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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

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ABSTRACT

Betanodaviruses are one of the serious pathogens in nervous necrosis viral (NNV) disease that brings about mortality in the larval stage of grouper (*Epinephelus coioides*). In this study, the efficacy of pretreatment, co-treatment, and posttreatment with the antimicrobial epinecidin-1 and hepcidin 1–5 peptides against a betanodavirus was evaluated by intraperitoneal inoculation in grouper. The results showed that co-treatment of epinecidin-1 or hepcidin 1–5 with the virus was effective in promoting a significant decrease in grouper mortality. Re-challenge with virus again after 30 day in co-treated grouper groups showed high survival suggesting that epinecidin-1 and hepcidin 1–5 enhanced fish survival. However, grouper inoculated with NNV and then inoculated with epinecidin-1 8 h later showed significantly different survival from the group inoculated with virus alone, suggesting that epinecidin-1 can be used as a drug to rescue infected grouper. Infection after pretreatment, co-treatment, and posttreatment with epinecidin-1 or hepcidin 1–5 was verified by RT-PCR which showed downregulation of Mx2 and Mx3 gene expressions. All these data strongly suggest that epinecidin-1 and hepcidin 1–5 are effective peptides for protecting grouper larvae by reducing NNV infection.

1. Introduction

Orange-spotted grouper, *Epinephelus coioides*, is a high-value aquaculture species in Taiwan and other countries. The annual market for grouper larvae is worth more than US$300 million. However, there is a critical phase in grouper rearing between weaning and metamorphosis when the larvae are vulnerable to viral infection and insufficient nutrient ingestion [1,2]. In recent years, mass mortalities of hatchery-reared grouper larvae and juveniles in Taiwan and other countries have frequently occurred due to nervous necrosis viral (NNV) disease, and these have produced severe economic impacts on the aquaculture industry. Various chemicals [3], vaccines [4], broodstock detection, and ozone sterilization of water and eggs [5,6] have been reported to inhibit the vertical and horizontal transmission of NNV. However, most of the available antiviral drugs often lead to the development of viral resistance which is coupled with problems of side effects, recurrence, and viral latency.

Recent findings in the field of innate immunity are shedding new light on the importance of host defence antimicrobial peptides (AMPs). Recently, more than 1200 AMPs from different origins were identified or predicted (http://aps.unmc.edu/AP/main.php). Most of these peptides maintain definite general characteristics, such as a positive charge and an amphipathic structure [7,8]. The amphipathic and cationic characteristics are important for their antimicrobial ability to bind to anionic microbial surfaces such as lipopolysaccharide (LPS). Although an important function of AMPs is their capacity to directly kill or inhibit the growth of microbes, other studies support the role of AMPs not only as antibacterial effectors of the innate immune response but also as antiviral peptides [9]. For instance, α-defensins exhibit activity in the gut and in granulocytes of being able to inactivate certain enveloped viruses (such as the human immunodeficiency virus) [10]. Daher et al. [11] presented results of the ability of human neutrophil peptide (HNP)-1 to directly inactivate the herpes simplex virus (HSV) and other enveloped viruses, including the influenza A virus. Those results suggested that this might be due to this AMP's ability to destabilize viral envelopes. The activity of defensins as antivirals involves mechanical membrane disruption [12] and also inhibition of influenza viral replication by a cell-mediated mechanism [13].

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To the present, there are no publications on AMPs derived from fish sources with antiviral ability determined in *in vivo* tests (in fish). Thus, one interesting result demonstrated the *in vivo* immunomodulatory capacity of human alpha defensin 1 in the rainbow trout immune system as a first approach to evaluating the possible use of human alpha defensin 1 to increase fish resistance by enhancing non-specific defence mechanisms [14,15]. *In vitro* characterization of native cecropin B and a synthetic analogue, CF17, against several important fish viral pathogens by the methods of co-incubation of these peptides and viruses caused reductions in viral titers yielded in fish cells from several fold to 104-fold. These results suggested inhibition of viral replication by the peptides by direct disruption of the viral envelope and disintegration of the viral capsids [16,17]. Fish nodaviruses are icosahedral, naked, non-enveloped, single-stranded RNA viruses of 25–30 nm which cause up to 100% mortality in fry and fingerling stages of grouper. To understand this vexing problem, the antiviral abilities of aquatic AMPs must first be examined.

