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Peptide P3 Selected from Phage Display Screen Shows Antiviral Activity against Porcine Reproductive and Respiratory Syndrome Virus

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Abstract

Background: Porcine reproductive and respiratory syndrome (PRRS) is an economical devastating disease of swine industry worldwide, especially in China. The pathogen responsible for this disease calls porcine reproductive and respiratory syndrome virus (PRRSV), against which, we developed a specific drug from phage display screen.

Methods: We selected a peptide SPHIIRNHRLSK (P3) through phage screening technology against PPRSV polymerase. Adopting Real-time PCR and tissue culture infective dose (TCID50) assay, we assessed the antiviral activity and cytotoxicity of peptide P3. Moreover, we defined the mechanism of inhibition effect of P3 by using Biomolecular fluorescence complementation assay (BiFC). Rhodamine labelling peptide, combined with the confocal fluorescence microscopy helped us visualize the absorption kinetics of P3 in cells directly. All the experiments were conducted in MARC-145 cells.

Results: P3 shared the same structure and positive charge with the anti-bacterial peptides. We proved that P3 indeed exhibited high antiviral activity through directly binding with PRRSV polymerase and exhibited low toxicity to cells. P3 inhibited PRRSV replication in MARC-145 cells in a dose-dependent manner and could pass through the surface of the cells to directly bind with PRRSV polymerase, which indicated the prevention and therapy effect of P3. All these results explained the specific mechanism of antiviral ability of P3.

Conclusion: All of the above facts suggested that the peptide P3 might be a potential therapeutic drug for PRRSV infection.

Keyword: Porcine reproductive and respiratory syndrome virus; Anti-PRRSV peptide

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Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a main cause of porcine reproductive and respiratory syndrome (PRRS), which is a wide-spread infectious disease of farm pigs [1]. Currently, the prevention and treatment of PRRS are becoming a more and more challenging research topic in veterinary viral immunology. However, the specific mechanism of immune response against PRRS virus (PRRSV) still remains unknown [2]. At early stage of infections, pigs develop a rapid but strong humoral response. These initial immune reactions, however, do not bring about protection and may even cause harm through mediating an antibody-dependent enhancement of disease. Neutralizing these adverse antibodies is a slow process and the cell-mediated immune responses are always suppressed by the virus. So an effective vaccine against PPRSV is in great need, but the fact is that the development of vaccine often stuck into dilemma because of high diversity of the virus genome. Increasing evidences show that the immunoreactions induced by one strain may be less or little effective to another different strain, even within the same

genotype. Besides, the effect of commercial vaccines against PRRSV is quite limited [2]. And there is a potential risk that the modified-live PRRSV vaccine might revert to virulent virus under farm conditions, which poses great safety concern. Indeed, it has been demonstrated that a conversion from a commercial modified-live PRRSV vaccine to pathogenic phenotype in vaccinated pigs did happened [3-6]. Therefore, it is urgent to explore new antiviral method to control the PRRS. So searching for the virus inhibitors as drugs might be preferred.

PRRSV is divided into two major genotypes: the European (EU; type 1) genotype and the North American (NA; type 2) genotype [7]. The latter is the main type isolated in China. PRRSV is a single-stranded positive-sense RNA virus with 15KB length of genome. The genome is compose of a cap structure at 5'-end and a poly(A) tail at 3'-end, containing nine open reading frames (ORF1a, ORF1b, ORF2a, ORF2b, ORF3–ORF7). ORF1 and ORF2 encode virus replication-related proteins, and account for 80% of the virus genome [8,9]. ORF1a and ORF1b encode polyproteins, which are proteolytic processed into 12 non-structural proteins (NSPs). Among these, proteins from ORF1b perform as the RNA-dependent RNA polymerase (NSP9), helicase (NSP10) and the conserved C-terminal domain (CTD) (NSP11) [10], which are important factors in the process of PRRSV infection [11]. Given the facts above, ORF1b can be treated as a potential target for antiviral drug screening [12-16].

Phage technology is an effective method for developing new drugs, and it has been applied in drug development trials increasingly [12,17,18]. Several successful cases of PRRSV inhibitors through phage screening technology were reported. Peptides obtained from phage screened of purified PRRSV showed low inhibition effect against PRRSV [19]. Except for antiviral peptides screen, phage was also used for pathogen detection [20]. In previous study, we obtained several peptides from phage display screen against PPRSV polymerase [21], instead of the whole virus. One of these peptides, P3 showed higher antiviral activity *in vitro* and lower cytotoxicity *in vivo*. Properties assay indicated that P3's strong solubility and positive charge make itself exhibit cationic anti-bacterial peptide characters. These evidences indicate that P3 has the potential possibility to become an antiviral peptide drug.

