Fish & Shellfish Immunology 28 (2010) 434–439

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Antimicrobial peptides (AMP) with antiviral activity against fish nodavirus

Ta-Jui Chia [a], Yu-Chi Wu [a], Jyh-Yih Chen [b,*], Shau-Chi Chi [a,c,**]

[a] Institute of Zoology, National Taiwan University, Taipei 106, Taiwan
[b] Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, 23-10 Dahuen Rd., Jiaushi, Ilan 262, Taiwan
[c] Department of Life Science, National Taiwan University, Taipei 106, Taiwan

ARTICLE INFO

Article history:
Received 11 September 2009
Received in revised form
23 November 2009
Accepted 23 November 2009
Available online 8 December 2009

Keywords:
Antimicrobial peptides
AMP
NNV
Fish nodavirus
Antiviral activity

ABSTRACT

Nervous necrosis virus (NNV) is classified as betanodavirus of *Nodaviridae*, and has caused mass mortality of numerous marine fish species at larval stage. Antimicrobial peptides (AMPs) play an important role of innate immunity either against bacterial pathogens or viruses. Up to date, little is known if any AMP could effectively inhibit fish nodaviruses and its mechanism. In this study, the antiviral activities of three antimicrobial peptides (AMPs) against grouper NNV (GNNV) were screened in the fish cell line. Two of the three AMPs, tilapia hepcidin 1-5 (TH 1-5) and cyclic shrimp anti-lipopolysaccharide factor (cSALF), were able to agglutinate purified NNV particles into clump, and the clumps were further confirmed to be viral proteins by TEM and Western blot. The NNV solution, separately pre-mixed with AMP (TH 1-5 or cSALF) or deionized-distilled water for 1 h, was used to infect GF-1 cells, and the levels of capsid protein in the GNNV-AMP-infected cells at 1 h post infection were much lower than that in the GNNV-H_2O-infected cells, indicating that only a small portion of viral particles in the GNNV-AMP mixture could successfully infected the cells. Treatment of cBB cells with TH 1-5 and cSALF did not induce Mx gene expression; however, grouper epinecidin-1 (CP643-1) could induce the expression of Mx in the pre-treated cBB cells. This study revealed three AMPs with anti-NNV activity through two different mechanisms, and shed light on the future application in aquaculture.

1. Introduction

Coexisting with diverse pathogens in nature, living organisms have evolved various strategies to combat against them. Despite species variation, antimicrobial peptides, a robust weapon in innate immunity, were widely found in plants and animals. To date, about 1500 AMPs have been identified [1]. In addition to the AMPs reported in insects and mammals, for example, cecropins and defensins, more AMPs were found in marine teleosts, such as epinecidin in groupers [2] and pleurocidin in flounders [3]. AMPs reports in marine fish have been published, including hepcidin [4], misgurain [5], pardaxin [6], parasin [7], and LEAP-2 [8]. AMPs can be constitutively expressed or induced upon pathogen invasion [9]. These small peptides, usually ranging from 12 to 50 amino acids, are mostly cationic, amphipathic, small peptides with low molecular weight ranging approximately from 1 to 5 kDa, although some AMPs consist of anionic peptides [10]. These AMPs possess broad-spectrum antimicrobial abilities against Gram-positive or -negative bacteria, fungi, parasites and viruses. Apart from interacting directly with pathogens and lead to membrane disruption via interaction with certain bacterial components [11], AMPs also exert their effects by modulating the adaptive immune response [12] or acting as chemokines to recruit other effector cells [13]. Many reports have indicated their antiviral activities, for example, defensins can inhibit infection of enveloped viruses including HIV-1 [14], influenza A virus [15], herpes simplex virus [16], as well as non-enveloped viruses such as human adenovirus (HAdV) [17] and human pappilomavirus [18]. However, little is known about if any AMP exerts its antiviral activity against NNV – the pathogen that primarily causes mass mortality of many marine cultured fish species.

NNV is a non-enveloped virion with diameter of 25–34 nm and two segments of single-stranded RNA genomes, and is classified as β-nodavirus (or fish nodavirus) of *Nodaviridae*. The RNA-dependent RNA polymerase (RdRp) of NNV is translated from RNA1, and its capsid protein is translated from the RNA2. A subgenomic RNA (RNA3) transcribed from the 3′ end of RNA1 was found to encode B2 protein, which is able to antagonize cellular RNA interference [19,20]. Clinical signs of NNV-infected fish include abnormal swimming behavior, darkened body coloration, and vacuolation of brain and retina [21]. Studies against NNV were focused on the development of

* Corresponding author. Fax: +886 39883197.
** Corresponding author at: Institute of Zoology, National Taiwan University, Taipei 106, Taiwan. Tel.: +886 2 33662505; fax: +886 2 23673852.
E-mail addresses: zoocjy@gate.sinica.edu.tw (J.-Y. Chen), shauchi@ntu.edu.tw (S.-C. Chi).

1050-4648/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2009.11.020

vaccine, immuno-stimulants and other means [22,23]. However, ever since the first report on the antiviral activities of cecropin B against infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), snakehead rhabdovirus (SHRV), and infectious pancreatic necrosis virus (IPNV) [24], more emerging studies are highlighting the benefits of AMP application on disease control, for example, not eliciting resistance in bacteria, compared with conventional antibiotic therapeutics. In this study, we examined the antiviral activities of three AMPs against NNV in fish cells, and elucidated their anti-NNV mechanisms *in vitro*.