So far, several AMPs have been reported in fish, including epinecidin-1 [18], hepcidin [19], misgurin [20], pardaxin [21], parasin I [22], pleurocidin [23], LEAP-2 [24], etc. In our laboratory, we cloned epinecidin-1 from grouper (*E. coioides*) [18] and hepcidin 1–5 from tilapia (*Oreochromis mossambicus*) [19] using molecular approaches. Synthetic peptides derived from these sources are able to destroy bacterial membranes according to scanning electron microscopic (SEM) and transmission electron microscopic (TEM) observations, suggesting that the abilities of epinecidin-1 and hepcidin 1–5 may be much broader than previously assumed. In order to explore potential applications of epinecidin-1 and hepcidin 1–5 as therapeutic agents and/or vaccines for grouper, in the present study, we evaluated the ability of synthetic epinecidin-1 and hepcidin 1–5 to inhibit the infectivity of a betanodavirus using different treatment methods. Our results demonstrated that these synthetic peptides inhibited the mortality of grouper inoculated with a betanodavirus. We also detected Mx2 and Mx3 gene expressions after different treatments, which suggested that after 21 day of treatment with epinecidin-1 or hepcidin 1–5, Mx3 showed decreased expression. This report is the first attempt to explore the use of synthetic fish peptides to protect grouper from a virus infection. Our results suggest that epinecidin-1 and hepcidin 1–5 may be good candidates for treating viral infections.

2. Materials and methods

2.1. Peptides, fish, virus, and challenge test

The tilapia hepcidin TH1-5 sequence was GIKCRFCCGCCTPGICGV CCRF, and the epinecidin-1 sequence was GFIFHIIKGLFHAGKMIHGLV. Both peptides were synthesized with an amidated C-terminus by GL Biochem (Shanghai, China) at >90% purity. Synthetic peptides were reconstituted in phosphate-buffered saline (PBS; pH 7.4) for the experiments. Grouper (*E. coioides*) larvae were obtained from a private hatchery farm in Jiaushi, Taiwan. Before the challenge test, the fish were acclimated for 14 day in a tank supplied with ozone-treated seawater and fed commercial fish fodder (Golden Prawn Enterprise, Kaohsiung, Taiwan) twice a day. The GNNV 9508 betanodavirus strain was a gift from Dr. Shau-Chi Chi's laboratory (Institute of Zoology, National Taiwan University, Taipei, Taiwan). Briefly, the virus (GNNV 9508 strain) purification procedure followed previous publications [1,25], and the purified virus was stored in phosphate-buffered saline (PBS) buffer for further experiments.

The first test for every group used 33 grouper (with an average body weight of 0.7 g and body length of 3.7 cm): (a) group 1 was only inoculated with virus; (b) in group 2, the virus and epinecidin-1 (100 μg ml^{-1}) or hepcidin 1–5 (1000 μg ml^{-1}) were inoculated at the same time; (c) group 3 was treated with the virus and epinecidin-1 (50 μg ml^{-1}) or hepcidin 1–5 (100 μg ml^{-1}) at the same time; (d) group 4 was treated with the virus and epinecidin-1 (10 μg ml^{-1}) or hepcidin 1–5 (10 μg ml^{-1}) at the same time; (e) group 5 was treated with the virus and epinecidin-1 (5 μg ml^{-1}) or hepcidin 1–5 (1 μg ml^{-1}) at the same time; and (f) group 6 was only inoculated with PBS.

For the second test, each group contained different numbers of fish that had survived the first test after a 30-day experimental period from the first day of co-inoculation with the AMP and virus mixture, and each fish was inoculated with virus again. The PBS and NNV groups used 33 new fish each, which were of the same age as those from the first test, but had received no previous treatment.

For the third test, each group contained 33 grouper (with an average body weight of 0.7 g and body length of 3.7 cm): (a) group 1 was only inoculated with virus (NNV); (b) group 2 was pretreated with the virus, and after 8 h, epinecidin-1 (100 μg/ml) (8 h-Epi) or hepcidin 1–5 (1000 μg ml^{-1}) (8 h-Hep) was inoculated; (c) group 3 was pretreated with the virus, and after 24 h, epinecidin-1 (100 μg ml^{-1}) (24 h-Epi) or hepcidin 1–5 (1000 μg ml^{-1}) (24 h-Hep) was inoculated; (d) group 4 was pretreated with the virus, and after 48 h, epinecidin-1 (100 μg ml^{-1}) (48 h-Epi) or hepcidin 1–5 (1000 μg ml^{-1}) (48 h-Hep) was inoculated; and (e) group 5 was only inoculated with PBS (PBS).