Methods

Cells and virus

Monkey Kidney cells (MARC-145 ATCC No. CRL-12231) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Gibco). PRRSV polymerase and helicase genes were cloned from PRRSV SY0608 strain. pAPRRSV (Genbank No. GO330474) and pSHE (Genbank No. GO461593) strains were kindly gift from Dr. Wei.

Peptides synthesize

All peptides in this study were chemically synthesized by TASH Biotechnology Co. Ltd with purity of more than 95%, and were dissolved in DMSO.

Real-time polymerase chain reaction (PCR) and tissue culture infective dose (TCID50) assay

MARC-145 cells were inoculated with viruses at 0.01 MOI for 1.5 h at 37°C, and treated with or without peptides at different concentration. The effects on PRRSV of selected peptides treatment were determined by both real-time PCR and TCID50 methods. Simply, total RNA was prepared using TRizol reagent (Invitrogen) from cells treated with or without peptides or drugs. Reverse Transcription was performed following manufacturer protocols (Invitrogen). Briefly, 2 µl total RNA, 1 µl 10 mM dNTP Mix, 2 pmol gene-specific reverse primer and 8.8 µl distilled water were added to a nuclease-free micro-centrifuge tube. Mixtures were heated to 65°C for 5 min and quickly chilled on ice for 5 min. Then 4 µl 5X First-Strand Buffer, 2 µl 0.1 M DTT, 1 µl Ribonuclease Inhibitor and 1 µl M-MLV were added. Tubes were incubated at 42°C for 50 min and 75°C for 15 min. To remove RNA complemented with cDNA, 1 µl *E. Coli* RNase H was added and incubate at 37°C for 20 min. Real-time PCR was performed using Taqman probe quantitative real-time PCR following manufacturer protocols (TaKaRa). The primers used for PCR are shown in **Table 1**. Results are expressed as virus RNA copies, which were normalized to β-actin copy numbers as the endogenous control [22].

TCID50 was determined by Reed-Muench method according to the previous report [23]. Briefly, MARC-145 cells were plated into a 96-well plate, 0.01 MOI PRRSV was added to 96-well plate containing a monolayer of MARC-145 cells for 1.5 h. After inoculation, peptide or DMSO was added. 24 h later, supernatant was collected and frozen at −70°C until use. Serial 10-fold dilutions were made of supernatant stocks, and 100 µl samples of each dilution were added to duplicate wells of a 96-well plate containing a confluent monolayer of MARC-145. Seven days were allowed for the appearance of cytopathology. The dilution causing cytopathology in half the cultures (the median tissue culture infective dose called TCID50) was then calculated as described by Reed and Muench method.

Cytotoxicity assay

CC50 was determined using MTT assay. Briefly, MARC-145 cells were incubated in 96-well plates in 200 µL of DMEM. MARC-145 cells were incubated with or without peptides (from 1-250 µM) for 24h. Incubation was terminated by aspirating the media and MTT solution (5 mg/ml in PBS) was added to each well. Formazane formation was terminated after 4 h by removing the MTT solution. Subsequently DMSO was added to each well to solubilize formazane, and the formazane-containing samples were measured at 590 nm in a microplate reader.

Table 1 Primers for Real time PCR, primers and probes used in this study.

Real time PCR Primers	Sequences (5'-3')
PRRSV F	AGTGGGTCGGCACCAGTT
PRRSV R	GCAGACAAATCCAGAGGCTCAT
MARC-145 actin F	CAGCACGATGAAGATCAA
MARC-145 actin R	GGGTGTAACGCAACTAAG

Absorption kinetics experiments

MARC-145 Cells were digested to single cell and resuspended in DMEM supplemented with 10% FBS. Approximately 1 x 106 cells were mixed with rhodamine labeled P3 in a culture dish (final concentration of rhodamine labeled P3 was 250 μM) and incubated in 37°C for different times. Cells were shaken in 20 min time interval. After incubation, cells were washed in phosphate buffered saline (PBS) and lysed in lysis buffer. Lysed supernatant was separated by centrifugation at 5,000 g for 10 min. 100 μL lysed supernatant was added to wells of 96-well plate.

Thereafter, the plate was transferred to BioTekSynergyTM2 and the fluorescence was measured (excitation wavelength 530/25 nm, emission wavelength 528/20 nm). The results were normalized with cells without treatment. Data was calculated and assayed by GEN 5.

