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Research Article

Protegrin-1 Inhibits Dengue NS2B-NS3 Serine Protease and Viral Replication in MK2 Cells

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Dengue diseases have an economic as well as social burden worldwide. In this study, the antiviral activity of protegrin-1 (PG-1, RGGRLCYCRRRFCVCVGR) peptide towards dengue NS2B-NS3pro and viral replication in Rhesus monkey kidney (MK2) cells was investigated. The peptide PG-1 was synthesized by solid-phase peptide synthesis, and disulphide bonds formation followed by peptide purification was confirmed by LC-MS and RPHPLC. Dengue NS2B-NS3pro was produced as a single-chain recombinant protein in *E. coli*. The NS2B-NS3pro assay was carried out by measuring the florescence emission of catalyzed substrate. Real-time PCR was used to evaluate the inhibition potential of PG-1 towards dengue serotype-2 (DENV-2) replication in MK2 cells. The results showed that PG-1 inhibited dengue NS2B-NS3pro at IC_{50} of 11.7 μM. The graded concentrations of PG-1 at nontoxic range were able to reduce viral replication significantly ($P < 0.001$) at 24, 48, and 72 hrs after viral infection. However, the percentage of inhibition was significantly ($P < 0.01$) higher at 24 hrs compared to 48 and 72 hrs. These data show promising therapeutic potential of PG-1 against dengue infection, hence it warrants further analysis and improvement of the peptide features as a prospective starting point for consideration in designing attractive dengue virus inhibitors.

1. Introduction

Dengue infection causes various clinical symptoms ranging from mild fever to severe hemorrhagic fever and dengue shock syndrome [1–3]. The virus uses host cell ribosomes to translate its genomic RNA to full-length precursor polyprotein. Subsequently, the host cell furin and dengue NS2B-NS3 serine protease (NS2B-NS3pro) cleave viral polyprotein at various regions to produce structural and nonstructural viral proteins [4–6]. The NS3 protein is one of the viral nonstructural proteins that possess enzymatic activities. The N-terminal of this protein contains 180 amino acid residues that represent NS3 protease [7, 8], while C-terminal region contains amino acid residues that represent RNA helicase and RNA-stimulated NTPase [9, 10]. The activity of NS2B-NS3pro depends on the interaction with its cofactor NS2B to form a NS2B-NS3pro complex [11]. It has been found that the disruption of NS2B-NS3pro functions inhibits viral replication [12]. Therefore, NS2B-NS3pro is considered as a potential target for the design of antiviral drugs [13].

At present, a legitimate vaccine or treatment to prevent or to cure this disease is unavailable [14]. These facts emphasize the need for a better understanding of the mechanism of viral infection and propagation in the host cell to combat this disease. Recently, computational studies indicated that cationic cyclic peptides have potential inhibition towards dengue NS2B-NS3pro [15, 16]. Protegrin-1 (PG-1, RGGRLCYCRRRFCVCVGR) is an eighteen amino acids cationic cyclic peptide with high content of basic residues and two disulphide bonds. The peptide is originally isolated from porcine white blood cells and considered as an antibiotic agent against a broad range of microorganisms [17, 18]. The formation of two disulphide bonds between cysteine residues endows PG-1 with a stabile β-hairpin secondary structure

that is crucial for antimicrobial activity [19, 20]. Therefore, removal of these two disulphide bonds is found to noticeably reduce the antimicrobial activity of PG-1 [21]. The peptide is able to penetrate and disrupt the cell membrane by homodimerization [22, 23]. The mechanism of this activity depends on the secondary structure and the cationic nature of PG-1 molecules which are essential to generate pores in the cell membrane of microorganism [24–27]. In this study, our objective was to examine the efficacy of PG-1 as cationic cyclic peptide to inhibit dengue serine protease and subsequently reduce viral replication in host cells.

