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Inhibitory effects of a peptide-fusion protein (Latarcin–PAP1–Thanatin) against chikungunya virus

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ABSTRACT

Chikungunya virus (CHIKV) outbreaks have led to a serious economic burden, as the available treatment strategies can only alleviate disease symptoms, and no effective therapeutics or vaccines are currently available for human use. Here, we report the use of a new cost-effective approach involving production of a recombinant antiviral peptide-fusion protein that is scalable for the treatment of CHIKV infection. A peptide-fusion recombinant protein LATA–PAP1–THAN that was generated by joining Latarcin (LATA) peptide with the N-terminus of the PAP1 antiviral protein, and the Thanatin (THAN) peptide to the C-terminus, was produced in *Escherichia coli* as inclusion bodies. The antiviral LATA–PAP1–THAN protein showed 89.0% reduction of viral plaque formation compared with PAP1 (46.0%), LATA (67.0%) or THAN (79.3%) peptides alone. The LATA–PAP1–THAN protein reduced the viral RNA load that was 0.89-fold compared with the untreated control cells. We also showed that PAP1 resulted in 0.44-fold reduction, and THAN and LATA resulting in 0.78-fold and 0.73-fold reductions, respectively. The LATA–PAP1–THAN protein inhibited CHIKV replication in the Vero cells at an EC_{50} of 11.2 μg/ml, which is approximately half of the EC_{50} of PAP1 (23.7 μg/ml) and protected the CHIKV-infected mice at the dose of 0.75 mg/ml. We concluded that production of antiviral peptide-fusion protein in *E. coli* as inclusion bodies could accentuate antiviral activities, enhance cellular internalisation, and could reduce product toxicity to host cells and is scalable to epidemic response quantities.

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1. Introduction

Chikungunya virus (CHIKV) is an alphavirus that is transmitted to humans via infected *Aedes* mosquitoes (Jupp and McIntosh, 1988). The name chikungunya derived from the Makonde language of Tanzania, means "that which bends up". This name reflects the severe and debilitating rheumatic manifestations experienced by most infected individuals (Ross, 1956). CHIKV has recently re-emerged as an important pathogen causing disease epidemics in several countries including the 2000–2005 epidemics in the Congo, Indonesia and India. In addition, outbreaks of 3500 confirmed cases of CHIKV have been recorded in the Maldives in December 2006, and ~60,000 suspected cases and ~80 deaths reported in Sri Lanka. Furthermore, an East Central South African (ECSA) strain of CHIKV has been associated with the 2006 CHIKV outbreak in Asia, first in the Indian subcontinent followed by Thailand. The World Health Organisation (WHO) reported two locally-acquired CHIKV disease cases in the French Caribbean isle of Saint Martin (WHO, 2007, 2013). In Malaysia, the CHIKV outbreaks were recorded in suburban areas (1998), rural areas (2006) and urban Malaysia in 2008 (Azami et al., 2013).

The pathognomonic features of clinical CHIKV infection are marked by pyrexia and excruciating arthralgia (Solignat et al., 2009). Interestingly, macrophages have recently been identified as the key players in CHIKV immunopathogenesis (Lidbury et al., 2008; Labadie et al., 2010). The pro-inflammatory cytokines produced by macrophages appear to be strongly associated with the musculoskeletal and rheumatic manifestations that follow an alphavirus infection (Lidbury et al., 2008; Labadie et al., 2010). Importantly, lack of an effective vaccine and CHIKV-specific antiviral therapeutics remains a major concern, and therefore the available options focuses on amelioration of symptoms that includes a recommendation of rest, fluid replacement, and use of analgesics and antipyretics. Thus, CHIKV infection creates a considerable economic burden during outbreaks especially in the tropical and sub-tropical world. Therefore, development of cost-effective vaccines, antiviral drugs and diagnostic tests are largely reliant on

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novel strategies and technological innovations to attract global interest (Peeling et al., 2010; Hotez, 2011).

One of the current strategies for developing newer antiviral therapeutics uses short cationic peptides (Zasloff, 2002; Mygind et al., 2005) that reportedly show high specificity and selectivity in their interactions leading to minimal side-effects and greater potencies (Craik et al., 2013). Cationic peptides with considerable inhibitory effects have been reported against the human immunodeficiency (Owen et al., 2004), influenza (Liang et al., 2009), herpes simplex (Yasin et al., 2004), and dengue viruses (Rothan et al., 2012a,b, 2013). However, these antiviral peptides remains to be promoted as commercially available drugs largely owing to the expensive chemical infrastructure necessary to achieve the required volumes for an epidemic response. Given that the recombinant forms of these peptides could be considered as an alternative manufacturing strategy, there remains certain limitations to their large-scale production, one being the low efficiency of *Escherichia coli* (*E. coli*) as a common expression system in the formation of disulphide bonds for cysteine-rich peptides necessary for exerting antimicrobial bioactivity (Lai et al., 2002). In addition, short peptides are almost always produced in soluble, often misfolded forms, which warrant additional steps such as in-column refolding and purification representing a considerable challenge to large-scale production efforts (Rothan et al., 2012b).