Confocal fluorescence microscopy

MARC-145 cells were treated with or without peptide at different time points. After fixation in 80% ice-cold acetone at -20°C for 10 min, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. After washing, cells were detected and photographed with confocal fluorescence microscopy.

Bimolecular fluorescence complementation (BiFC) assay

A variant of yellow fluorescent protein plasmid, Venus, was obtained by reforming of pEYFP-N1 (Genbank Accession: U55762.1). Four polyclone sites (Bgl II, EcoRI, BamHI and XhoI) and (GGGGS)x3 linker were added in front of EYFP first. Then, EYFP was truncated in N-terminus part (VC 174-239) or C-terminus part (VN 1-173), respectively. As shown in **Figure 4a**, target gene fragments were fused to truncated EYFP via a (GGGGS) X 3 linker [24,25]. Helicase gene from PRRSV was amplified and fused to the N-terminus of VN and mutant P3 (mP3) (STQRPTLMRTRP) was amplified and fused to the N-terminus of VC, which was treated as negative controls. All genes were cloned to plasmid by Bgl II and XhoI sites. The primers used for BiFC are shown in **Table 1**. About 60% confluency of MARC-145 cells cultured in 6-well plates were co-transfected with 350 ng of each BiFC plasmid using liposome 2000 Transfection Reagent. After 18h post-transfection, living cells were visualized through an Olympus inverted fluorescence microscope.

Statistical analyses

All assays described here were repeated at least twice, and all the measurements were made in triplicate. Mean values±SD (Standard Deviation) were calculated using Microsoft Excel. Student's t-test or one-way analysis of variance was used to test for significant differences between means, with $P<0.05$ being considered statistically significant. Figures were performed using the GraphPadTM Prism 5.0 software.

Results

Compare of antiviral peptides against PRRSV

As described before, proteins encoded by PRRSV ORF1b play vital roles during PRRSV replication. In our previous study, we had used phage screening technology to screen out several effective peptides targeting ORF1b proteins. Among these peptides, SPHIIRNHRLSK (P3) exhibited highest antiviral activity. To further explore P3's comprehensive antiviral ability, we compared the physical properties of it. P3's IC50 is 67.79μM, determined by Real-time PCR assay. P3 also showed strong solubility (GRAVY-1.100) and positive charge (PI 12.01) in culture media. Strong solubility indicated that P3 is easy to be absorbed at a high concentration *in vivo* and *in vitro*. Positive charge of P3 implied it performed antiviral effect just like cationic anti- microbial peptide **(Table 2)**. These two properties facilitated P3's inhibition effect on PRRSV *in vitro*.

Inhibitory effect of P3 peptide against PRRSV infection *in vitro*

The inhibition ability of P3 was determined by real-time PCR. MARC-145 cells were incubated with viruses at 0.01 MOI for 1.5 h at 37°C, and then treated with P3 peptides at 250 μM for 24 h. The cells treated with DMSO were used as the negative control. P3 showed high antiviral activity in MARC-145 cells, meanwhile

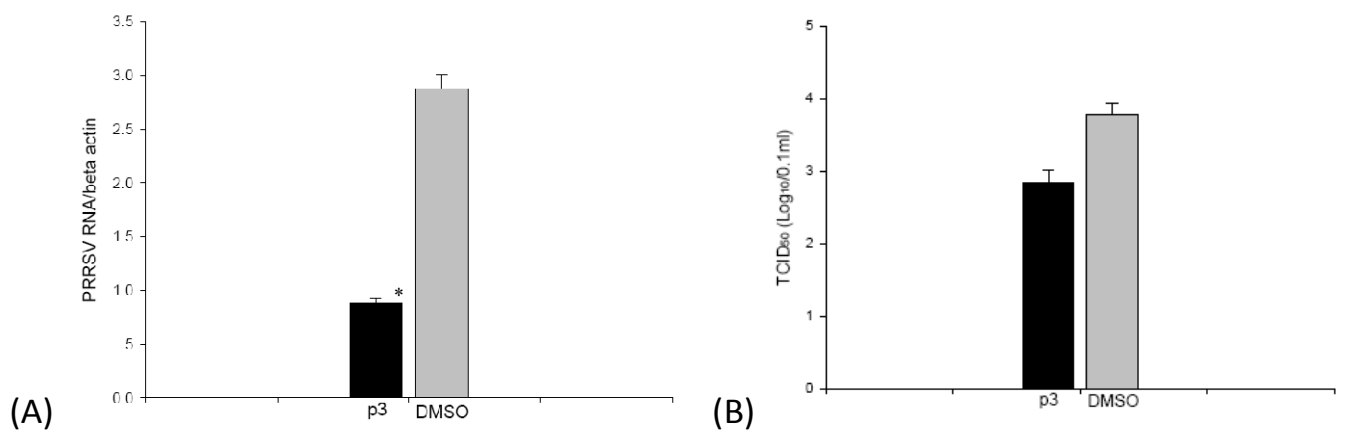

Figure 1 P3 inhibited PRRSV replication. (A) Real-time PCR method was used to detect antiviral activity of P3. (B) Antiviral activity of P3 determined by TCID50, 250 μM P3 was added and CPE was recorded at 24 h. ($p < 0.05$, indicated by *).