2. Methods

2.1. Synthesis of Cyclic PG-1. The linear peptides were prepared by automated peptide synthesis using Symphony parallel synthesizer (Protein Technologies, Tucson, AZ, USA) by standard solid-phase peptide synthesis. The crude peptides were lyophilized before proceeding to folding experiments. The lyophilized peptide was dissolved in 20% DMSO solution in a round bottom flask and stirred on a magnetic stirrer to produce a peptide concentration of 1.1 mM. The progress of the reaction was monitored by HPLC and LCMS. The formation of the first disulphide bond was completed within 24 hrs. The solution was then lyophilized to proceed for second disulfide formation. The lyophilized peptide with the first disulphide bond was dissolved in acetic acid water (4 : 1) so that the peptide concentration was 1.2 mM and iodine (10 equivalents) was added in one portion. The reactions were stirred at 25°C for 60 min and then quenched by diluting with water and extracting with CCl_4 to remove excess iodine. The aqueous phase was then lyophilized to give the crude peptide. The identity of the crude peptide was confirmed by LC-MS. Purification of crude cyclised peptide was accomplished by RP-HPLC (Agilent 1200 series). The identity of the purified peptide with 98% purity was confirmed by LC-MS (Shimadzu LC/MS 2020, single quad).

2.2. Production of NS2B-NS3pro in E. coli. To produce single chain protease *NSB2-* (G_4-*T*-G_4)-*NS3*, the *NS2B* fragments were amplified individually by PCR using the primer pairs NS2B-F (5′-ATACTGAGGATCC GCCGATTTGGAACTG-3′) and NS2Blinker-R (ACCTACTAGGTACCTCCT-CCACCCAGTGTCTGTTCTTC). The *NS2B-NS3* was amplified by NS3linker-F (5′-ATCTATAGGTACCGGC-GGTGGAGGTGCTGGAGTATTGTGG-3′) and NS3-R (5′-AGCATAAGCTTAAGCTTCAATTTTCT-3′). The linker sequence was added to NS2Blinker-R and NS3linker-F primers which included the site for *Kpn*I restriction enzyme (all restriction sites are underlined). The PCR product of *NS2B* fragment was digested with *BamH*I and *Kpn*I while *NS2B* fragment was digested with *Kpn*I and *Hind*III. Purified fragments were cloned into pQE30 plasmid downstream of 6×His tag. The *Escherichia coli* X-blue strain (Promega, USA) was transformed with pQE30-*cNSB* -(G_4TG_4)-*NS3* plasmid. 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 brief, 10 ml of overnight grown culture was added to 1000 mL of medium and incubated with shaking at 37°C until the optical density at 600 nm reached 0.5. Subsequently, isopropylthio-β-D-galactoside (IPTG) was then added to a final concentration of 0.5 mM and the bacteria were cultured for an additional 5 hrs at 37°C in a shaking incubator to induce protein expression. Finally, bacterial cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C.

2.3. Protein Purification. The recombinant NS3pro was produced as soluble proteins and, therefore, the purification had been performed by His GraviTrap Flow precharged Ni Sepharose 6 Fast column (Amersham Biosciences, USA) according to the manufacturer's instructions. In brief, the column was normalized with phosphate buffer (20 mM sodium phosphate buffer and 500 mM NaCl, pH 7.4). The sample was loaded into the column and the column was washed with binding buffer (phosphate buffer containing 20 mM imidazole, pH 7.4). The recombinant protein was eluted with elution buffer (phosphate buffer containing 200 mM imidazole, pH 7.4). Further purification was applied using gel affinity chromatography to achieve more than 95% purity.

2.4. Protease Assay. The bioassay used in this study was modified from the method published by Kiat and coworkers [28]. Reaction mixtures with total volume of 200 μL were prepared. These reaction mixtures consisted of 100 μM fluorogenic peptide substrate (Boc-Gly-Arg-Arg-MCA), 2 μM NS2B-NS3pro complex, with or without PG-1 of varying concentrations, buffered at pH 8.5 with 200 mM Tris-HCl. The PG-1 was initially prepared in Tris-HCl buffer and assayed at five different concentrations. The reaction mixtures without fluorogenic peptide substrate were firstly incubated at 37°C for 30 minutes. Subsequently, the substrate was added and the mixture was further incubated at the same temperature for 30 minutes. Triplicates were performed for all measurements and the readings were taken using Tecan Infinite M200 Pro fluorescence spectrophotometer. Substrate cleavage was optimized at the emission at 440 nm upon excitation at 350 nm. The readings were then used for calculating K_m values of peptide substrate and IC_{50} values of peptide inhibitors using nonlinear regression models in GraphPad Prism 5.01 software.