Here, we evaluated three peptides with different structures: the Latarcin (LATA), an amphiphilic helix peptide isolated from the venom of *Lachesana tarabaevi*, a spider (Kozlov et al., 2006); Thanatin (THAN), a loop-structure peptide, isolated from the insect *Podisus maculiventris* (Matejuk et al., 2010); and Tachyplesin I (TACH1), a beta-sheet structure peptide derived from the hemocytes of the horseshoe crab (*Tachypleus tridentatus* and *Limulus polyphemus*) (Murakami et al., 1991). We found that the LATA and THAN peptides showed higher inhibitory effects against CHIKV compared to the TACH peptide. We constructed an antiviral peptide-fusion protein LATA–PAP1–THAN to harbour the cationic peptides LATA and THAN fused to a central antiviral protein. The central PAP1, a pokeweed-derived antiviral protein, has previously been produced in *E. coli* as inclusion bodies (Rajamohan et al., 1999). PAP1 is a ribosome-inactivating protein (RIP) from *Phytolacca americana* that has been shown to exhibit antiviral effects against herpes simplex (HSV) (Aron and Irvin, 1980) and Japanese encephalitis (JEV) viruses (Ishag et al., 2013). The antiviral activity of PAP1 depends on the RNA N-glycosidase functions against rRNA that leads to a slow pace of protein synthesis (Domashevskiy et al., 2012). The current investigation represents a new approach to produce functional recombinant cationic peptides as part of the peptide-fusion strategies employed using *E. coli*. This protein was produced in an insoluble form (inclusion bodies) and then refolded to retrieve its biological activity.

2. Materials and methods

2.1. Virus propagation in mosquito cells and titration

A clinical isolate of CHIKV recovered from serum (CHIKV isolate, SGEHICHS277108, Accession FJ445510) was used for propagation by a single passage in C6/36 mosquito cells. The CHIKV-infected C6/36 mosquito cells with obvious cytopathic effects (CPE) were lysed by a freeze and thaw cycle. The culture medium was centrifuged at 1800 rpm for 10 min to remove the cell debris, filtered (0.2 μm), portioned into aliquots, and stored at −80 °C until use.

2.2. Peptide synthesis

The TACH1, LATA and THAN peptides were manufactured chemically using a standard automated solid-phase peptide synthesis technique in a Symphony parallel synthesiser (Protein Technologies, Tucson, AZ, USA).

2.3. Design and production of the peptide-fusion protein in E. coli

The recombinant PAP1 with and without fusion with the LATA and THAN peptides was produced in *E. coli* as inclusion bodies as previously described (Rajamohan et al., 1999; Rothan et al., 2013). The DNA sequence of *PAP1* and the peptide-fusion protein (*LATA-PAP1-THAN*) was obtained by reverse translating the amino acid sequence and was optimised to *E. coli* preferred codons using software available online as previously described (Puigb et al., 2007, 2008). Alternating sense and antisense oligos of 60-mers in length (with a 15 bp overlap region) were designed to span the entire *LATA-PAP1-THAN* expression cassette (Table S1). The splicing and synthesis of the entire *LATA-PAP1-THAN* expression cassette was achieved using the Klenow-*Pfu* DNA polymerase method (Holowachuk and Ruhoff, 1995). The *LATA-PAP1-THAN* expression cassette (and the individual *PAP1* gene) was amplified using the forward and reverse primers that were designed to include both *Bam*HI and *Hind*III restriction sites. Then, the *LATA-PAP1-THAN* expression cassette or *PAP1* by itself was digested with the *BamHI* and *Hind*III enzymes to facilitate cloning into an appropriate *E. coli* expression vector (pTrc-His-A, Invitrogen). To isolate the inclusion bodies, the bacterial cells were harvested and lysed by sonication in lysis buffer. Following a centrifugation step, the isolated inclusion bodies were subjected to excessive washing steps, and were solubilised with NaOH. This step was then followed by protein refolding steps as described previously (Sijwali et al., 2001). Further purification was carried out using a His Gravi-Trap™ Flow pre-charged Ni Sepharose™ 6 Fast column (Amersham Biosciences, USA) according to the manufacturer's instructions.

2.4. Maximum non-toxic dose test (MNTD)

Vero cells were seeded at 1×10^4 cells/well in triplicate under optimal conditions (37 °C, 5% CO_2 in a humidified incubator) in 96-well plates Each peptide was diluted to serial concentrations (12.5, 25, 50, 100, 200 μg/ml) with DMEM media supplemented with 2% FBS. The cell culture was analysed after 72 h using a commercial non-radioactive cell proliferation assay (Promega, USA) according to the manufacturer's protocol. The peptide concentration that showed >90% cell viability was considered as the MNTD value, assuming that ~90% of the cells were healthy.

2.5. Real-time cell proliferation assay (RTCA assay)

Cell proliferation was measured using the xCELLigence Real-Time Cellular Analysis (RTCA) system (Roche, Germany) as described previously (Atienzar et al., 2011). Briefly, background measurements were taken after adding 100 μl of the culture medium to the wells. Next, the cells were seeded at a density of 1×10^4 cell/well in a 16-well plate with electrodes for 18 h to allow the cells to progress to the log phase. The cells were treated with different concentrations of the peptides dissolved in cell culture medium, and were monitored continuously for 100 h. The cell sensor impedance was expressed as an arbitrary unit called the cell index. The cell indices were recorded every 5 min by the RTCA analyser. To eliminate variation between the wells, the cell index values were normalised to the value at the beginning of the treatment.

