication, and immune evasion during dengue pathogenesis (Iglesias et al., 2011; Smit et al., 2011). Anti-dengue drugs are mostly investigated for their viral replicating proteins, such as NS2B-NS3 (protease), NS3 (helicase), and NS5 (RNA-dependent RNA polymerase) (Schleich et al., 2011; Nitsche et al., 2014). Peptide drug development has become a novel pharmaceutical research field (Huang et al., 2018). Schmidt et al. (2010a) showed that peptide DV2 (419-447) inhibited DVI through non-specific binding to the viral membrane and a specific interaction with the E protein to undergo a conformational rearrangement at pH less than 7. Schmidt et al. (2010b) also confirmed that residues at 441-447 amino acids mainly interact with the viral membrane, whereas those at 419-440 bind to the E protein. These results revealed that the synthetic peptides at residues 441-447 can result in marked viral enhancement.

In this study, the anti-dengue activity of synthetic DV2 (413-440), DV2 (413-447), and DV2 (419-447) peptides in LLC-MK2 cells was investigated at the cellular and molecular levels. Moreover, the effects of these synthetic peptides on apoptotic caspase-10 (CASP10), pro inflammatory IL-1β, and TNF-α and antiviral IFN-β gene expression in LLC-MK2 cells were also sought.

2. MATERIALS AND METHODS

2.1 Synthetic peptides

Exactly 14 mg synthetic DV2413-440, DV2413-447, and DV2 (419-447) peptides (Table 1) were purchased in the form of white lyophilized powder from GenScript Biotech (Piscataway, NJ, USA). A total of 5 mg of each synthetic peptide was prepared in 1 mL dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) and then stored in -20°C.

2.2 Cell lines and virus

Aedes albopictus C6/36 (American Type Culture Collection, Manassas, VA, USA) and Rhesus monkey kidney LLC-MK2 cell lines (National Institute for Biological Standards and Control, Hertfordshire, England) were cultivated in minimum essential medium (MEM, GIBCO, Grand Island, NY, USA), 10% fetal bovine serum (FBS, GIBCO, Grand Island, NY, USA), 2% L-glutamine (Hyclone, South Logan, UT, USA), 1% non-essential amino acid (Hyclone, South Logan, UT, USA), and 1% NaHCO3 (Sigma-Aldrich, St. Louis, MA, USA). C6/36 and LLC-MK2 cells were grown at 28°C and 37°C with 5% CO_2, respectively (Rabablert et al., 2020).

DV2 (16681 strain), which was first isolated from a Thai child suffering from DHF in 1959, was serially passaged in suckling mice, *Toxorhynchites ambonensis* mosquitoes, and C6/36 cells (Yoksan et al., 1986). DV2 (1 ml) was added to the C6/36 cell monolayer (1×10^6 cells per flask) and gently shaken for 1 h to obtain a stock of the DV. A total of 4 mL MEM with 10% FBS was added to the cells, which were then kept at 28°C for 5-7 days. The supernatant was collected, aliquoted, and frozen at -80°C. This process was repeated several times until a sufficient amount of virus stock was collected.

Table 1. List of synthetic peptides against DV2 (New Guinea strain) in C6/36 cells

Synthetic Peptide names	Amino acid sequences	IC_{50} (μM)	References
DV2(413-440)	AIL-GDT-AWD-FGS-LGG-VFT-SIG-KAL-HQV-F	0.250	Schmidt et al. (2010a)
DV2(413-447)	AIL-GDT-AWD-FGS-LGG-VFT-SIG-KAL-HQV-FGA-IYG-AA	0.275	Schmidt et al. (2010a)
DV2(419-447)	AWD-FGS-LGG-VFT-SIG-KAL-HQV-FGA-IYG-AA	0.125	Schmidt et al. (2010a, 2010b)