2.5. Maximum Nontoxic Dose Test (MNTD). The MK2 cell lines were seeded at 1×10^4 cells per well in triplicate at optimal conditions (37°C, 5% CO_2 in humidified incubator) in 96 well plates. PG-1 was diluted to serial concentrations 2.5, 12.5, 25, 50, 100 and 200 μM with DMEM media supplemented with 2% FBS. The cell culture was analyzed at 24, 48 and 72 hours using nonradioactive cell proliferation assay (Promega, USA) according to the manufacture protocol.

2.6. Treatment of DENV-2-Infected Cells with PG-1 Peptide. The MK2 cell lines were grown in a 24-well tissue culture

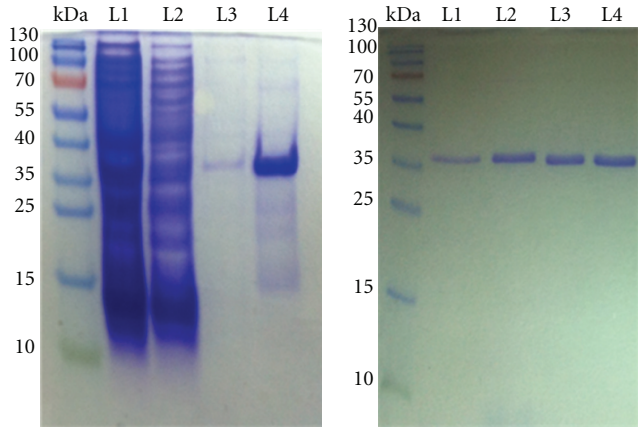

Figure 1: Production of single chain protease *NSB2-* $(G_4\text{-}T\text{-}G_4)$*-NS3pro* in *E. coli*. (a) The recombinant NS2B-NS3pro was produced as soluble proteins and purified by nickel column. L1: cell lysate, L2: washing flow through, L3: elution 1, and L4: elution 2. (b) Further purification was applied using gel affinity chromatography (95% of enzyme purity) L1 to L4: Elution 1 to 4. The activity of purified enzyme was assessed by catalyzing the fluorogenic peptide substrate (Boc-Gly-Arg-Arg-MCA).

plate (1×10^5 cells/well), incubated 24 hrs under optimal conditions (37°C and 5% CO_2). DENV-2 was added to the wells (MOI of 2) followed by incubation for 1 hr with gentle shaking every 10 min for optimal virus to cell contact. The virus supernatant was removed, and the cells were washed twice with fresh serum free DMEM media to remove residual virus. New complete DMEM media containing 2.5, 7.5 and 12.5 μM of PG-1 were added and the cultures were incubated for 24, 48 and 72 hrs. Afterwards, cellular supernatants were collected and stored at −80°C for viral quantification by real-time PCR.

2.7. Real-Time PCR. For quantification of DENV-2 copies, the standard curve was generated by 10-fold serial dilution of known copies of DENV-2 RNA. Viral RNA was extracted from culture supernatant using QIAmp viral RNA minikit (QIAGEN, Germany) according to the manufacturer's instructions. A fragment located at the 5′UTR region of the virus genome was used to generate the primers. One-step RT-PCR using SyBr Green Master Kit (Qiagen, Germany) was used to conduct absolute quantification using ABI7300 machine from Applied Biosystems (Foster City, CA). The PCR programme included 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Dissociation curve analysis was added to the end of each run. Results were analyzed using Sequence Detection Software Version 1.3 (Applied Biosystems, Foster City, CA, USA).

2.8. Statistical Analysis. All the assays were done in triplicates and the statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). P values <0.05 were considered significant. Error bars are expressed as ± SD.

Table 1: The parameters of PG-1 Inhibition potential towards NS2B-NS3pro.