Table 2 Antiviral activity and cytotoxicity of p3 peptide against PRRSV. The molecular weight (MW), pI, and grand average of hydropathicity (GRAVY) were predicted with the ProtParam algorithm. IC50: peptide concentration required to inhibit virus infection by 50%; CC50: peptide concentration required to reduce cell viability by 50%, as determined by the MTT method; SI (selectivity index) = CC50/IC50.

Name	Amino acid sequence	MW	pI	GRAVY	IC_{50} (μM)	CC_{50} (μM)	SI
P3	SPHIIRNHRLSK	1457.7	12.01	–1.100	67.79	618.73	9.12

DMSO showed no activity against PRRSV **(Figure 1a).** The results indicated that P3 peptide exerted high antiviral activity *in vitro*, and the antiviral activity was dependent on the amino acid structure of P3. TCID50 showed strong inhibition of P3 against PRRSV **(Figure 1b).** The results of Real-time PCR and TCID50 showed that P3 was an effective antiviral peptide against PRRSV in vitro.

Time- and dose-course test of antiviral activity.

To further investigate the antiviral activity of P3, time-course effect of P3 on inhibition of PRRSV replication was performed. As shown in **Figure 2a**, during PRRSV replication (12 - 48 h), P3 showed significant inhibition on PRRSV replication compare to control. Furthermore, P3 inhibited PRRSV replication by a dose-dependent manner. The intracellular PRRSV numbers were gradually decreased when peptides concentrations were gradually increased **(Figure 2b).** These results suggested that P3 exhibited inhibition on PRRSV replication in a time and dose-dependent manner.

P3 exert its prevention and therapy effect through inhibiting PRRSV replication.

To explore the prevention and therapy effect of P3, we treated MARC-145 cells with peptide for 4 h before and after virus infection, respectively. The Real-time PCR results showed that pre-incubated with P3 for 4 h, the intracellular PRRSV numbers was violently decreased after 24 h infection, compared to control which was pre-incubated with DMSO **(Figure 2c).** Although PRRSV RNA numbers in infected cells treated with P3 were lower than that of DMSO control, it was still significantly higher than that of pre-incubated with P3 for 4 h. These results suggested that P3 pre-incubate before virus infection could strongly inhibit PRRSV replication in MARC-145 cells.

Absorption kinetics of P3

As a virus polymerase inhibitor, it is important to be absorbed by cells to execute its antiviral function. To monitor the process of uptake, the labeled P3 was incubated with MARC-145 cells for 1 hour. Cells were washed and then incubated for another 120 minutes before fixation for confocal fluorescence microscopy **(Figure 3a).** The results showed that the P3's uptake efficiency was very high. Basically all the cells showed fluorescence throughout cytoplasm and nucleus. Meanwhile, we measured intracellular concentration of labeled P3 after different incubation. Surprisingly, P3 could penetrate into cells in a very short time after exposure to cells at 37°C **(Figure 3b).** In the same way, we examined the stability of P3 in MARC-145 cells. After 24 hours exposure, P3 still maintained high level of concentration *in vivo* **(Figure 3c).**

P3 interacted with PRRSV polymerase in cells directly

To further demonstrate that P3 could directly bind with polymerase intracellular, the biomolecular fluorescence complementation (BiFC) had been conducted. The principle of BiFC assay is based on structure complementation between two non-fluorescent N- and C-terminal fragments of an intact fluorescent protein. If the proteins of interest do interact, the non-fluorescent fragments can be brought into close proximity and the fluorescence of intact fluorescent protein can be visualized using fluorescence microscopy. BiFC assay allows for the visualization of specific protein-protein interactions within the living cells **(Figure 4a).**