2.3 *In vitro* cytotoxicity study

LLC-MK2 cells were initiated by seeding 5×10^4 cells per well into 96-well plates. The plates were then incubated in a CO_2 incubator (Euroclone, Italy) for 24 h to complete the monolayer. The synthetic peptides were serially diluted by two-fold with MEM/2% FBS. Exactly 100 mL of each dilution was added to the cells in triplicate and kept at 37°C at 1, 2, and 3 day(s) post-incubation. The negative control cells were treated with MEM/2% FBS and MEM/2% FBS/5% DMSO. Then, 10 mL 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazoliumbromide (MTT, Sigma-Aldrich, St. Louis, MA, USA) solution was added to each well, and the plates were kept at 37°C for 2 h. A total of 100 mL DMSO was added to each well for 30 min, and the OD_{565} was measured with a microplate reader (Tecan, Austria). The 50% cytotoxic concentration (CC_{50}) and maximum non-toxic dose (MNTD) for each synthetic peptide were determined using the Predictive Analytic Software Statistics 18 (SPSS: IBM, Stanford, CA, USA).

2.4 Plaque assay

LLC-MK2 cells were initiated by seeding 5×10^5 cells per well into 12-well plates. Then, the cells were incubated in a CO_2 incubator set at 5% for 24 h to complete the monolayer. Next, 200 mL of 10-fold dilutions of DV2 with MEM/2% FBS were added to each well in duplicate, and the plates were kept at 37°C with 5% CO_2 for 90 min. The negative control cells were treated with MEM/2% FBS and MEM/2% FBS/5%DMSO. The medium containing MEM, 2% FBS, 2% carboxymethyl cellulose (CMC), and 0.02% neutral red was overlaid onto each well, and the plates were incubated at 37°C with 5% CO_2 for 7 days. The plaque size and numbers were recorded and calculated as plaque-forming units per milliliter (pfu/mL) (Rabablert et al., 2020).

2.5 Time-of-addition experiment

To determine the effect of synthetic peptides on prophylaxis, 100 mL of each synthetic peptide of different concentrations were added to each well of the LLC-MK2 cells, in triplicate, and kept the plates at 37°C for 1 h before DV2 infection. MEM/2% FBS/2.5% DMSO and MEM/2% FBS were used as the negative and positive controls, respectively. The cells were washed twice with phosphate buffered saline (PBS; pH 7.4). A total of 100 pfu DV2 was inoculated into each well and kept at 37°C for 1 h.

A total of 100 mL DV2 (100 pfu) was added to the LLC-MK2 cells, except for the negative control, in triplicate. Then, the plates were kept at 37°C for 1 h after infection to determine the effect of synthetic peptides on viral replication. The cells were washed with PBS, followed by the addition of different concentrations of each synthetic peptide to the LLC-MK2 cells, incubated, and washed further twice.

To investigate the effects of the synthetic peptides on direct virucidal activity, 200 pfu DV2 suspension with an equal volume of each peptide at different concentrations were incubated for 1 h at 37°C at the same time during infection. The LLC-MK2 cells were subsequently infected with the treated viral suspension at 37°C for 1 h in triplicate. The cells were washed twice with PBS to remove the unabsorbed viruses.

After the indicated times, the medium containing MEM, 2% FBS, 2% CMC, and 0.02% neutral red was overlaid onto each well, and the plates were kept at 37°C under 5% CO_2 for 7 days. The plaque size and number were recorded as pfu/mL, and the percentage of plaque inhibition was calculated.

2.6 RNA and reverse transcription-polymerase chain reaction (RT-PCR)

Given that synthetic peptides inhibit DV2 directly in simultaneous treatment, the effect of synthetic peptides on DV2, CASP10, IL-1β, TNF-α, and IFN-β gene expression in LLC-MK2 cells were also investigated. The 200 pfu DV2 and different concentrations of each peptide at a ratio of 1:1 were kept at 37°C for 1 h. For the negative and positive controls, 200 pfu DV2 was incubated with 2.5% DMSO and MEM/2%FBS, respectively. The LLC-MK2 cells were infected with the treated viral suspension and absorbed at 37°C for 1 h in triplicate. The cells were washed with PBS to eliminate the unabsorbed viruses. MEM/2% FBS was added to the cells, which were then kept at 37°C for 12, 24, and 48 h. The dengue viral RNA was determined by RT-PCR.