2. Material and methods

2.1. Cells, viruses and AMPs

Grouper fin-1 (GF-1) [25] and cured barramundi brain (cBB) [26] cell lines were grown at 28 °C in Leibovitz's L-15 medium (GIBCO) separately supplemented with 5% and 10% foetal bovine serum (FBS). The NNV strain, G9508KS, isolated from grouper larvae infected with NNV [27], was propagated on GF-1 cells, and was titrated in GF-1, using L-15 medium supplemented with 1% FBS.

Three AMPs, including grouper epinecidin-1 (CP643-1) (Accession number AY294407) [28], tilapia hepcidin 1-5 (TH 1-5) [4], and cyclic shrimp anti-lipopolysaccharide factor (cSALF) [29], were gifts from Dr. J.Y. Chen. Each AMP was dissolved in sterilized deionized-distilled water (ddH_2O) to the final concentration 1000 μg ml^{-1}, and stored at −80 °C until use.

2.2. Determination of the maximal non-cytotoxic concentration of AMPs to GF-1 and cBB cells

Each AMP was subjected to serial 2-fold dilution with L15 medium supplemented with 1% or 2% FBS, and each dilution was applied on GF-1 or cBB cells pre-seeded in 96-well plates. After 6 days of incubation, cell morphology was examined, and the maximal non-cytotoxic concentration of each AMP to GF-1 or cBB cells was determined and used for the following antiviral activity assay.

2.3. Antiviral activity assay

NNV was pre-treated with each AMP in the presence or absence of 1% FBS and titrated in the GF-1 cells to determine the effects of FBS on the antiviral activities of three AMPs. NNV (10^{10} $TCID_{50}$ ml^{-1}) was 100-fold diluted in L15 medium with or without 1% FBS, and the diluted NNV (10^8 $TCID_{50}$ ml^{-1}) solutions were separately treated with the same volume of AMP (1000 μg ml^{-1}) at 28 °C for 24 h. The AMP-treated NNV solution was then diluted to the maximal non-cytotoxic concentration of each AMP, and titrated in GF-1 cells.

To test the sole effect of AMP on GF-1 cells, the cells in 96-well plate were treated with AMP for 24 h, and then infected with 10-fold serial diluted NNV after removing AMP.

During viral titration, cells were incubated with L-15 medium containing 1% FBS. Viral titres were recorded on the 6th day post infection (dpi). The neutralization activity of AMP against NNV was expressed as logarithm of the neutralization index (NI = viral titre without AMP treatment/viral titre with AMP treatment). Neutralization activity is considered valid when log NI value is above 1.7 [30].

2.4. Dose-dependent effect of TH 1-5 and cSALF

To examine if the antiviral activity of TH-1 and cSALF is dose-dependent, NNV (10^{10} $TCID_{50}$ ml^{-1}) was separately pre-treated with 250, 500 and 1000 μg ml^{-1} of TH 1-5 or cSALF with a volume ratio of 1:1 at 28 °C for 24 h. The AMP-NNV mixtures were 100-fold diluted to bring down the AMP concentration to a level that would be non-toxic to GF-1 cells in each set. The 100-fold diluted AMP-NNV mixtures and the 100-fold diluted NNV solution were then titrated in GF-1 cell. The log NI values were determined 6 dpi. The experiments were performed in triplicate.

2.5. The effect of AMP on the adsorption and entry of NNV to GF-1 cells

The NNV (10^{10} $TCID_{50}$ ml^{-1}) was pre-treated with TH 1-5 or cSALF (1000 μg ml^{-1}) or ddH_2O with a volume ratio of 1:1 at 28 °C for 24 h, and applied for viral adsorbing on GF-1 cells for 1 h prior to western blot analysis. Thereafter, the NNV-infected GF-1 cells were washed with PBS for 3 times, and the harvested cells were lysed in the buffer containing 1% NP-40, 50 mM Tris (pH 8.0), 1 mM DTT and 1× protease inhibitor (Roche). The cell lysate was laid on ice for 30 min, and cleared by centrifugation at 12,000 × *g* at 4 °C for 15 min. The protein concentration of the samples were adjusted to 10 μg, and applied for 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Following SDS-PAGE, proteins were eletrophoretically transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% skim milk in TBS buffer containing 0.1% Tween 20 for 1 h at room temperature, and reacted with purified antibodies against NNV capsid protein. After TBS buffer washing, the membrane was reacted with peroxidase-labeled goat anti-rabbit IgG (KPL), and the signals were developed by LumiGLO chemiluminescent substrate (KPL), and visualized by autoradiography. The internal control, actin, was reacted with anti-actin mAb (CHEMICON), and then with alkaline-phosphatase (AP)-conjugated goat anti-mouse IgG antibody (KPL). The colour of protein bands was developed by reaction with BCIP/NBT substrate Kit (KPL).