2.6. Treatment of CHIKV-infected cells with antiviral peptides

To infect the Vero cell lines with CHIKV, the cells were cultured in 24-well plates (1.5×10^5 cells/well) for 24 h at 37 °C and 5% CO_2.

The virus supernatant was added to the cells at an MOI of 0.2, followed by incubation for 1 h with gentle shaking every 15 min for optimal virus to cell contact. The cells were washed twice with fresh serum-free DMEM after removing the virus supernatant. Then, new complete DMEM containing the 50 μg/ml of each peptide was added to the cultures and incubated for 72 h and viral load quantification was performed by plaque assay.

2.7. Time-of-addition assay

The peptides were added to Vero cells at various time points relative to viral inoculation. The cells were pre-incubated with the peptides at final concentration of 50 μg/ml for 24 h before viral inoculation at a MOI of 1. In simultaneous studies, both CHIKV and the peptide were added into cells for 1 h followed by replacement of medium without peptide. For post-infection studies, Vero cells were infected with CHIKV for 1 h before the peptide were applied. The viral titers for each experiment were quantitated after 72 h by plaque assays.

2.8. Virus quantification by plaque formation assay

A 10-fold serial dilution of medium supernatant was added to new Vero cells grown in 24-well plates (1.5×10^5 cells) and incubated for 1 h at 37 °C. The cells were then overlaid with DMEM containing 1.1% methylcellulose. The viral plaques were stained with crystal violet dye after 5-days incubation. The virus titres were calculated according to the following formula: Titre (PFU/ml) = number of plaques × volume of the diluted virus added to the well × dilution factor of the virus used to infect the well in which the plaques were enumerated. The percentage of the PFU was calculated using the formula: % PFU = (Viral titre of treated cells/viral titre of untreated cells) × 100.

2.9. Quantitative real-time PCR

Viral RNA was extracted using a QIAmp viral RNA mini kit (QIAGEN, Germany), and the qRT-PCR was carried out using a SYBR Green Master Kit (Qiagen, Germany) in quadruple experiments using forward primer (5′-TGGCAGCCCTGATTGTTCTA-3′) and reverse primer (5′- TGGCTCCAAAGTGACTGACA-3′). The absolute quantification was performed using an ABI7500 machine (Applied Biosystems, Foster City, CA). The results were analysed using Sequence Detection Software Version 1.3 (Applied Biosystems, Foster City, CA). The percentage of viral inhibition (%) was calculated as follows: 100 − (Viral copy number of treated cells/viral copy number of untreated cells) × 100. The data are expressed as the relative fold expression compared with the untreated controls, which was defined as 1.0 (100%).

2.10. Fluorescence ELISA-like cell-based assay and immunostaining

Vero cells were seeded within each well of a black 96-well plate with transparent bottom and treated with 0 (control), 5, 10, 15, 20, 25, 30 μg/ml of the peptide-fusion protein or PAP1 for 24 h in quadruplicate. Later, the cells were fixed with ice-cold methanol for 15 min at −20 °C and then incubated with a coating buffer for 1 h at room temperature. A mouse anti-6X His tag antibody (Abcam, UK) was added, and the cells were incubated overnight at 4 °C. The cells were incubated for 30 min with an anti-mouse IgG labelled with FITC fluorescent dye (Invitrogen, USA). The fluorescence signals were measured using Tecan Infinite M200 Pro fluorescence spectrophotometer (Tecan Group Ltd., Switzerland). For imaging, cell nuclei were stained with Hoechst dye (Invitrogen, USA) for the final 15 min of incubation followed by examination under the fluorescence microscopy. The same protocol was used to visualise the viral particles after treating the infected cells with the peptides using a mouse anti-CHIKV antibody (Abcam, UK).

2.11. In vivo evaluation of antiviral activity

In vivo evaluation experiments were carried out in accordance with the University of Malaya guidelines on the Care and Use of Laboratory Animals following approval of the animal ethics protocols used in the investigation by the Animal Ethics Committee of the University of Malaya. The ICR mice (5–6 weeks of age) with an average body weight of ~33 g were used in the acute toxicity and viral challenge experiments. The experiment of acute toxicity was performed by administering the peptide-fusion protein intraperitoneally at low dose (0.25 mg/kg) and high dose (2.5 mg/kg) to two groups of animals, while the third group was kept without treatment ($n = 6$ each). The range of the doses was selected based on previous *in vivo* studies on PAP1 protein (Benigni et al., 1995; Ishag et al., 2013). The animals were observed for 24 h for signs of toxicity, after 14 days post-treatment, the animals were sacrificed for histological examinations. The experiment of viral challenge was performed using four groups of animals ($n = 6$ each). The animals were intraperitoneally inoculated with 1×10^6 plaque-forming units (PFU) of the purified CHIKV as described previously (Parashar et al., 2013). Concurrently, three groups were individually administrated with 0.5, 0.75 and 1.0 mg/kg of the peptide-fusion protein by intraperitoneal administration; while the forth group was administrated with PBS as a mock-administrated group. Mice were observed for 7 days post-infection and the death cases were recorded.

2.12. Statistical analysis

All of the assays were performed in triplicate, and the statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). *P* values of <0.05 were considered significant.