The total RNA was prepared from uninfected and D2V-infected LLC-MK2 cells treated with synthetic peptides using an E.Z.N.A.® Total RNA Kit (Omega BIO-TEK, Norcross, GA, USA). The extracted dengue viral RNAs were reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) at 42°C for 1 h. Table 2 lists the sequences and product size for each pair of PCR primers. The primers were purchased from Bioline Meridian Bioscience (United Kingdom). Subsequently, the cDNA was amplified using the Dream Taq PCR Master Mix (Thermo Fisher Scientific, USA). The reactions with cDNA began with an initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 10 min. The gene expressions of DV2, CASP10, IL-1β, TNF-α, and IFN-β were facilitated by the amplification of PCR product size on 2% agarose gels (Yoksan et al., 2018).

3. RESULTS AND DISCUSSION

3.1 Determination of CC_{50} and MNTD

In this study, the CC_{50} and MNTD of each synthetic peptide was determined by testing in LLC-MK2 cells, in triplicate. The studies were initiated using a 2-fold dilution of synthetic peptides, followed by further optimization to achieve the specific cytotoxic concentration. The CC_{50} and MNTD of each synthetic peptide obtained through the optimization steps are presented in Table 3.

Cytotoxicity studies revealed that the CC_{50} and MNTD for DV2 (413-447) were 68.59±1.39 and 47.01±1.32 μM, respectively, and these values were the highest among all three synthetic peptides. By contrast, the CC50 and MNTD of DV2 (413-440) were the lowest, with a minute concentration of 102.74±0.78 and 77.88±0.52 μM, whereas DV2 (419-447) recorded the second lowest CC_{50} (77.98±1.35 μM) and MNTD (63.82±1.97 μM).

Table 2. List of primer names used in this study for RT-PCR

Primer names	Primer sequences	Base pairs
DV2- Forward (F)	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	119
DV2-Reverse (R)	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	
TS2-R	5'-CGCCACAAGGGCCATGAACAG-3'	
CASP10-F	5'-ATTAGCGGCAACTCAGCACT-3'	200
CASP10-R	5'-TGTGCAGGTCACAAGGGATA-3'	
TNF-α-F	5'-CAGAGGGAAGAGTTCCCCAGGGACC-3'	325
TNF-α-R	5'-CCTTGGTCTGGTAGGAGACGG-3'	
IL-1β-F	5'-TCCTGTGCTGAATGTGGACTCAATCC-3'	291
IL-1β-R	5'-GACAGAGGAGGGTTTCTTAGAACC-3'	
IFN-β-F	5'-TAGCACTGGCTGGAATGAGA-3'	186
IFN-β-R	5'-TCCTTGGCCTTCAGGTAATG-3'	

Table 3. Cytotoxicity assays performed using MTT assay

Synthetic peptide names	CC_{50} (μM)	MNTD (μM)
DV2(413-440)	102.74 ± 0.78	77.88 ± 0.52
DV2(413-447)	68.59 ± 1.39	47.01 ± 1.32
DV2(419-447)	77.98 ± 1.35	63.82 ±1.97

3.2 Anti-dengue synthetic peptides on DV2 by plaque assay

Using various concentrations of each synthetic peptide, three different experimental strategies (pre-, simultaneous, and post-treatment) were evaluated regarding their effects on processes before, during, and after the viral entry. In the pre- and post-treatment strategies, at 25 μM DV2 (413-440), DV2 (419-447), and DV2 (413-447) synthetic peptides significantly decreased the infection by ≤50% (Figures 1a and 1c).