2.6. Purification of NNV

GF-1 cells were infected with NNV (MOI = 1). After 6 days, NNV-infected cells with the culture medium were collected and centrifuged at 8000 × *g* for 20 min at 4 °C. The cell pellet was resuspended in TET buffer (100 mM Tris HCl, 1 mM EDTA, 0.1% Triton X-100, pH = 7.2), and then centrifuged at 8000 × *g* for 20 min at 4 °C. The supernatants derived from the first and the second centrifugations were mixed, laid over 20% sucrose, and centrifuged at 100,000 × *g* for 3 h at 4 °C. All the sediments were resuspended in 2 ml of TET buffer at 4 °C overnight, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The viral stocks were layered on 36% CsCl, and centrifuged at 157,000 × *g* for 20 h at 16 °C. The visible virus band was collected and dialyzed with PBS at 4 °C for overnight. The dialyzed NNV was then stored at −80 °C.

2.7. Electron microscopy

The purified NNV (9.3 mg ml^{-1}) was treated with the same volume of TH 1-5, cSALF, Hep 2-2 (1000 μg ml^{-1}) or sterilized ddH_2O, and incubated at 28 °C for 24 h. Each mixture was added on the grid, stained with 1% phosphotungstic acid (PTA) and observed under TEM (HITACHI, H-7650, Japan).

2.8. Detection of NNV in the supernatant and the agglutinates of AMP-NNV mixture

To examine if the NNV virions were agglutinated by AMP treatment, NNV (10^{10} $TCID_{50}$ ml^{-1}) was treated with TH 1-5 or cSALF (1000 μg ml^{-1}) with a volume ratio of 1:1 at 28 °C for 24 h. The supernatants and agglutinates were further separated by centrifugation at 12,000 × *g* at 4 °C for 10 min. The supernatant and the insoluble agglutinates which were resuspended in 8 M urea were analyzed by western blot using anti-NNV polyclonal antibodies.

2.9. The effect of salinity on the anti-NNV activity of TH 1-5- or cSALF

To demonstrate the effect of increasing salt concentration on the solubility and the titre of AMP-treated viruses, NNV ($10^{8.5}$ $TCID_{50}$ ml^{-1}) was treated with TH 1-5 or cSALF (1000 μg ml^{-1}) in L-15 medium (1% FBS) supplemented with 0, 150, 350 and 750 mM NaCl at 28 °C for 24 h. The AMP-NNV mixtures were 100-fold diluted to bring down the AMP concentration to a non-toxic level for GF-1 cells. The 100-fold diluted AMP-NNV mixtures and the 100-fold diluted NNV solutions were then titrated in GF-1 cell, and the log NI values were determined 6 dpi.

2.10. Detection of Mx gene expression by RT-PCR

To elucidate if AMPs can induce Mx gene expression and modulate immune system in cBB cells, three AMPs (epinecidin, TH 1-5 and cSALF) were separately applied to cBB cells at non-cytotoxic concentrations, and incubated at 28 °C for 24 h. NNV-infected (MOI = 10), and poly I:C-treated cBB cells were served as positive controls. The extraction method of cellular RNA and the RT-PCR program for detecting Mx RNA were accorded to the published paper [26]. The forward primer sequence of Mx gene is (5′-CACCATGAACACCCTGAACC-3′) and the reverse primer sequence is (5′-CTTCTGACCCCTGCACCTGACGA-3′). Actin served as an internal control, and the primer sequences for actin gene include forward primer (5′-CACTCAACCCCAAAGCCAACAGG-3′) and reverse primer (5′-AAAGTCCAGCGCCACGTAGCACAG-3′) [31].

3. Results

3.1. The maximal non-cytotoxic concentration of three AMPs in GF-1 and cBB cells

In GF-1 cells, the maximal non-cytotoxic concentration was determined to be 4 μg ml^{-1} for epinecidin (CP643-1), 16 μg ml^{-1} for tilapia hepcidin (TH 1-5) and cyclic shrimp anti-lipopolysaccharide factor (cSALF), In cBB cells, the maximal non-cytotoxic concentration was revealed to be 8 μg ml^{-1} for CP643-1, 64 μg ml^{-1} for TH 1-5, and 32 μg ml^{-1} for cSALF.

3.2. FBS affected the antiviral activity of some AMPs

The effects of serum supplement (1% FBS) on the antiviral activities of 24-h treated AMPs against NNV at 28 °C were examined, and only TH 1-5 and cSALF showed antiviral activities against NNV (Fig. 1). When FBS was absent in the medium, the antiviral activity of TH 1-5 was significantly increased, while the changes in the antiviral activity of cSALF was negligible in the absence of FBS. However, the log NI of epinecidin either in the presence or absence of FBS was all below 1.7, indicating that treatment of epinecidin on NNV could not block the infectivity of the virions.

In addition, no difference was found among the NNV titres determined in the GF-1 cells, with or without the pretreatment of AMPs, indicating that TH 1-5, cSALF or epinecidin would not block the viral receptor of GF-1 cells.

3.3. TH 1-5 and cSALF inhibited NNV infection in a dose-dependent manner

To confirm if TH 1-5 and cSALF inhibit NNV infectivity in a dose-dependent manner, the concentrations of the two AMPs were adjusted to 250, 500 and 1000 μg ml^{-1}. The antiviral activities of both peptides increased with rising concentrations (Fig. 2). However, unlike TH 1-5, which was effective against NNV (10^{10} $TCID_{50}$ ml^{-1}) at all concentrations, cSALF exhibited valid antiviral activity only when the concentration was 1000 μg ml^{-1} (log NI > 1.7). The statistical analysis revealed that the difference between 500 and 1000 μg ml^{-1} of cSALF is significant ($p < 0.01$).