3. Results

3.1. Antiviral activity of the peptides using the maximal non-toxic dose (MNTD)

The MNTD value for each peptide was estimated to be 50 μg/ml, and was used in the antiviral activity analyses (Fig. 1A). The 50% effective concentration (EC_{50}) of the THAN peptide was estimated to be 8.0 ± 0.5 μg/ml and the LATA peptide was 15.2 ± 1.1 μg/ml, while the TACH showed an EC_{50} of >50 μg/ml (Fig. 1B). Based on these results, the two peptides that exhibited the higher inhibition potential against CHIKV (the THAN and LATA peptides) were selected for further investigation. The percentage of viral load (PFU%) was reduced after the treatment with THAN peptide at pre-treatment (23.5 ± 5.1) while the higher reduction was observed when the peptide was simultaneously added to the cells with the virus inoculums (2.4 ± 1.1) and at post-treatment (9.8 ± 2.3). Intriguingly, the LATA peptide showed a decreased inhibition potential against CHIKV at pre-treatment (76.7 ± 7.2) compared with the simultaneous addition with the virus inoculums (29.1 ± 3.0) and post-infection treatment (16.5 ± 3.4) as presented in Fig. 1C.

3.2. Production of peptide-fusion protein LATA–PAP1–THAN

The LATA and THAN peptides were fused with PAP1 to construct the antiviral peptide-fusion protein. The LATA peptide was joined

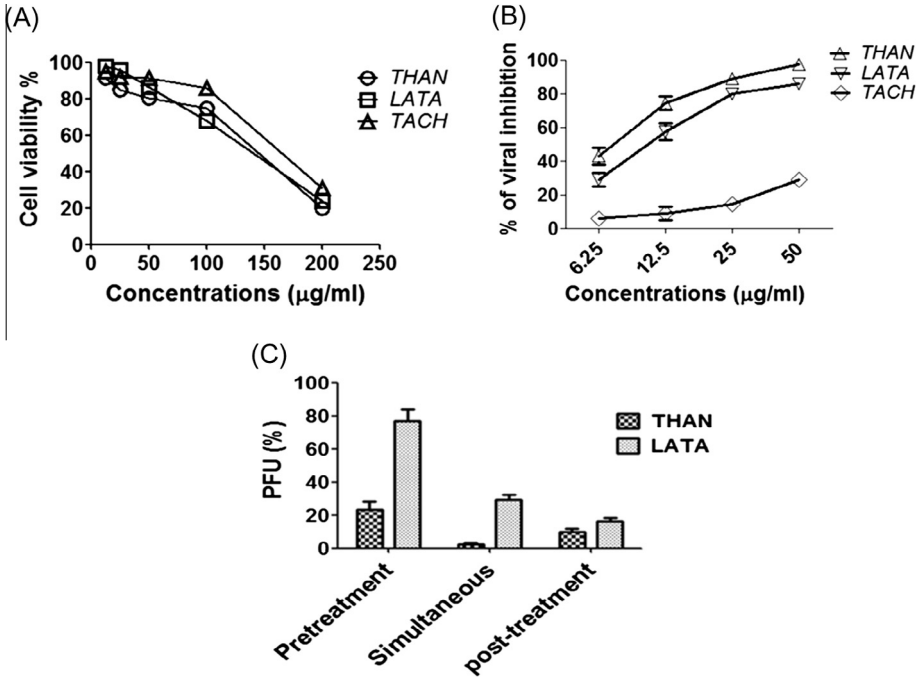

Fig. 1. Maximal non-toxic dose (MNTD) and antiviral activity of the peptides. (A) Evaluation of peptides cytotoxic effect. The MNTD value for the peptides was estimated to be approximately 50 µg/ml. (B) Determination of peptides antiviral activities using plaque formation assay. The EC_{50} of the THAN peptides was estimated to be 8.0 ± 0.5 µg/ml and the LATA peptides was 15.2 ± 1.1 µg/ml, while the TACH showed an EC_{50} of more than 50 µg/ml. (C) The mode of inhibition assay showed a considerable reduction in the viral load at the simultaneous and post-infection treatments (two-way ANOVA with a Bonferroni' post-test (Mean ± SD)).

to the N-terminal portion of PAP1, and the THAN peptide was linked to the C-terminal portion using 10-amino acid linkers to leave the C-terminus of the THAN peptide free to form a hairpin structure. (Fig. 2A and B). The molecular weight of the resulting peptide-fusion protein was ~37.4 kDa including the 6X His-tag (Fig. 2C). The peptide-fusion protein was produced at high levels as insoluble inclusion bodies reaching approximately 75% of the total bacterial protein. The total amount of the recombinant peptide-fusion protein was approximately 27 mg per litre of *E. coli* cell culture.