Conversely, in the simultaneous treatment strategy, the DV2 (419-447) at concentrations of 12.5 and 25 μM showed 100% plaque inhibition. DV2 (413-447) and DV2 (413-440) showed 100% and 45% plaque inhibition, respectively, at 25 μM concentration (Figure 1b). These results suggest that DV2 (413-447) and DV2 (419-447) can directly neutralize the DV2 infection. Nevertheless, nine of the synthetic peptides can interact with cellular receptors and/or cofactors to interfere with the virus–receptor binding during pre-treatment. Furthermore, nine of the synthetic peptides inhibited DV2 infection at post-entry (viral replication, assembly, or budding).

3.3 Effect of synthetic peptides on DV2-RNA, apoptotic CASP10, pro-inflammatory TNF-α and IL-1β, and antiviral IFN-β genes in DV2-infected LLC-MK2 cells

As shown in Figures 2a and 2b, DV2-RNA and CASP10 amplicons from DV2-infected LLC-MK2 cells had a size of 119 and 200 bp, respectively, at 12, 24, and 48 h. Similarly, the amplicons from DV2-infected LLC-MK2 cells treated with 2.5% DMSO showed similar patterns of DV2-RNA and CASP10 amplicons at the indicated times. Conversely, the DV2-RNA and CASP10 amplicons from DV2-infected LLC-MK2 cells treated with DV2413-440, DV2413-447, or DV2 (419-447) disappeared at the indicated times. These findings suggest that these peptides inhibited DV2-RNA and apoptotic CASP10 genes at the indicated times.

Figures 2c and 2d reveal that the pro-inflammatory TNF-α (325 bp) and IL-1β amplicons (291 bp) of DV2-infected cells treated with MEM/2% FBS or MEM/2% FBS/2.5% DMSO were presented at 12, 24, and 48 h. The TNF-α and IL-1β amplicons of infected cells treated with DV2 (413-440), DV2 (413-447), or DV2 (419-447) disappeared at the indicated times. Thus, at 12, 24, and 48 h, these synthetic peptides inhibited the pro-inflammatory TNF-α and IL-1β genes.

Figure 2e shows that the antiviral IFN-β amplicon (186 bp) of DV2-infected cells treated with MEM/2% FBS or MEM/2% FBS/2.5% DMSO appeared at 12 h and disappeared at 48 h. On the other hand, the IFN-β amplicon from DV2-infected cells treated with DV2 (413-440), DV2 (413-447), or DV2 (419-447) continually increased at 12, 24, and 48 h. The results reveal that these synthetic peptides stimulated the antiviral IFN-β genes at the given times.

4. DISCUSSION

Dengue disease is caused by DV1-DV4, genus *Flavivirus*, Family Flaviviridae. Recovery from the infection is believed to provide lifelong immunity against the serotypes. However, cross-immunity to other serotypes after recovery is partial and temporary. Subsequent infections (secondary infection) by other serotypes increase the risk of developing severe dengue. Numerous antiviral candidates have failed to reach clinical trials due to their poor pharmacokinetic properties (Chew et al., 2017). This study identified the antiviral activity of synthetic DV2 (413-440), DV2 (413-447), and DV2 (419-447) peptides in LLC-MK2 cells at the cellular and molecular levels. Furthermore, the effects of these synthetic peptides on apoptotic CASP10, pro-inflammatory TNF-α, and IL-1β and antiviral IFN-β genes in LLC-MK2 cells were also studied.