Parameter*	Values	Std. Error
V_{max}	0.28 μmol/min	±0.01
Alpha	3.14	±0.81
K_i	5.85 μM	±1.82
IC_{50}	11.70 μM	±2.23
K_m	109.10 μM	±7.50

*V_{max}: maximum enzyme velocity (μmol/min). K_m: Michaelis-Menten constant (μM). K_i: inhibition constant (μM). Alpha: constant that determines mechanism. If alpha = 1, this is the same as noncompetitive. If alpha is very high, then the model approaches a competitive model. If alpha is very low (but greater than zero), the model approaches an uncompetitive model. All parameters were calculated by GraphPad Prism 5.0 software.

3. Results and Discussion

The recombinant NS2B-NS3pro was produced as a soluble protein in *E. coli* and purified by nickel column (Figure 1(a)). Further purification was applied using gel affinity chromatography to achieve more than 95% of enzyme purity (Figure 1(b)). The activity of purified enzyme had been assessed at 37°C by catalyzing the fluorogenic peptide substrate t-Butyloxycarbonylglycyl-L-arginyl-L-arginyl-L-4-methylcoumaryl-7-amide (Boc-Gly-Arg-Arg-MCA). The PG-1 peptide was added to the protease reaction at different concentrations and the inhibition profile was plotted as shown in Figure 2.

It was observed that the inhibition potential increased with PG-1 concentration and the highest inhibition (95.7%) with low concentrations of PG-1 was at 40 μM. The kinetic assay study indicated that PG-1 competitively inhibited NS2B-NS3pro activity (alpha value 3.14) with K_i value 5.85 μM (Table 1). Intriguingly, the maximum enzyme velocity decreased threefold when PG-1 concentration was 20 μM

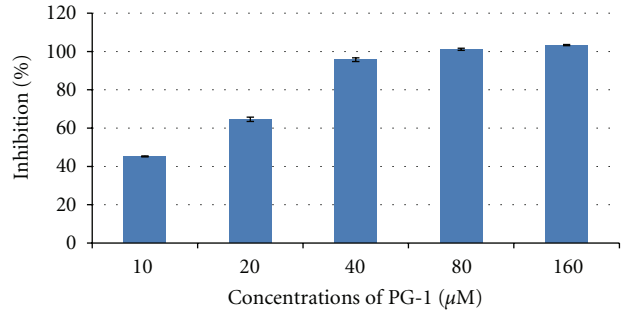

Figure 2: Dengue protease inhibition assay. The reaction mixtures consisted of fluorogenic peptide substrate and NS2B-NS3pro, with or without PG-1 of varying concentrations. The PG-1 was initially prepared in Tris-HCl buffer and assayed at five different concentrations, 10–160 μM. The best inhibition with low concentration of PG1 was observed at 40 μM with 95.7% inhibition.

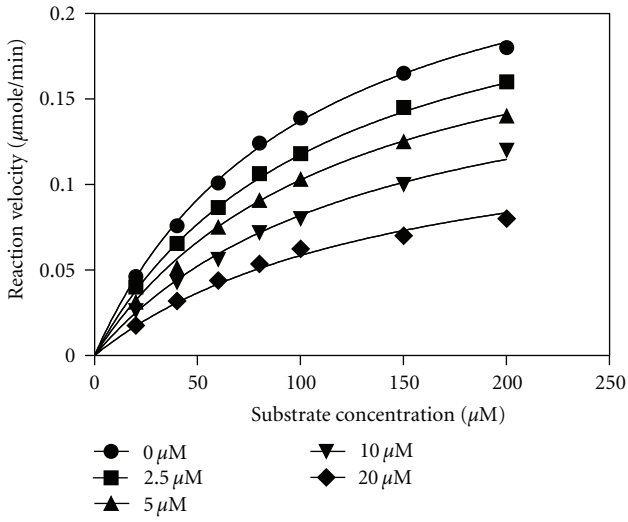

Figure 3: Kinetic assay plot for the inhibition of the NS2B-NS3pro from dengue virus by PG-1 peptide. This assay was carried out using increasing concentration of inhibitor while all other conditions were kept constant. The data was analysed by Michaelis-Menten model under nonlinear regression curve fit in GraphPad Prism 5 software. The peptide concentrations were 0, 2.5, 5, 10, and 20 μM. The maximum enzyme velocity decreased from approximately threefold when PG-1 concentration was 20 μM.