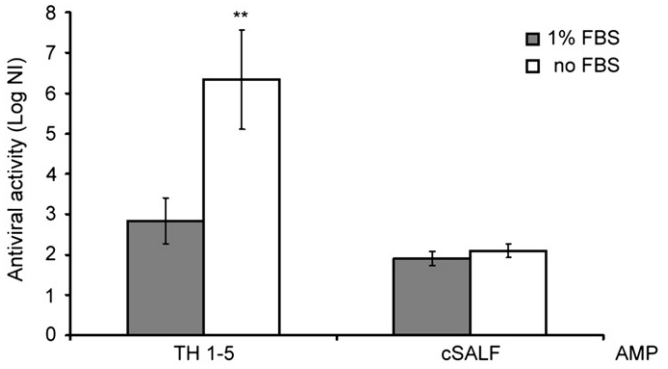

Fig. 1. The impact of FBS on the antiviral activities of TH 1-5 and cSALF was different. NNV in L-15 medium with or without 1% FBS, treated with TH 1-5 or cSALF at 28 °C for 24 h, and then titrated in GF-1 cells. Log NI values were determined 6 dpi. Each value is the mean ± SD of three independent experiments conducted in triplicates. **, $p < 0.01$.

3.4. TH 1-5 or cSALF treatment reduced the adsorption level of NNV to GF-1 cells

To investigate if TH 1-5 and cSALF directly act on NNV virions and hamper their adsorption or entry into GF-1 cells, western blot was applied to examine the level of NNV capsid protein in GF-1 cells 1 h after the infection by AMP-treated NNV. The results indicated that the levels of viral capsid protein in GF-1 cells infected by AMP-treated NNV were much lower than that in control cells (Fig. 3), suggesting that TH 1-5 and cSALF might inhibit viral particles adherence or penetration into host cells.

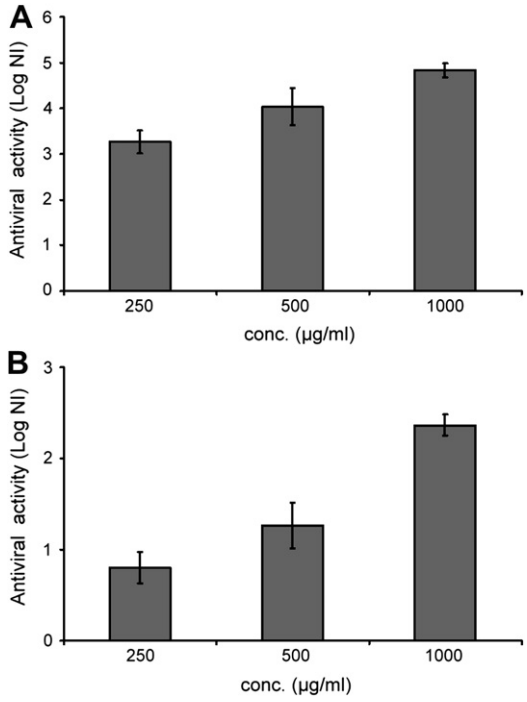

Fig. 2. The antiviral activity of TH 1-5 and cSALF against NNV was dose-dependent. NNV was respectively treated with 250, 500 and 1000 μg ml^{-1} of (A) TH 1-5 and (B) cSALF at 28 °C for 24 h, and then titrated on GF-1 cells. Log NI values were determined 6 dpi. Each value is the mean ± SD of three independent experiments conducted in triplicates.

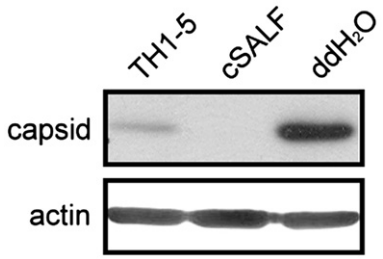

Fig. 3. TH 1-5 and cSLAF inhibited the binding of NNV and its entry into GF-1 cells. NNV (10^{10} $TCID_{50}$ ml^{-1}) was treated with TH 1-5, cSALF or ddH_2O at 28 °C for 24 h, and then inoculated on GF-1 cells for 1 h. The cells were washed with PBS for three times and harvested for western blot analysis.

3.5. TH 1-5 and cSALF inhibited NNV infection by agglutinating virions

The antiviral mechanism of TH-1 and cSALF was examined in this study. When NNV derived from the culture supernatant of infected cells was treated with TH-1 or cSALF for 24 h, the sediment was observed in the mixture. To confirm that the sediment was not cellular or medium components, purified NNV (pNNV) was used for AMP treatment. The sediment was observed once again in TH 1-5- or cSALF-treated pNNV, but not in non-treated pNNV, ddH_2O-treated NNV, nor in the mixture of pNNV and Hep 2-2 which did not exhibit anti-NNV activity. The mixtures of each treatment were further examined under TEM, and viral clumps were observed in TH 1-5 or cSALF-treated pNNV (Fig. 4), indicating that TH 1-5 or cSALF could agglutinate virions. On the other hand, free virions were observed in pNNV, ddH_2O-treated pNNV and epinecidin-treated pNNV, indicating that epinecidin could not agglutinate viral particles.