From the simultaneous treatment, DV2 (413-447) and DV2 (419-447) peptides are promising candidates for their anti-dengue viral activity against DV2, reaching the highest peptide concentrations of 25 and 12.5 μM, respectively (Figure 1b). The results suggest that peptide residues have anti-dengue activity in viral envelope destabilization, which results in decreased infection. These data were correlated with the previous reports of Schmidt et al. (2010a) and Schmidt et al. (2010b). Our data showed seven amino acids at the 419-447 positions, and they are involved in viral envelope destabilization. RT-PCR showed

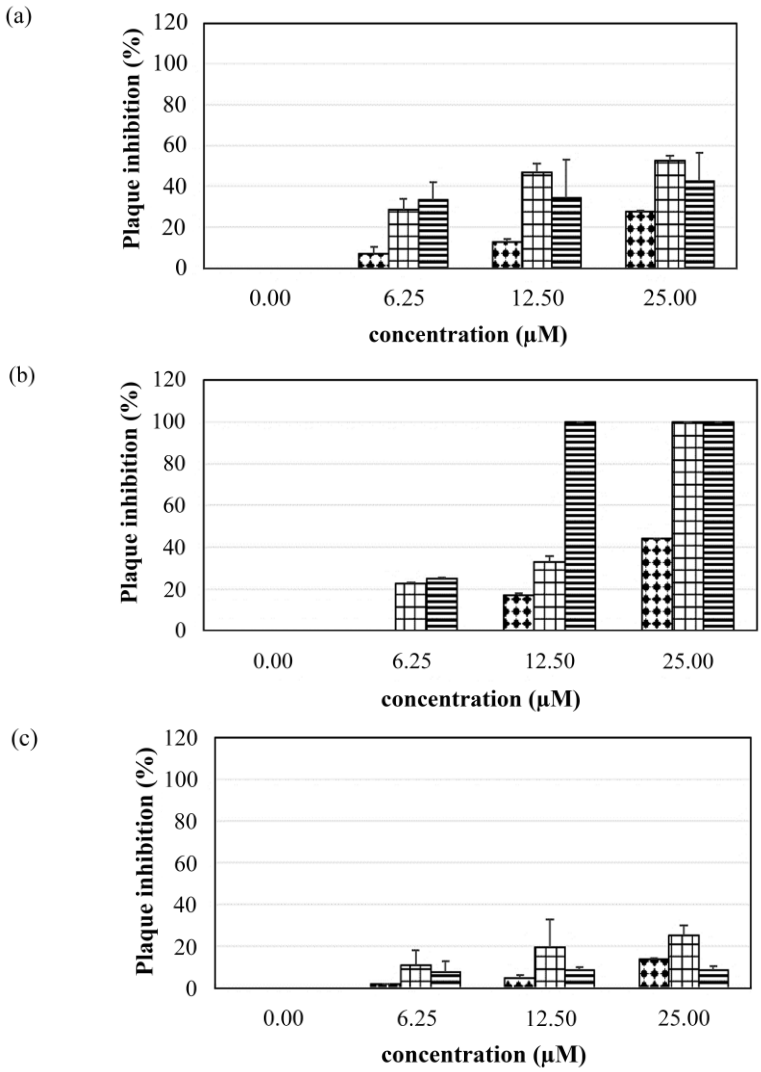

Figure 1. Plaque inhibition (%) of DV2(413-440), DV2(413-447), and DV2(419-447) peptides against DV2 in LLC-MK2 cells in (a) pre-treatment, (b) simultaneous treatment and (c) post-treatment, in triplicate

a reduction in the number of dengue viral genome copies (119 bp); DV2 was preincubated with DV2 (413-440), DV2 (413-447), or DV2 (419-447) peptides (Figure 2a). Although the results of DV2 (413-447) and DV2 (419-447) corroborated the plaque assay, the viral genome does not indicate an infectious virus (Monterio et al., 2018). Our data differed from the report of Schmidt et al. (2010a), which proposed that the IC_{50} of synthetic DV2 (413-440), DV2 (413-447), and DV2(419-447) peptides at 0.25 µM especially inhibited DV2 (New Guinea C strain) in C6/36 cells. Two reasons were given to explain this phenomenon: (i) C6/36 is more susceptible to DVs than LLC-MK2 cells (Sydow et al., 2000); (ii) DV2 (16681 strain) was isolated from a patient with DHF, whereas DV2 was isolated from a patient with DF (Martínez-Betancur and Martínez-Gutierrez, 2016). Additionally, studies of rhesus monkeys (*Macaca mulatta*) DV2 (16681 strain) showed various degrees of dengue severity, including hemorrhage, decline in platelet count, and leucopenia (Onlamoon et al., 2010; Clark et al., 2013). DV2 presented the highest levels of T cell response to anti-NS proteins, which indicates viral replication and immune evasion (Mladinich et al., 2012).