(Figure 3). Previous studies have shown that various types of natural and chemical compounds were able to inhibit dengue NS2B-NS3pro activity. For example, some of plant natural compounds, such as Chalcones, have shown good inhibition potential against the dengue protease (K_i value 21–25 μM) [28]. The synthesized peptidic α-keto-amide compound inhibited dengue NS2B-NS3pro with K_i value of 47 μM [29]. Most recent study indicated that the retrotripeptides with an arylcyanoacrylamide group as N-terminal cap exhibited high inhibition potential against dengue protease with K_i value of 4.6 μM [30]. Most recent study showed that the inhibition potential of some chemical compounds towards NS2B-NS3pro measured by IC_{50} was 15.4, 20.4, and 27.0 μM [31].

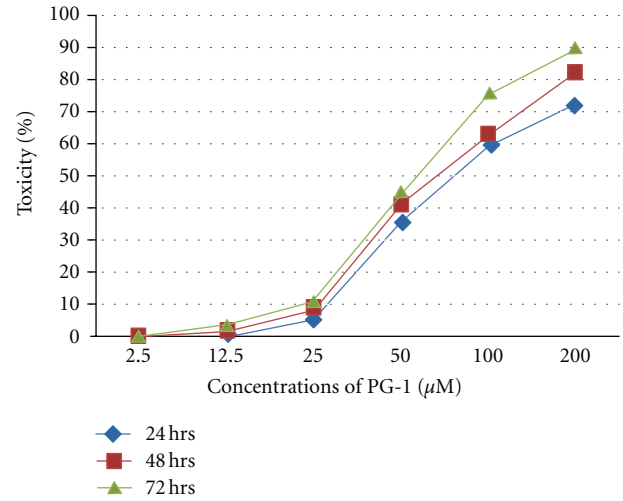

Figure 4: Maximum nontoxic dose (MNTD) of PG-1.The MK2 cell lines were seeded at 1×10^4 cells per well in triplicate at optimal conditions (37°C, 5% CO_2 in humidified incubator) in 96 well plates. PG-1 was diluted to serial concentrations 2.5, 12.5, 25, 50, 100, and 200 μM with DMEM media supplemented with 2% FBS. The cell culture was analyzed at 24, 48, and 72 hrs using Non-Radioactive Cell Proliferation assay (Promega, USA) according to the manufacture protocol. The toxic dose of MK2 cells was identified to be greater than 12.5 μM.

Cytotoxicity and compound stability can be considered as major limitations of the practical application of protease inhibitors. In this study, to test PG-1 toxicity, MK2 cell lines were incubated with increasing concentrations of PG-1 for 24, 48, and 72 hrs. The PG-1 peptide showed toxic effect against MK2 cell lines at concentrations greater than 12.5 μM (Figure 4). Other studies indicated that PG-1 was also toxic to 293A cell lines (human embryonic kidney cells) at 50 μg/ml (25 μM) [32] and more than 50% of human red blood cells lyses were observed at 80 μg/ml (40 μM) [33]. Therefore, three concentrations at nontoxic range were used to test PG-1 stability and ability to reduce dengue viral replication in MK2 cell lines. The results showed that the viral copy number significantly ($P < 0.001$) reduced with increasing concentrations of PG-1 (Figure 5(a)). Furthermore, the highest inhibition percentage was observed when the PG-1 concentration was 12.5 μM at 24, 48 and 72 hrs (Figure 5(b)). However, the low concentrations exhibited less inhibition percentage at 48 and 72 hrs as compared with 24 hrs, indicating that the PG-1 stability declined with longer incubation in culture media (Figures 5(a) and 5(b)). Similarly, it has been showed that at low doses (4 μg/ml), PG-1 has significant *in vitro* antimicrobial activity with low *in vivo* toxicity (up to 8 mg/kg i.v. mouse injection) [34]. This may be accounted by its short half-life *in vivo* as its level in mice plasma that was injected with 4 mg/ml i.v. declined rapidly to 28 μg/ml after 5 min [34]. Although PG-1 showed significant inhibition profile towards dengue virus in this study and to human immunodeficiency virus 1 (HIV1) in another study [35], the peptide instability should be considered as a major concern. The results in this study may give a clear picture that would then help in engineering new