After the centrifugation of the AMP-NNV mixture, the supernatant and the pellet were separately examined by western blot, using NNV capsid protein-specific antibodies. Viral protein was detected only in the pellets, instead of supernatant of TH 1-5 or cSALF-treated NNV (Fig. 5A). On the contrary, the majority of viral protein resided in the supernatant of ddH_2O-treated NNV (Fig. 5B). This result reconfirmed that TH 1-5 and cSALF inhibited NNV infection through agglutinating viral particles into clump, interfering the viral adsorption and entry of cells.

3.6. The increasing salt concentration did not change the solubility and viral titre of AMP-treated NNV

To examine whether the agglutination of AMP-treated NNV was due to the electrostatic forces between AMP and NNV virions, AMP-treated NNV solution was supplemented with high concentrations of salt which was used to disrupt electrostatic forces and abrogate the agglutination of AMP-treated virions. However, after the supplement of high salinity, neither the agglutinates in AMP-NNV mixtures disappeared (data not shown), nor the viral titre of AMP-treated NNV restored (Table 1). This might imply other mechanism, besides electrostatic forces, may be involved in the binding between TH 1-5, cSALF and NNV.

3.7. Grouper epinecidin-1 could induce Mx gene expression in cBB cells

Mx gene expression can be induced in cBB cells by NNV infection or poly I:C transfection [26]. In this study, cBB cells were pre-treated with TH1-5, cSALF and grouper epinecidin-1 (CP643-1) for 24 h, and Mx gene expression was examined by RT-PCR. The results are demonstrated in Fig. 6. Minor Mx gene expression was found in the cBB cells treated with grouper epinecidin-1 but not TH 1-5 or cSALF, suggesting that the anti-NNV mechanism of grouper epinecidin-1 was different from that of TH 1-5 and cSALF.

4. Discussion

As a vital member in innate immunity, AMPs have a broad-spectrum of antimicrobial abilities including antiviral activity. After examining three AMPs in the present study, we found that tilapia hepcidin 1-5 (TH 1-5) and cSALF exhibited noticeable antiviral activity *in vitro* by agglutinating NNV virions into clump and preventing viral entry into cells, instead of modulating immune response of infected cells. However, grouper epinecidin-1 was the only AMP in this study to induce minor Mx gene expression response in the treated cBB cells. Therefore, the anti-NNV mechanism of TH 1-5 and cSALF was different from that of grouper epinecidin-1.

Hepcidin is an AMP first isolated from human blood [32] and urine [33], mainly expressed in liver with dual roles, namely as an antibacterial host peptide and iron homeostasis regulator [34].

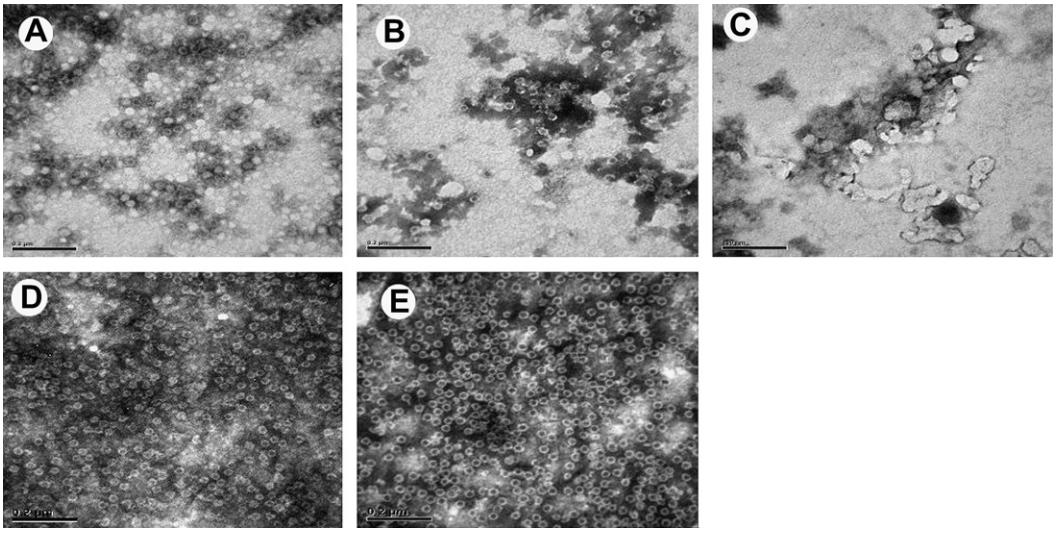

Fig. 4. TEM observation of NNV aggregation induced by TH 1-5 and cSALF. Purified NNV were separately mixed with TH 1-5, cSALF, epinecidin or sterilized ddH_2O at 28 °C for 24 h. The mixtures were then negatively stained and examined by TEM. A, purified NNV; B, TH 1-5-treated NNV; C, cSALF-treated NNV; D, Hep 2-2-treated NNV; E, ddH_2O-treated NNV. (bar = 0.2 μm).