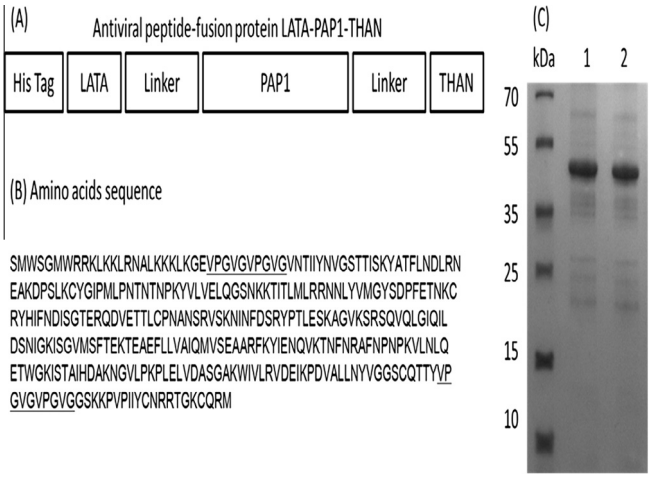

Fig. 2. Production of the recombinant peptide-fusion protein (LATA–PAP1–THAN) in *E. coli* as inclusion bodies. (A) The design of the peptide-fusion protein. (B) Amino acids sequence of the peptide-fusion protein. (C) The isolation of the inclusion bodies of the peptide-fusion protein (Lane1) and PAP1 (Lane 2).The molecular weight of the resulting peptide-fusion protein was approximately 37.4 kDa.

3.3. Cellular internalisation, uptake of the peptide-fusion protein and the real-time cell proliferation assay (RTCA)

Better internalisation of the peptide-fusion protein compared with PAP1 was observed by immunostaining showing the recombinant proteins were distributed around the cell nuclei (Fig. 3A). In addition, the cellular uptake of the peptide-fusion protein was higher than PAP1 alone (Fig. 3B). The central protein of our peptide-fusion protein is PAP1, which possesses ribosome-inactivating activities (Rajamohan et al., 1999). This activity of PAP1 could induce cellular alterations that decrease the formation of plaques, leading to a false interpretation of the antiviral activity. The results of Real-Time Cellular Analysis showed that the effects of PAP1 on cell proliferation were insignificant at the dose of 62.5 µg/ml for 53 h as the cell index was almost similar to the untreated control cells (Fig. 4A). Interestingly, the peptide-fusion protein showed non-toxic effects on cell proliferation at the dose of 31.25 µg/ml for the same time (Fig. 4B).

3.4. Antiviral activity of LATA–PAP1–THAN against CHIKV

The CHIKV load expressed as the percentage of the plaque forming units (PFU%) was significantly reduced after the treatment with PAP1 ($P < 0.001$), LATA, THAN and LATA–PAP1–THAN ($P < 0.0001$) compared with the untreated cells (Fig. 5). Interestingly, the peptide-fusion protein showed an 89.0% ± 2.1 reduction in plaque formation compared with PAP1 (46.0% ± 8.2), LATA (67.0% ± 4.4) and THAN (79.3% ± 2.4). The peptide-fusion protein was able to inhibit virus replication in the Vero cells that led to a considerable reduction in the viral RNA (0.89-fold ± 0.01) compared with the untreated control cells (Fig. 6A). While PAP1 showed a lower inhibition against the CHIKV virus (0.44-fold ± 0.05) compared with the THAN peptide (0.78-fold ± 0.01) and the LATA peptide (0.73-fold ± 0.02). The peptide-fusion protein inhibited CHIKV replication in Vero cells at an EC_{50} of

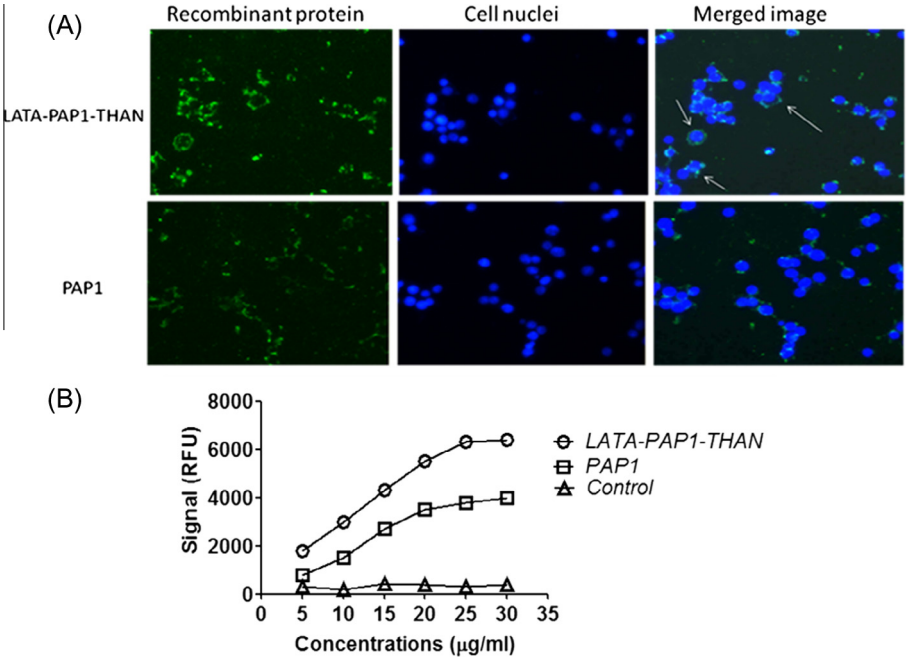

Fig. 3. Cellular internalisation and uptake of the peptide-fusion protein and PAP1. (A) Florescence images show recombinant proteins internalisation and localisation around the cells nuclei. (B). Fluorescence ELISA-like cell-based assay was used to determine cellular uptake of the recombinant proteins. The results showed higher cellular uptake of the peptide-fusion protein compared with PAP1 alone.