The fluorescent protein between amino acid residues 173 and 174 was split to generate two fragments, VN and VC. As expected, neither VN nor VC expressed alone produced a fluorescent signal when expressed in MARC-145 cells (data not shown). Likewise, co-expression of VN and VC yet also produced no fluorescence in MARC-145 cells **(Figure 4b)**. We then generated full PRRSV polymerase and helicase gene to fuse into the N-terminus of VN with a flexible linker and designated VN-polymerase and VN-helicase, respectively. And we generated P3 and mutant P3 gene (HIRHRKSIPSNL) to fuse into N-terminus of VC with a flexible linker and designated VC-P3 and VC-mP3. In order to determine whether BiFC can efficiently detect the polymerase-P3 interactions in living cells, cells were co-transfected with the different combinations of VN-polymerase, VN-helicase, VC-P3 and VC-mP3, and ultimately were examined for fluorescence. After 18 hours transfection, as expected, VN and VC co-expression produced no fluorescence signal. Meanwhile, the combination of VN-helicase and VC-P3 also failed to generate fluorescence, demonstrating that P3 couldn't bind with PRRSV helicase. In the pairs of VN-polymerase and VC-P3, fluorescence was observed in the cytoplasm **(Figure 4b),** representing the p9-polymerase strong interaction. When P3 was mutated, no positive BiFC signals had been captured. The VN-helicase/VC-P3 and VN-polymerase/VC-mP3 pairs were employed as negative controls here.

Antiviral activity of combined peptides

In previous study, we find P9, APP-1, P3 and APP-3 showed highest antiviral activity in vitro. To determine the potentially combined antiviral effect of these peptides, two, three or four peptides were used in antiviral experiment. We found that P3 combined with p9 exerted synergistic antiviral activity against PRRSV *in vitro* **(Figure 5)**.

Discussion

In recent years, PRRSV infection has seriously harmed the pig industry. PRRSV infection has caused great economic loss,