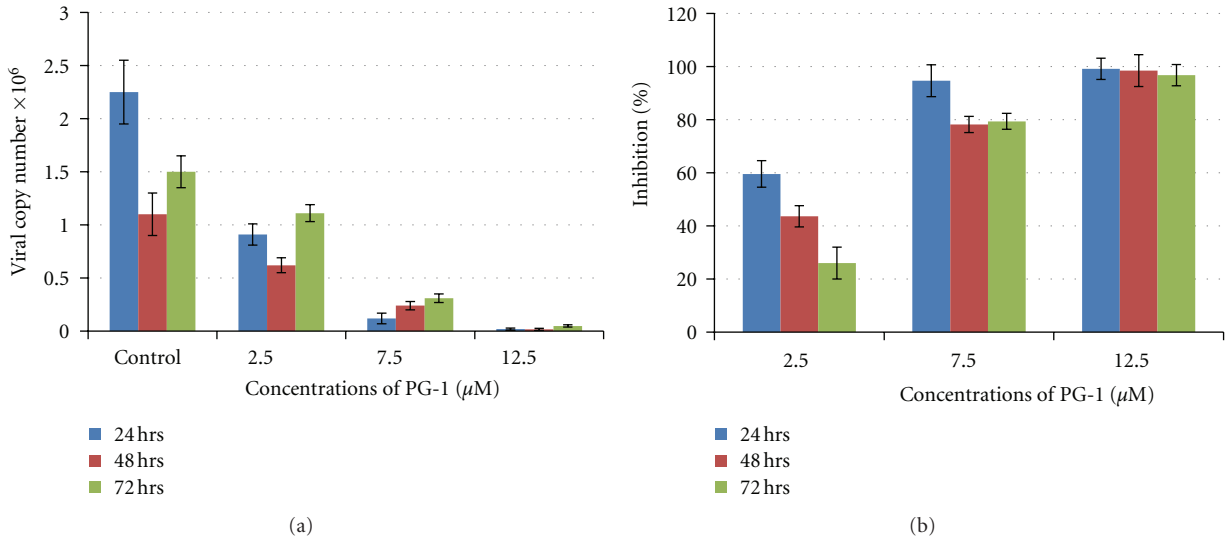

FIGURE 5: RNA quantification and the percentage of inhibition after applying different concentrations of PG-1 on MK2 cell lines infected with dengue virus. The standard curve was generated by 10-fold serial dilution of known copies of dengue RNA. Viral RNA was extracted from cellular supernatant and the fragment located at the 5′UTR region of the virus genome was amplified using one-step RT-PCR. Significant reduction ($P < 0.001$) in viral copy number was observed with increasing PG-1 concentration (a). The highest inhibition percentage was observed with 12.5 μM at 24, 48, and 72 hrs (b). PG-1 significantly ($P < 0.01$) inhibited DENV-2 replication in a dosedependent manner with the greatest inhibition effects at 24 hrs (two-way ANOVA with Bonferroni posttest, mean ± SD).

sequence of peptides to retain the antiviral activity against dengue while increasing its stability and eliminating the toxic characteristics of PG-1.

Abbreviations

PG-1:	Protegrin-1 (RGGRLCYCRRRFCVCVGR)
NS2B:	NS2B cofactor amino acids sequence 49–95 in DEN2 NS2B and 1394–1440 in DENV-2 polyprotein
NS3:	NS3 protease amino acids sequence 1–185 in NS3 protease and 1476–1660 in DENV-2 polyprotein
DENV-2:	Dengue virus serotype 2
NS2B-NS3pro:	NS2B fused to NS3 via 9 amino acids (G4-T-G4)
MCA:	fluorogenic peptide substrate (Boc-Gly-Arg-Arg-AMC)
MK2 cells:	Rhesus monkey kidney cell lines.

Conflict of Interests

The authors have declared that no competing interests exist.

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