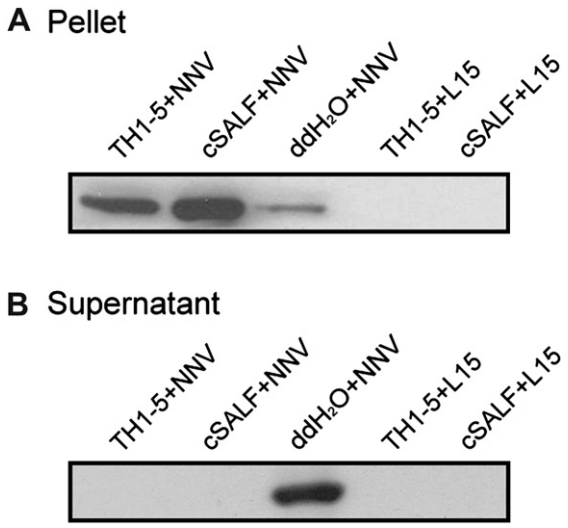

Fig. 5. Western blot analysis of NNV capsid proteins agglutinated by TH 1-5 and cSALF. NNV was treated with TH 1-5 or cSALF for 24 h, and then centrifuged. The pellets (A) and the supernatants (B) were analyzed by western blot using NNV-specific antibodies.

In fish, hepcidins were obtained by expressed sequence tags (EST) from more than 20 species [35]. Hepcidins are highly conserved among fish and human in their genome similarity and secondary structure [36]. Moreover, they were both found in the liver of bass and human, suggesting their similar physiological functions. Hepcidin has been reported to be induced and expressed as an acute phase protein upon bacterial and viral infection [37]. In our study, tilapia hepcidin 1-5 (TH 1-5) (GIKCRFCCGCCTPGICGVCCRF) [4], apart from its antibacterial function reported previously [34], exhibited anti-NNV activity *in vitro,* either in serum-present or -absent medium. Nevertheless, the log NI value of TH 1-5 decreased when serum was present, as opposed to when it was absent, indicating that serum to be a critical factor for the antiviral activity of TH 1-5. It is still unknown which component(s) in the serum would interact with a certain domain of TH 1-5 and perturbs its ability to agglutinate NNV. However, the impact of serum was less important on another AMP, cyclic shrimp (*Penaeus monodon*) anti-lipopolysaccharide factor cSALF, while exerting its anti-NNV activity.

The amino acid sequence of cSALF is ECKFTVKPYLKRFQ-VYYKGRMWCP [29]. It exhibits antimicrobial ability against both Gram-positive and -negative bacteria and is able to form an amphipathic hairpin loop in the region of 20–21 amino acids in helical length, which can form an LPS-binding motif, or disrupt viral functions at the phospholipid membrane bilayer [29]. However, NNV is non-enveloped, and the anti-NNV mechanism of cSALF was achieved by agglutinating NNV capsid protein, similar to that of TH 1-5, and the agglutination was irreversible, indicating that the two peptides might change NNV virion or capsid protein from hydrophilic to hydrophobic, causing the formation of aggregates. Some studies have reported increased salt concentrations abrogate the binding abilities of cationic peptides to viral proteins due to the interference of

Table 1
Increasing salt supplement did not restore the viral titre of NNV treated with TH 1-5 or cSALF at 28 °C for 24 h. The original titre of NNV was $10^{8.5}$ $TCID_{50}$ ml^{-1}.

AMP	NaCl			
	0 mM	150 mM	350 mM	750 mM
TH 1-5	5.5[a]	6.5	6.0	5.7
cSALF	6.7	6.5	6.3	6.0

[a] Viral titre (Log $TCID_{50}$ ml^{-1}).

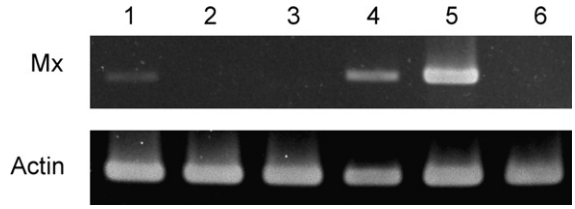

Fig. 6. Detection of Mx gene expression in cBB cells after the treatment of three AMPs. 1-3, cBB cells separately treated with epinecidin, TH1-5 and cSALF; 4, NNV-infected cBB cells; 5, poly I:C-transfected cBB cells; 6, cBB cells without any treatment.

electrostatic forces between them [38,39]. Since the antiviral mechanism of TH 1-5 and cSALF is achieved via agglutination, if increasing salt could abrogate the binding between them, it would reflect on the viral titre of AMP-treated NNV. However, NNV titres were not restored after the supplement of high salinity in the AMP-treated NNV mixtures, indicating that the agglutination of NNV through the treatment of TH 1-5- and cSALF was not due to the electrostatic forces or the binding between AMP and NNV.

Because the antiviral mechanism of TH 1-5 and cSALF is to agglutinate NNV virions and reduce the level of free virions to infect the cells, they might be exploited as pretreatment agents for VNN disease control. The reported antiviral mechanisms of other AMPs are diversified. For example, cecropin B is demonstrated to inhibit IHNV, VHSV, SHRV and IPNV by direct disruption of viral envelope and disintegration of viral capsids [24]; human α-defensins HD5 inhibits BK virus, by agglutinating its virions and forming aggregates [39]; some defensins are shown to act like lectins, binding to both HIV glycoprotein gp120 and cell surface receptor CD4, resulting in the inhibition of infection [40]; however, a member of α-defensins, human neutrophil peptide 1 (HNP1), is proved to up-regulate type I interferon response gene of trout head kidney leukocytes [41].