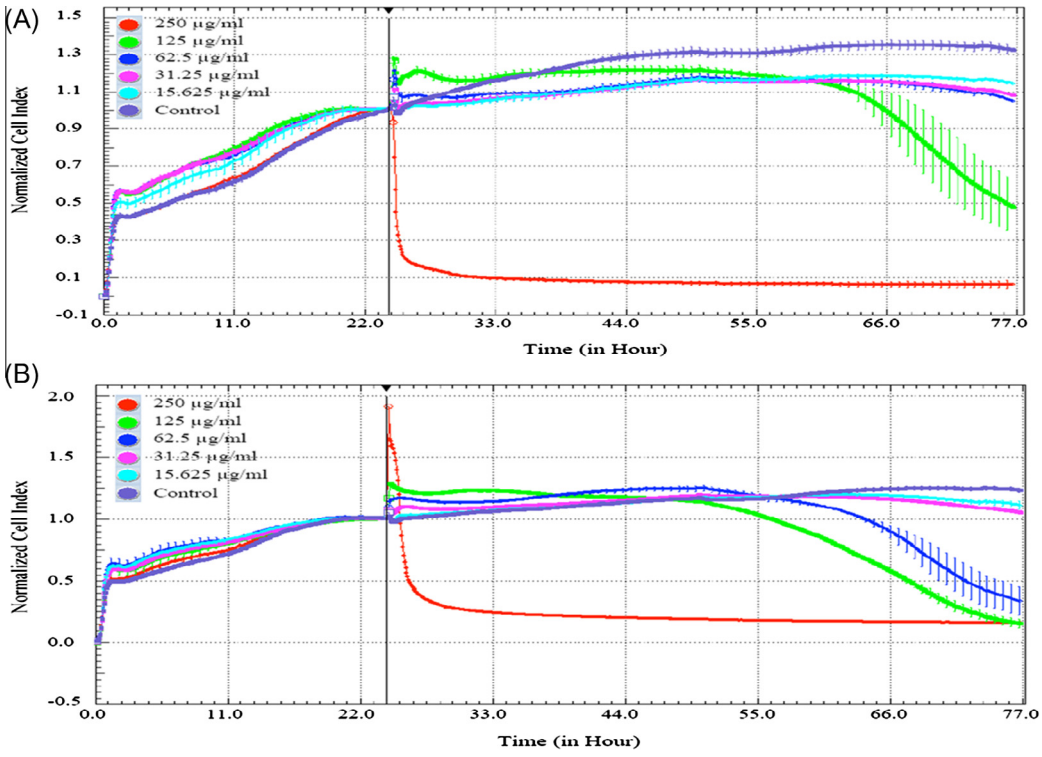

Fig. 4. The effect of the increased concentrations of recombinant proteins on real-time cell proliferation using the Real-Time Cellular Analysis (RTCA) system. (A) Cell proliferation after 53 h incubation with increased concentration of PAP1. The effects of PAP1 on cell proliferation were insignificant at the dose of 62.5 µg/ml (B) Cell proliferation after the treatment with increased concentration of peptide-fusion protein (LATA–PAP1–THAN). The peptide-fusion protein showed non-toxic effects on cell proliferation at the dose of 31.25 µg/ml.

11.2 ± 6.1 µg/ml, which was almost half the EC_{50} of PAP1 (23.7 ± 4.3 µg/ml) as presented in Fig. 6B. In addition, the peptide-fusion protein, THAN and LATA showed reduced viral particles compared to the PAP1 peptide (Fig. 6C).

3.5. Protection against viral challenge

The acute toxicity experiment showed that the peptide-fusion protein was non-toxic at doses <2.5 mg/kg. The animals after

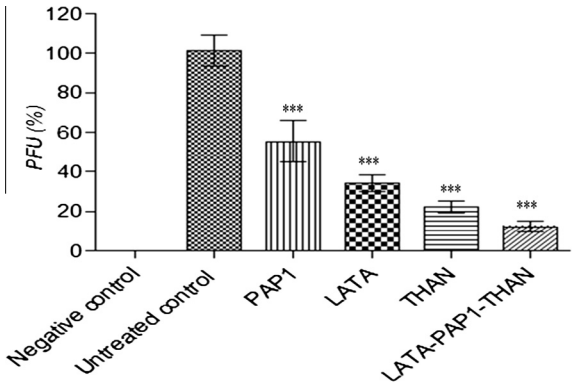

Fig. 5. Evaluation of the antiviral activity of the peptide-fusion protein LATA–PAP1–THAN compared with the individual components. Virus load expressed as the percentage of plaque forming units (PFU%) was significantly ($P < 0.0001$) reduced after treatment with all of the peptides compared with the untreated cells The asterisks denote statistically significant differences between the untreated control and infected cells treated with the peptides (one-way ANOVA with Dunnett's post-test, $P < 0.0001$(Mean ± SD)).