Man and Kanneganti (2016) proposed that CASP10 gene is recruited into apoptosis pathways, especially those involving TRAIL receptors 1 and 2. TRAIL receptors 1 and 2 promote the FAS-associated death domain protein-dependent apoptosis through the nuclear factor-kB pathway. CASP10 can initiate Fas- and TNF-dependent apoptosis via ligand-receptor (Wang et al., 2001). The DV may activate apoptosis and disseminate its viral progenies to neighbor cells through apoptotic bodies; alternatively, it may inhibit cell death for viral survival and replication (Torrentes-Carvalho et al., 2009; Thepparit et al., 2013). The present study demonstrated that the CASP10 gene was expressed in DV2-infected LLC-MK2 (Figure 2 b), in accordance with the report of Conceição et al. (2010), which showed the increased transcription in DV2-infected human hepatocyte (HepG2) cells. This study is the first report to explain the induction of apoptosis by DV2 via the CASP10 gene in kidney cells. The downregulation of CASP10 gene from DV2-infected LLC-MK2 treated with DV2 (413-440), DV2 (413-447), and DV2 (419-447) peptides was observed. The result shows the specific interaction of the virus. Given that the virus can be blocked

by these synthetic peptides, these peptides therefore function as caspase inhibitors. These data are consistent with the report of Mohr et al. (2018), which showed that the downregulation of CASP10 in human colorectal tumor HCT116 cells increased the efficacy of treatment in cancer cells. Therefore, these synthetic peptides are likely to have potential against apoptosis in DV2-infected LLC-MK2 cells.