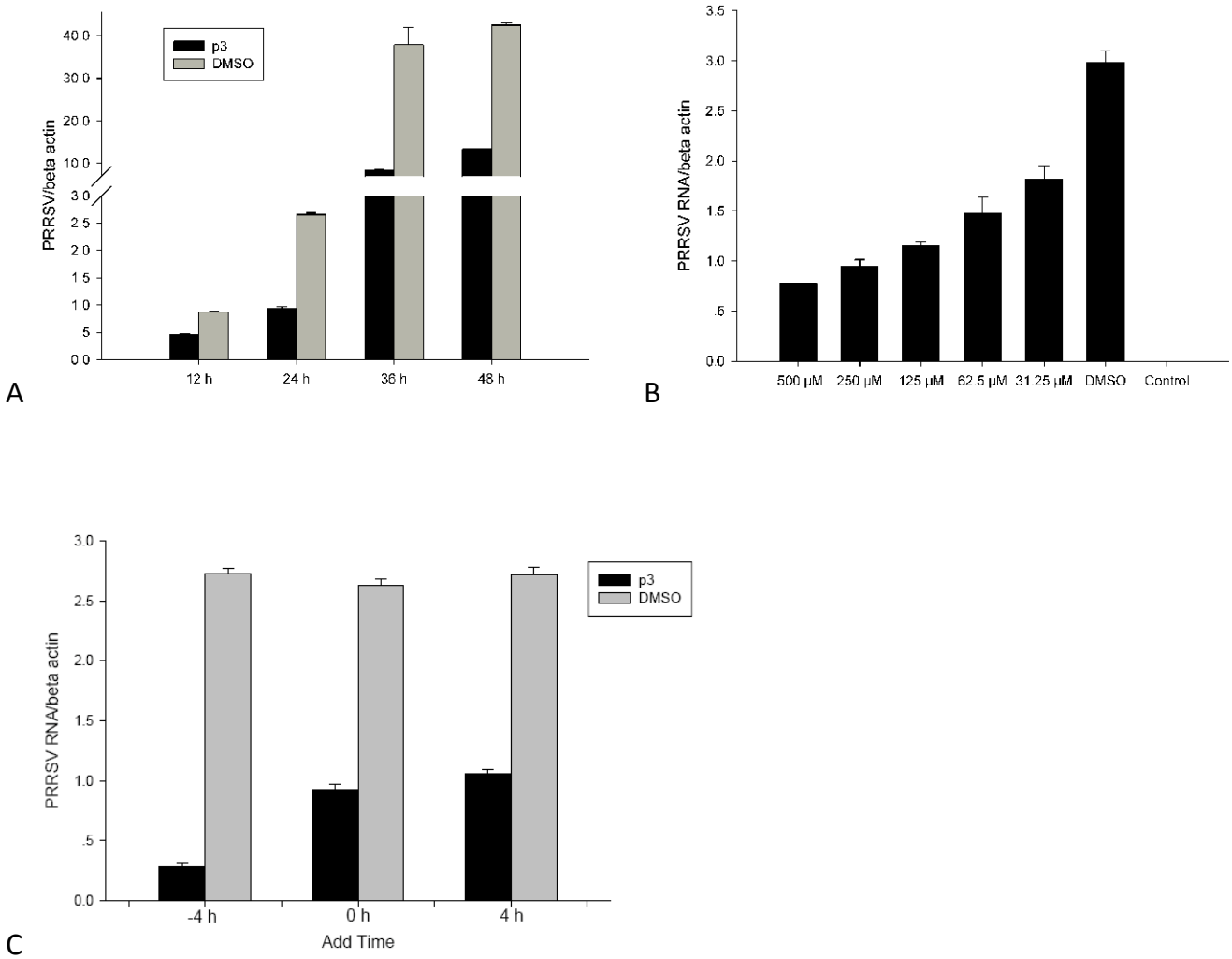

Figure 2 Determination of the time–course and dose–course of P3 inhibition. (A) Time-course of P3, DMSO was selected as control at different times. (B) P3 antiviral activity preformed in a dose-dependent manner. (C) Determination of prevention and therapy effect of P3. Peptide was added at 4 h before and after virus inoculated, and detected in 24 h. ($p < 0.05$, indicated by *).

especially in the years 2005-2008 [2,6]. To date, there is no effective drug that can control PRRSV infection, and PRRSV vaccination is ineffectively. To investigate the novel strategy against PRRSV infection, in this study, the key protein involved in PRRSV replication was expressed and its high-affinity peptides were screened following phages display. One of selected peptide P3 showed very high extracorporeal antiviral activity against PRRSV replication.

PRRSV polymerase is an RNA-dependent RNA polymerase and is an enzyme specific to viruses. Therefore, peptides screened by phages could play a role in the inhibition of this specific virus enzyme in cells without affecting cell enzyme activity [10,14,26]. Several peptides were obtained from phage display in previous study. Based on antiviral test, it was found that the antiviral ability of P3 was high. P3's pI value was 12.48, which indicated some characters like cationic anti-microbial peptides with antiviral activity. This pI value got close to some animal cathelicidin. The cationic character of antimicrobial peptides facilitated them to get closer to target; it is an important character to anti-microbial peptides. Our uptake experiments showed P3 could be effectively absorbed by cell.

Peptide inhibitors can be degraded and metabolized by intracellular proteases after they enter the cell [27,28]. Stability in cells is an important index to evaluate antiviral peptide [29,30]. After invaded cells, viruses persist and form new virus particles, which results in transmission of infection from one cell to another. This process requires that virus inhibitors exist stably in cells for a prolonged time [29]. Our study detected and observed the time-course of P3 peptide in infected cells. Our results also indicated that P3 played an inhibitory role in cells in a dose-dependent manner. Virus concentration in cells increased with time, but when compared with the PBS control group, the quantity of virus and speed of virus were both lower in the presence of P3 [18].

We further tested the inhibitory effect of P3 added at 4 h before and after virus infection, which represented the virus prevention and the treatment effects of P3 [31,32]. The results showed that P3 added before cell infection had higher antiviral ability than P3