14 days post-treatment were healthy with no signs of toxicity or death in comparison to the untreated control. The histological examinations on the liver and kidney of the treated animals showed no evidence of tissue damage following treatment with the peptide-fusion protein (Fig. S1). The results showed the peptide-fusion protein protected the animals in a dose-dependent manner. All the animals of the mock-administrated group died 5 days post-infection, and mean morality was observed after day 3 in animals administrated with 0.5 mg/ml of the peptide-fusion protein. Interestingly, the peptide-fusion protein showed 100% survival at the doses, 0.75 and 1.0 mg/kg (Fig. 7).

4. Discussion

Our current research represents a novel approach to produce functional recombinant cationic peptides as part of the peptide-fusion strategies using *E. coli* expression system. Therefore, the present strategy is a convenient method for the large-scale and rapid production of an efficient anti-CHIKV therapeutic in volumes necessary to control a potential outbreak of CHIKV epidemic. Our previous studies have shown the cationic peptides displayed high antiviral activities. However, the high cost of chemical manufacturing infrastructure and the cumbersome recombinant production processes appears to remain a major roadblock in their large-scale production (Rothan et al., 2012a,b, 2013). The current method presents a new approach for producing these antiviral peptides based on the fusion of two antiviral peptides to a central antiviral protein. For large-scale production, the peptide-fusion protein can be produced in *E. coli* as inclusion bodies, which can easily be retrieved from native bacterial proteins and properly refolded (Heiker et al., 2010). The advantages of this method compared with other conventional methods are the production of the cationic peptide in a cost-effective way, maximisation of antiviral potentials and the efficient facilitation of the delivery of the antiviral protein into virally-infected cells for an optimal antiviral response (Lim et al., 2013). Furthermore, the cytotoxic effect of the cationic peptides on the host cells continues to remain a major challenge in the production of these recombinant peptides (Shlyapnikov et al., 2008). The biological activity of the peptide-fusion protein was demonstrated by its higher antiviral activity compared with its individual components. It is known the secondary structure of the cationic peptides is important for their optimal antimicrobial activity (Jenssen et al., 2006). Therefore, this issue has been addressed in the current study by reducing the exiting disulphide bonds during the solubilisation of the inclusion bodies and their reforming in the

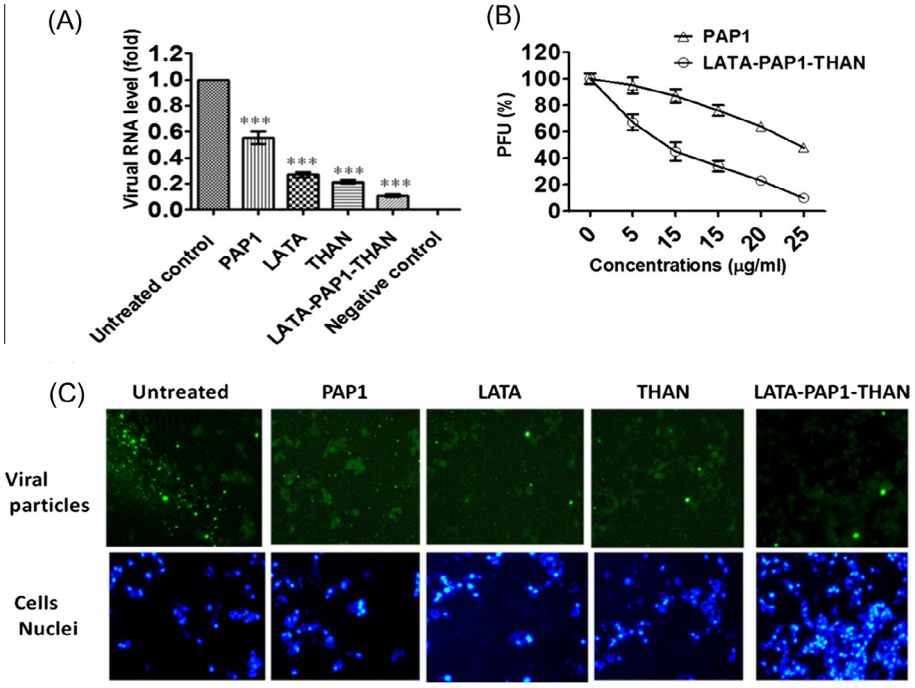

Fig. 6. Evaluation of the antiviral activity of the LATA–PAP1–THAN fusion protein by qRT-PCR, a dose–response curve and immunostaining. (A) The viral RNA load was reduced after treatment with the peptide-fusion protein. The asterisks denote statistically significant differences between the untreated control and infected cells treated with the peptides (one-way ANOVA with Dunnett's post-test, $P < 0.0001$(Mean ± SD)). (B) The dose–response curves show a significant reduction in viral load at all concentrations after the infected cells were treated with LATA–PAP1–THAN compared with PAP1 (two-way ANOVA with a Bonferroni's post-test, $P < 0.0001$(Mean ± SD)). (C) The immunostaining images show a considerable reduction in viral particle numbers after the infected cells were treated with the peptides.