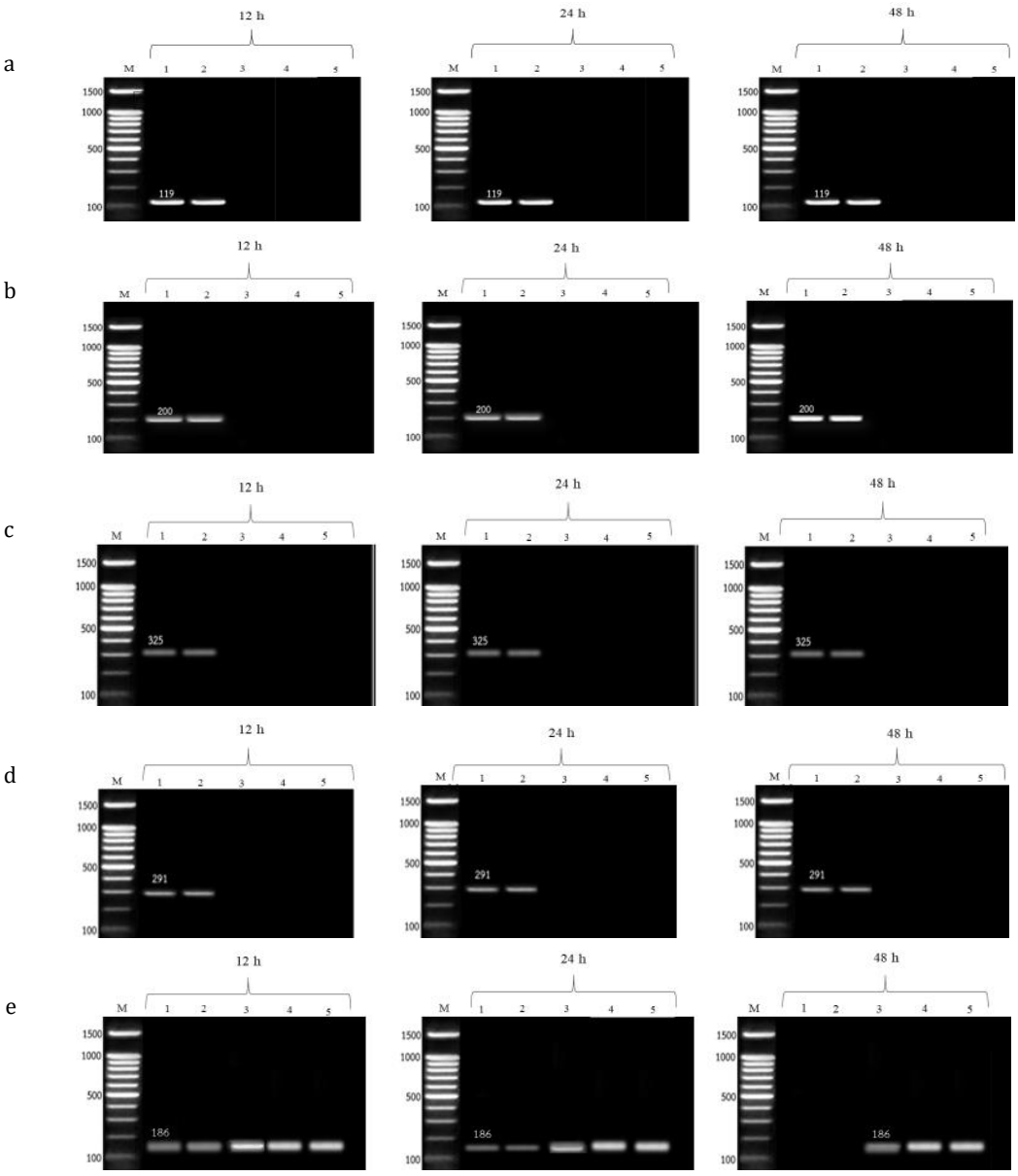

Figure 2. Agarose gel electrophoresis of generic RT-PCR amplicons of (a) DV2-119 bp, (b) CASP10-200 bp, (c) TNF-α-325 bp, (d) IL-1β-291 bp and (e) IFN-β-186 bp genes from D2V-infected LLC-MK2 treated with Lane-1, MEM+2%FBS; Lane-2, 2.5% DMSO; Lane-3, DV2(413-440); Lane-4, DV2 (413-447); and Lane-5, DV2(419-447)

Kittigul et al. (2000) proposed that the increase in TNF-α levels is associated with the increase in vascular permeability in dengue-infected patients. Increased C-X-C motif chemokine ligand 10 and TNF-α levels were also correlated with an increased clinical severity of dengue disease due to inflammation and vascular changes in the observed cases (Masood et al., 2018). TNF-α induces reactive oxygen and nitrogen intermediates, whereas apoptotic cell death increases the vascular permeability and hemorrhage development (Yen et al., 2008; Jeewandara et al., 2015). High levels of IFN-γ and TNF-α are correlated with the inflammation of the liver, lung, and kidney samples from dengue fatal cases, suggesting that TNF-α is involved in dengue immunopathogenesis (Póvoa et al., 2016). The role of pathogenic TNF-α and its activity have been shown in the models of immune complex-mediated glomerulonephritis, antineutrophil cytoplasmic antibody-associated glomerulonephritis, diabetic nephropathy, obstructive uropathy, allograft rejection, and AKI (Mehaffey and Majid, 2017; Akash et al., 2018). In this study, DV2 increased the TNF-α gene in LLC MK2 cells (Figure 2c), suggesting that this virus is involved in the inflammation of kidney cells. These data are similar to those of Conceição et al. (2010), who showed DV2 involvement in the inflammation of HepG2 cells. It is described that DV2 (413-440), DV2

(413-447), and DV2 (419-447) synthetic peptides can suppress the pro-inflammatory TNF-α gene. Possibly, these synthetic peptides can be used in the treatment of other pro-inflammatory diseases.