The mice transfected with human β-defensin gene, show higher antibacterial activity [42], and the administration of cSALF into mice liver and spleen elevates the expression of certain cytokine genes, including tumor necrosis factor (TNF) and Toll-like receptor 4 (TLR4) [29]. Therefore, transfection of certain AMPs into appropriate cell lines might be an approach to evaluate the potential of gene therapy.

Wang et al. (2009) [43] have demonstrated that coincubation of 10 μl of TH 1-5 (1000 μg ml^{-1}) or epinecidin-1 (100 μg ml^{-1}) with 10 μl of NNV (10^9 $TCID_{50}$ ml^{-1}) for 10 min before injection of grouper larvae (body weight: 0.7 g), can increase 5-fold or 2-fold survival rate compared to the control fish which were injected with the same volume of non-treated NNV. Moreover, the re-challenge of the AMP-NNV-injected fish with NNV after 30 days show a higher survival rate, suggesting that these two AMPs might be applicable to the preparation of attenuated NNV vaccine. The present study highlights the antiviral activities and anti-NNV mechanism of TH 1-5 and cSALF. Better understanding of antiviral activity of AMPs and their biological actions will be beneficial to their future possible application in the aquaculture practice.

Acknowledgement

This study is supported in part by a grant from the National Scientific Council (NSC 95-2313-B-002-042-MY3). The authors appreciate Mr. C.-J. Cheng for reviewing this manuscript.

References

[1] Wang G, Li X, Wang Z. APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 2009;37:933–7.
[2] Yin ZX, Wei H, Chen WJ, Yan JH, Yang JN, Chan SM, et al. Cloning, expression and antimicrobial activity of an antimicrobial peptide, epinecidin-1, from the orange-spotted grouper, *Epinephelus coioides*. Aquaculture 2006;31:204–11.