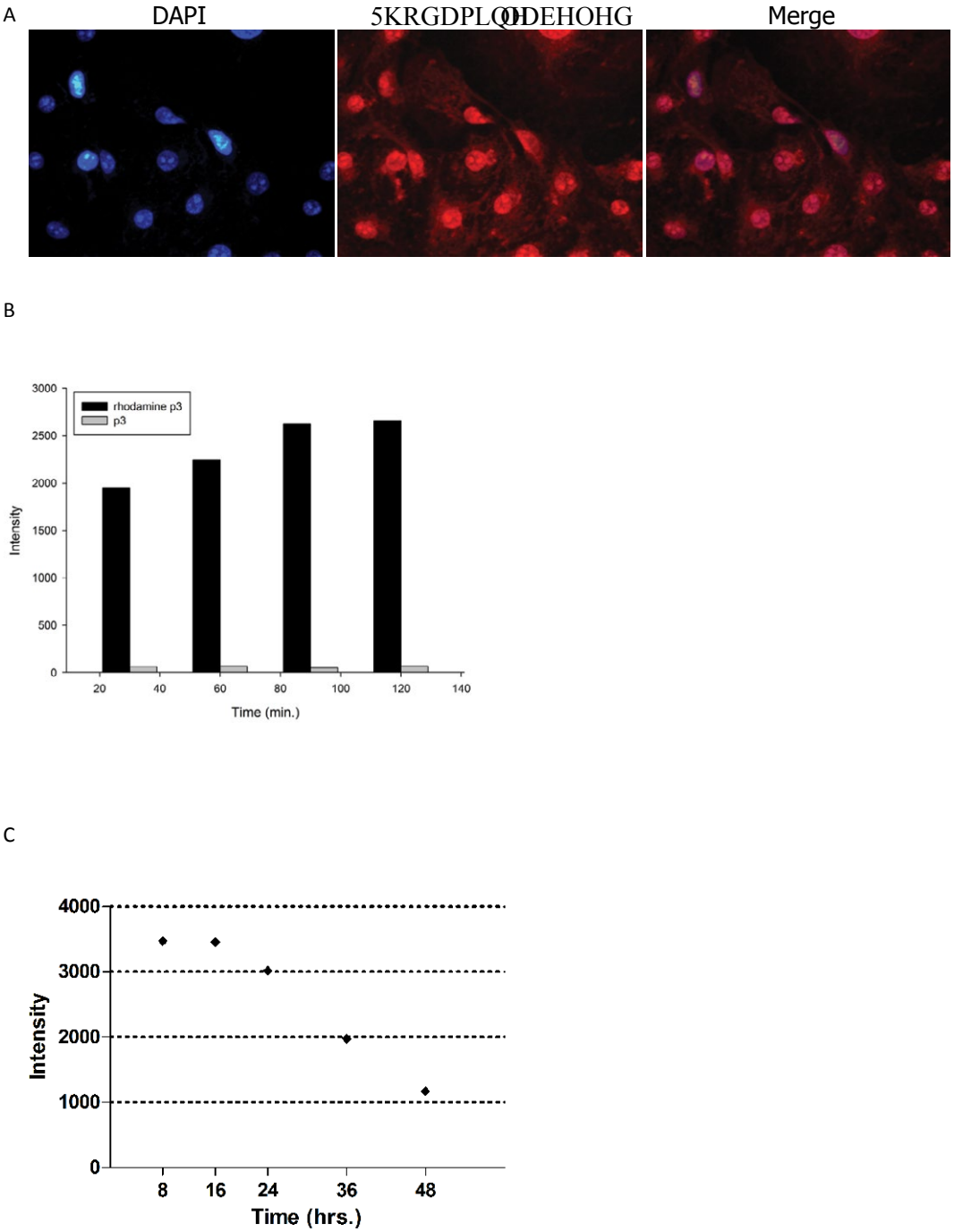

Figure 3 Absorption kinetics of P3 peptide. (A) Photomicrographs demonstrated the adsorption of P3 into MARC-145 cells after 12 h of p3 exposure at 37 °c. (B) Time course of P3 uptake into MARC-145 cells. (C) The stability of P3 in MARC-145 cells. Cells cultures incubated in medium containing 100 µM peptide added at time 0.

added at 0 h or 4 h after infection. In the +4 h group, P3 was added at 4 h after virus infection, and we calculated that P3 entered the cells probably at 5-6 h. In this 5-6 h period, viruses had enough time to complete intracellular capsid removal and polymerase expression [29, 33-35]. Based on these results, we suggest that P3 plays an inhibitory role, and its inhibition efficiency was comparatively much lower than that of the –4 h group and 0 h group.

Our BiFC experiment showed intensively interaction between P3 and PRRSV polymerase. These results proofed that P3 is interacting with polymerase intracellular but not others [25,36].

Our studies show that the PRRSV Polymerase is an attractive drug target. Furthermore, we found that P3 is potent antiviral peptide with low cytotoxic at different concentrations. Thus, further development of P3 as an antiviral agent may lead to a new type of polymerase inhibitors that is expected to inhibit PRRSV.