Dinarello (1999) proposed that an IL-1β cytokine, specifically fever-inducing pyrogen, is a regulator of systemic inflammation. The DV induced high levels of IL-1β cytokine in the sera of patients with DHF/DSS and infected C57BL/6 mice (Tan and Chu, 2013; Pan et al., 2019). The infections by DV and ZIKV activate the NLRP3 inflammasome and produced IL-1β in the kidney, thus leading to AKI (Wang et al., 2018; Liu et al., 2019). DV2 NS2A and NS2B proteins increase the NLRP3 inflammasome activation, apoptosis-associated speck-like protein, and IL-1β secretion through caspase-1 activation (Shrivastava et al., 2020). These results showed the induction of IL-1β (291 bp) gene expression in DV2-infected LLC-MK2 cells (Figure 2d), similar to the other reports, which showed the upregulated transcription in HepG2 cells (Conceição et al., 2010; Yoksan et al., 2013). DV2 (413-440), DV2 (413-447), and DV2 (419-447) decreased the IL-1β gene in DV2-infected LLC-MK2 cells, suggesting that these synthetic peptides have antiviral activity against viral replication in LLC-MK2 cells.

Seo and Hahm (2010) proposed that IFN-α or IFN-β is an important antiviral response to various viral infections. Mice lacking IFN-α and IFN-β receptors become more susceptible to mortality after intraperitoneal inoculation with the DV (Shresta et al., 2005; Shresta et al., 2006). Conversely, a strong IFN-α-mediated inhibitory effect on DV replication was observed when IFN-β-treated cell types were exposed to the DV (Shresta et al., 2004). Type-I IFNs inhibited the DVI of HepG2 (Diamond et al., 2000) and human lung adenocarcinoma cells (A549) (Uno and Ross, 2018). A reduction of the IFN-α/β producing plasmacytoid DCs has been observed in patients with DHF (Pichyangkul et al., 2003; Rodriguez-Madoz et al., 2010). The DV inhibits IFN-α/β signaling in numerous cells (Munoz-Jordan, 2010). IFNs bind the IFNα/β receptor on the surface of DV-infected cells, induce the Janus kinase/signal transducer, and activate the JAK/STAT signaling pathway, thus leading to virucidal activity (Morrison and García-Sastre, 2014; Uno and Ross, 2018). The present study demonstrated that the DV decreased IFN-β (186 bp) at 48 h (Figure 2e), suggesting that the inhibition of type-I IFN response contributes to the viral infection. By contrast, synthetic DV2 (413-440), DV2 (413-447), and DV2 (419-447) peptides inhibited dengue viral replication by upregulating IFN-β in LLC-MK2 cells at 12, 24, and 48 h, suggesting that these synthetic peptides stimulate type-I IFN response to promote antiviral activity.

5. CONCLUSION

DV2 (419-447) and DV2 (413-447) had complete plaque reduction, whereas DV2 (413-440) exhibited a 50% plaque reduction against dengue 2 viral particles at 7 days post-incubation (in simultaneous treatment). However, these peptides also inhibited DV2 RNA during early infection (12-48 h). These peptides downregulated apoptotic CASP10, pro-inflammatory TNF-α, and IL-1β genes during immunopathogenesis. On the other hand, these peptides upregulated the antiviral IFN-β gene during innate immunity. This study is the first report revealing that DV2 (419-447) and DV2 (413-447) synthetic peptides have anti-dengue activity in adaptive and innate immunities.

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