[3] Cole AM, Weis P, Diamond G. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J Biol Chem 1997;272:12008–13.
[4] Huang PH, Chen JY, Kuo CM. Three different hepcidins from tilapia, *Oreochromis mossambicus*: analysis of their expressions and biological functions. Mol Immunol 2007;44:1922–34.
[5] Park CB, Lee JH, Park IY, Kim MS, Kim SC. A novel antimicrobial peptide from the loach, Misgurnus anguillicaudatus. FEBS Lett 1997;411:173–8.
[6] Primor N, Tu AT. Conformation of pardaxin, the toxin of the flatfish *Pardachirus marmoratus*. Biochem Biophys Acta 1980;626:299–306.
[7] Park IY, Park CB, Kim MS, Kim SC. Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, Parasilurus asotus. FEBS Lett 1998;437:258–62.
[8] Zhang YA, Zou J, Chang CI, Secombes CJ. Discovery and characterization of two types of liver-expressed antimicrobial peptides 2 (LEAP-2) genes in rainbow trout. Vet Immunol Immunopathol 2004;101:259–69.
[9] Sarmaşik A. Antimicrobial peptides: a potential therapeutic alternative for the treatment of fish diseases. Turk J Biol 2002;26:201–7.
[10] Brogden KA, Ackermann M, McCray PB, Tack BF. Antimicrobial peptides in animals and their role in host defenses. Int J Antimicrob Agents 2003;22: 465–78.
[11] Hancock REW, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. FEMS Microbiol Lett 2002;206:143–9.
[12] Oppenheim JJ, Biragyn A, Kwak LW, Yang D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. Ann Rheum Dis 2003;62 (Suppl. 2):17–21.
[13] Chertov O, Michiel DF, Xu L, Wang JM, Tani K, Murphy WJ, et al. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. J Biol Chem 1996;271:2935–40.
[14] Chang TL, Vargas Jr J, Delportillo A, Klotman ME. Dual role of alpha-defensin-1 in anti-HIV-1 innate immunity. J Clin Invest 2005;115:765–73.
[15] Hartshorn KL, White MR, Tecle T, Holmskov U, Crouch E. Innate defense against influenza A virus: activity of human neutrophil defensins and interactions of defensins with surfactant protein D. J Immunol 2006;176:6962–72.
[16] Hazrati E, Galen B, Lu W, Wang W, Ouyang Y, Keller MJ, et al. Human alpha- and beta-defensins block multiple steps in herpes simplex virus infection. J Immunol 2006;177:8658–66.
[17] Bastian A, Schafer H. Human alpha-defensin 1 (HNP-1) inhibits adenoviral infection *in vitro*. Regul Pept 2001;101:157–61.
[18] Buck CB, Day PM, Thompson CD, Lubkowski J, Lu W, Lowy DR, et al. Human alpha-defensins block papillomavirus infection. Proc Natl Acad Sci USA 2006;103:1516–21.
[19] Sommerset I, Nerland AH. Complete sequence of RNA1 and subgenomic RNA3 of Atlantic halibut nodavirus (AHNV). Dis Aquat Organ 2004;58:117–25.
[20] Fenner BJ, Thiagarajan R, Chua HK, Kwang J. Betanodavirus B2 is a RNA interference antagonist that facilitates intracellular viral RNA accumulation. J Virol 2006;80:85–94.
[21] Yoshikoshi K, Inoue K. Viral nervous necrosis in hatchery-reared larvae and juveniles of Japanese parrotfish, *Oplegnathus fasciatus* (Temminck & Schlegel). J Fish Dis 1990;13:69–77.
[22] Tanaka S, Mori K, Arimoto M, Iwamoto T, Nakai T. Protective immunity of sevenband grouper, *Epinephelus septemfasciatus* Thunberg, against experimental viral nervous necrosis. J Fish Dis 2001;24:15–22.
[23] Kai YH, Chi SC. Efficacies of inactivated vaccines against betanodavirus in grouper larvae (*Epinephelus coioides*) by bath immunization. Vaccine 2008;26:1450–7.
[24] Chiou PP, Lin CM, Perez L, Chen TT. Effect of cecropin B and a synthetic analogue on propagation of fish virus in vitro. Mar Biotechnol 2002;4:294–302.
[25] Chi SC, Hu WW, Lo BJ. Establishment and characterization of a continuous cell line (GF-1) derived from grouper, *Epinephelus coicoides* (Hamilton): a cell line susceptible to grouper nervous necrosis virus (GNNV). J Fish Dis 1999;22:173–82.
[26] Wu YC, Chi SC. Persistence of betanodavirus in Barramundi brain (BB) cell line involves the induction of Interferon response. Fish Shellfish Immunol 2006; 21:540–7.
[27] Chi SC, Lo BJ, Lin SC. Characterization of grouper nervous necrosis virus (GNNV). J Fish Dis 2001;24:3–13.
[28] Pan CY, Chen JY, Cheng YSE, Chen CY, Ni IH, Sheen JF, et al. Gene expression and localization of the epinecidin-1 antimicrobial peptide in the grouper (*Epinephelus coioides*) and its role in protecting fish against pathogenic infection. DNA Cell Biol 2007;26:403–13.
[29] Pan CY, Chao TT, Chen JC, Chen JY, Liu WC, Lin CH, et al. Shrimp (*Penaeus monodon*) anti-lipopolysaccharide factor reduces the lethality of *Pseudomonas aeruginosa* sepsis in mice. Intern Immunophar 2007;7:687–700.
[30] Mahy BWJ, Kangro HO. Neutralization. In: Mahy BWJ, Kangro HO, editors. Virology method manual. London: Academic Press; 1996. p. 108–9.
[31] Larsen R, Rokenes TP, Robertsen B. Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein. J Virol 2004;78:7938–44.
[32] Krause A, Neitz S, Magert HJ, Schluz A, Forssmann WG, Schluz-Knappe P, et al. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett 2000;480:147–50.
[33] Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in liver. J Biol Chem 2001;276:7806–10.
[34] Verga Falzacappa MV, Muckenthaler MU. Hepcidin: iron-hormone and antimicrobial peptide. Gene 2005;364:37–44.
[35] Shi J, Camus AC. Hepcidins in amphibians and fishes: antimicrobial peptides or iron-regulatory hormones? Dev Comp Immunol 2006;30:746–55.
[36] Lauth X, Babon JJ, Stannard JA, Singh S, Nizet V, Carlberg JM, et al. Bass hepcidin synthesis, solution structure, antimicrobial activities and synergism, and in vivo hepatic response to bacterial infections. J Biol Chem 2005;280:9272–82.
[37] Nicolas G, Bennoun M, Porteu A, Mativet S, Beaumont C, Grandchamp B, et al. Severe iron deficiency anemia in transgenic mice expressing liver hepcidin. Proc Natl Acad Sci USA 2002;99:4596–601.
[38] Smith JG, Nemerow GR. Mechanisms of adenovirus neutralization by human alpha-defensins. Cell Host Microbe 2008;3:11–9.
[39] Dugan AS, Maginnis MS, Jordan JA, Gasparovic ML, Manley K, Page R, et al. Human α-defensins inhibit BK virus infection by aggregating virions and blocking binding to host cells. J Biol Chem 2008;283:31125–32.
[40] Furci L, Sironi F, Tolazzi M, Vassena L, Lusso P. Alpha-defensins block the early steps of HIV-1 infection: interference with the binding of gp120 to CD4. Blood 2007;109:2928–35.
[41] Falco A, Mas V, Tafalla C, Perez L, Coll JM, Estepa A. Dual antiviral activity of human alpha-defensin-1 against viral haemorrhagic septicaemia rhabdovirus (VHSV): inactivation of virus particles and induction of a type I interferon-related response. Antiviral Res 2007;76:111–23.
[42] Huang GT, Zhang HB, Kim D, Liu L, Ganz T. A model for antimicrobial gene therapy: demonstration of human β-defensin 2 antimicrobial activities in vivo. Hum Gene Ther 2002;13:2017–25.
[43] Wang YD, Kung CW, Chi SC, Chen JY. Inactivation of nervous necrosis virus infecting grouper (*Epinephelus coioides*) by epinecidin-1 and hepcidin 1–5 antimicrobial peptides, and downregulation of Mx2 and Mx3 gene expressions. Fish Shellfish Immunol 2009;28:113–20.