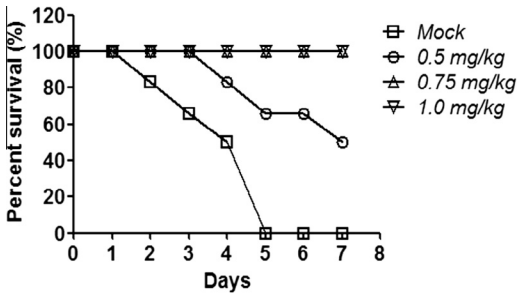

Fig. 7. The efficacy of the peptide-fusion protein (LATA–PAP1–THAN) against viral propagation *in vivo*. The animals were intraperitoneally inoculated with purified CHIKV. And concurrently administrated with 0.5, 0.75 and 1.0 mg/kg of the peptide-fusion protein by intraperitoneal administration. The survival rate of 0.5 mg/kg group decreased to 50% at day 4 post-infection while the survival rate of 0.75 and 1.0 mg/kg groups were 100% until the last day of the experiment.

presence of redox agents during the refolding step (Sijwali et al., 2001; Rothan et al., 2012b, 2013).

In this study, the cationic peptides Tachyplesin I (TACH1), Latarcin (LATA) and Thanatin (THAN), which have different secondary structures, exhibited various inhibitory effects against CHIKV. The results showed a greater inhibitory potential of LATA and THAN against CHIKV replication in infected cells compared with the TACH1 peptide. The insignificant antiviral activity of the TACH1 peptides against the influenza A (H1N1) virus, adenovirus 1, reovirus 2 and poliovirus 1 has been reported previously (Murakami et al., 1991). Although previous studies have reported considerable antibacterial and antifungal activities of the LATA (Idiong et al., 2011) and THAN (Matejuk et al., 2010) peptides, based on our knowledge, there are limited data on their antiviral activities. This study has reported for the first time the considerable antiviral activity of the LATA and THAN peptides against CHIKV replication in Vero cells. Other α-helical peptides similar to the LATA peptide have shown considerable inhibitory effects against HIV-1 due to an actual interference with the virus assembly stage in the viral life cycle (Zhang et al., 2011). In addition, peptides with a C-terminal loop possess a strong positive charge similar to THAN peptide have shown considerable antiviral activity against the dengue virus protease that is important for post-translational processes (Rothan et al., 2012a). The results of the time-of-addition assay showed the THAN peptides exhibited considerable inhibition against virus binding to the host cells. It could be possible the function of THAN peptide is similar to retrocyclin peptide in blocking viral attachment to host cell through its binding ability to heparan sulphate on cell membranes (Munk et al., 2003; Hazrati et al., 2006). Therefore, the antiviral activity of the LATA and THAN peptides individually or in fusion form (LATA–PAP1–THAN) against CHIKV appears to be associated with interference with the viral entry and replication stages in the host cells. However, further studies may be required to explore the actual mechanism of action of the LATA and THAN peptides against CHIKV replication.

Further, the current study also illustrates that PAP1 exhibited considerable antiviral activity against CHIKV but to a lesser extent compared with the LATA and THAN peptides. Previous studies have provided ample evidence for the further development of PAP1 as an antiviral therapeutic. This might be because the RNA N-glycosidase activity by which, specific purine residues are removed from the rRNA, arresting protein synthesis at the translocation step (Domashevskiy et al., 2012). In addition, PAP1 is also a cap-binding protein and is a potent antiviral agent against many plant, animal, and human viruses (Domashevskiy et al., 2012). Therefore, the PAP1 protein was selected in this study as the central protein in the peptide-fusion protein (LATA–PAP1–THAN) to take advantage of these attributes.

The combined effects of the LATA–PAP1–THAN fusion protein on the cell viability should be considered in evaluating its antiviral activity. The LATA–PAP1–THAN protein may induce cellular alterations that decrease the formation of plaques, leading to a false interpretation of the antiviral activity. The results of the real-time proliferation assay showed both PAP1 and the LATA–PAP1–THAN fusion protein inhibited CHIKV replication in infected cells with minimal effects on cell proliferation. Our data concluded that the antiviral activity of the peptide-fusion protein was independent of its possible ribosome-inactivating effects.

The current design of the peptide-fusion protein is pertinent for targeting different stages of the viral life cycle. As mentioned above, the THAN peptide showed significant inhibition against viral entry and replication, while the LATA peptide showed considerable interference with viral replication inside the infected cells. The central protein, PAP1 acted towards arresting protein synthesis, leading to slow down the synthesis of viral proteins. In addition, the cationic peptides facilitated the internalisation of the PAP1 fusion protein into the infected cells. For these reasons, the peptide-fusion protein (LATA–PAP1–THAN) showed improved antiviral activity against CHIKV compared with its individual components. The effectiveness of the peptide-fusion protein against CHIKV propagation was further verified in mice challenged with lethal dose of the virus. Interestingly, the peptide-fusion protein showed 100% survival at the dose, 0.75 mg/kg. These results support the *in vitro* findings which warrant a need for pharmacology and histopathology studies to examine the efficacy of the peptide-fusion protein in attenuating the syndromes of CHIKV infection in an expedient animal model.

5. Conclusion

Our study reports the inhibitory effect of PAP1, LATA and THAN against CHIKV replication in Vero cells. The anti-CHIKV activity was amplified in the recombinant peptide-fusion protein (LATA–PAP1–THAN). Our work also suggests the cost-effective production of antiviral peptide-fusion protein in *E. coli* as inclusion bodies. This strategy could accentuate antiviral activities, enhance cellular internalisation, reduce product toxicity to host cells and is scalable to epidemic response quantities.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.05.019.

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