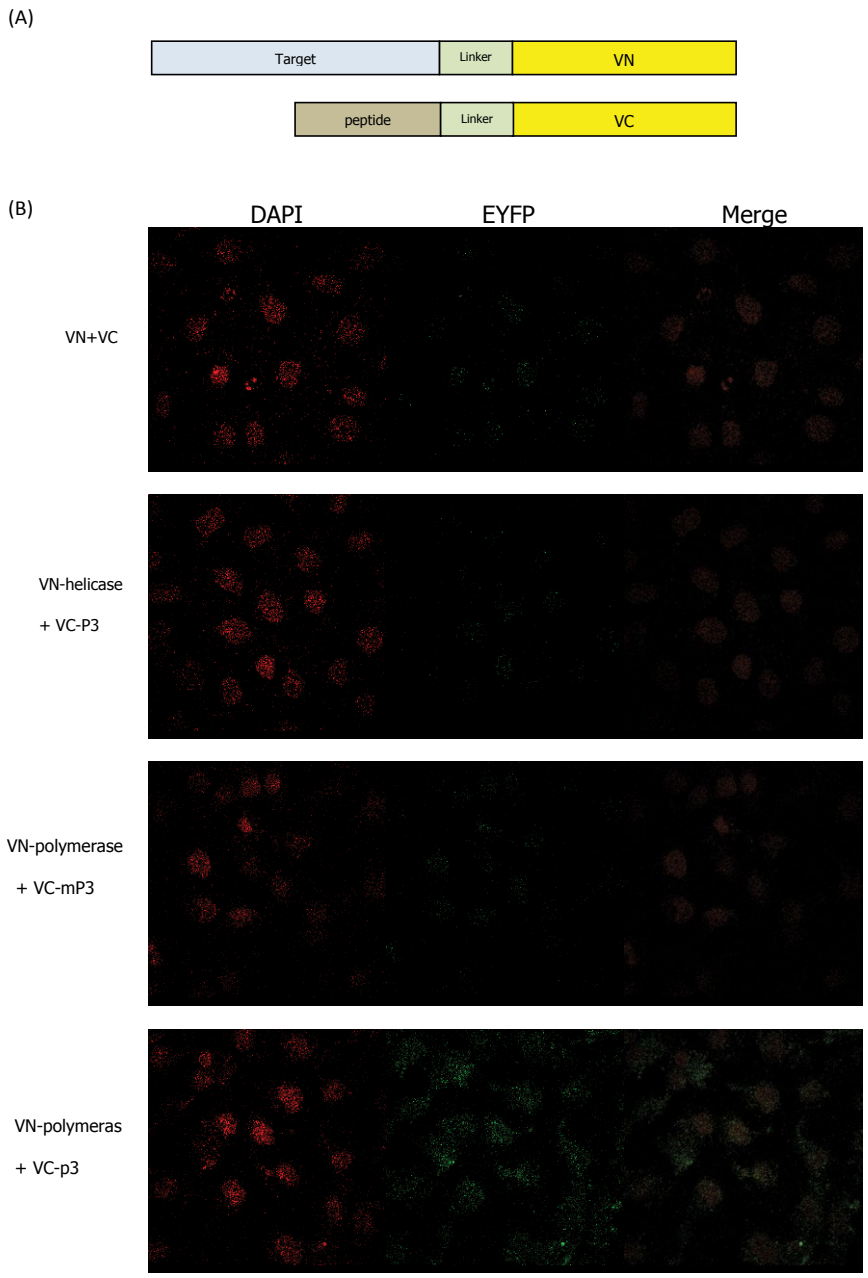

Figure 4 BiFC assay of P3 and polymerase's intracellular interaction. (A) The design of fluorescence plasmids. (B) The confocal fluorescence visualized in MARC-145 cells.

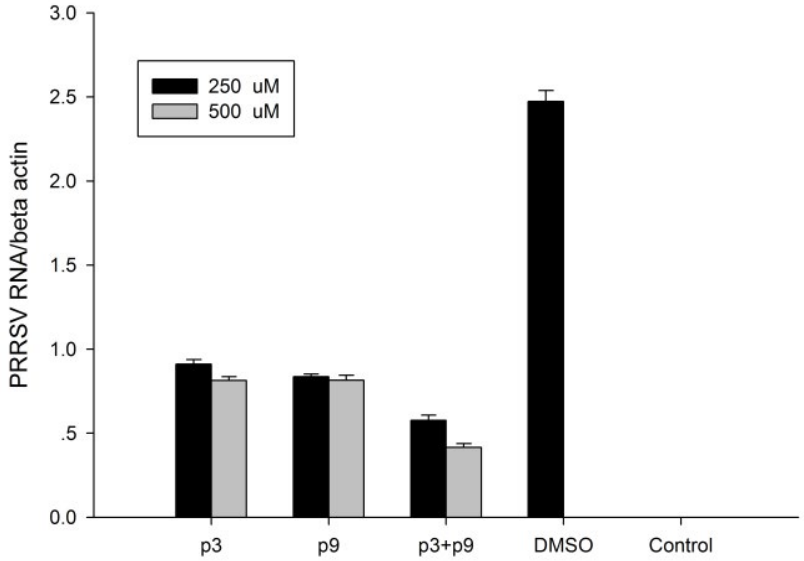

Figure 5 Antiviral activity of combined peptides. P3 and P9 (250uM, 500uM) inhibit PRRSV replication with same potency shown by Real-time PCR, but the combined use of P3 and P9 (125uM or 250uM respectively) shows synergistic effect against virus.

Acknowledgment

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