For the fourth test, each group contained 33 grouper (with an average body weight of 0.7 g and body length of 3.7 cm): (a) group 1 was only inoculated with the virus (NNV); (b) group 2 was pretreated with epinecidin-1 (100 μg ml^{-1}) (Epi-2 h) or hepcidin 1–5 (1000 μg ml^{-1}) (Hep-2 h), and after 2 h, the virus was inoculated; (c) group 3 was pretreated with epinecidin-1 (100 μg ml^{-1}) (Epi-4 h) or hepcidin 1–5 (1000 μg ml^{-1}) (Hep-4 h), and after 4 h, the virus was inoculated; (d) group 4 was pretreated with epinecidin-1 (100 μg ml^{-1}) (Epi-8 h) or hepcidin 1–5 (1000 μg ml^{-1}) (Hep-8 h), and after 8 h, the virus was inoculated; and (e) group 5 was only inoculated with PBS (PBS).

All administrations were given through the anal canal (cloaca), and the inoculation volume was 10 μl of the peptide for each fish. The inoculation of virus was at 1×10^7 TCID50/fish (10^9 TCID50 ml^{-1}), and 10 μl was inoculated. Fish were maintained in 10-L tanks at approximately 27 °C. Mortality was recorded daily, and each survival curve was composed of the average of three independent groups, and each group was composed of 33 fish. Mortality figures are presented as percentages (%).

2.2. Detection of the NNV, Mx2, and Mx3 by RT-PCR analysis

Samples ($n = 5$) were treated the same as described above. Viral RNA was extracted from a cut in the head of a tested fish, and was amplified by RT-PCR methods [26]. Relative quantification of messenger (m)RNA was carried out in a one-step RT-PCR procedure using the Fast-Run™ HotStart RT-PCR (AMV) kit (Protech Technology, Taipei, Taiwan). The resulting complementary (c)DNA was amplified by RT-PCR using specific forward (F) and reverse (R) primers for the target gene expression with β-actin as the housekeeping gene (Table 1) under the following conditions: 58 °C for 5 min, 42 °C for 30 min, and 94 °C for 2 min for 1 cycle; and then 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min for 35 cycles. The relative levels of each transcript after treatment were quantified by RT-PCR following our previous publication [26], after running 1.0% agarose gels, taking pictures of ethidium bromide (EtBr) staining with a Bio-Rad GelDoc machine, and using a Quantity One 4.5.2 analyzer (Bio-Rad, Hercules, CA, USA). Values were calculated by the Quantity One 4.5.2 analyzer using Excel software.

2.3. Statistical analysis

Statistical analysis was performed with *t*-test to compare two groups. Multiple group differences were defined as significant at

Table 1
Primers used in this paper.

Primer name	Sequences
NNV _5′	GCCCCTGATGGAGCAGTCT
NNV _3′	AGCACGGTCAACATCTCCAGTT
Grouper_Mx2_5′	CTTCACTGGATTTCCTAACCTCAT
Grouper_Mx2_3′	TGTCACTCAAACTGTTGCTGTAGGT
Grouper_Mx3_5′	CAAGGATAGAAACCGTAATGCGA
Grouper_Mx3_3′	CTTTATGGGAGTCTGTGCCTTTC
Grouper_rt_actin 5′	ATGTTAACTGCATTGTTCAGACAC
Grouper_rt_actin 3′	GGGAAGAAGAGGTCACGATT

$p < 0.05$ and <0.01. Different letters indicate significant differences between different groups, while the same letter indicates no difference between two groups.

3. Results

3.1. Co-treatment with virus and epinecidin-1 peptide or hepcidin 1–5 peptide reduced fish mortality and enhanced the survival after a second infection

Different concentrations of epinecidin-1 or hepcidin 1–5 were co-incubated with NNV (1×10^7 TCID50/fish) dissolved in PBS. The virus-epinecidin-1 and virus-hepcidin 1–5 mixtures were then put on a bench for 10 min and inoculated into grouper. Fig. 1a shows that epinecidin-1 inhibited virus infection in grouper in a dose-dependent manner, and survival for PBS, NNV, and 1, 0.5, 0.1, and 0.05 μg epinecidin-1/fish treatments were respectively 64%, 11%, 35%, 29%, 20%, and 17%. Re-challenge with the virus from first trial and after another 30 day showed that grouper respectively exhibited 62%, 20%, 51%, 52%, 55%, and 45% survival for the PBS, NNV, and 1, 0.5, 0.1, and 0.05 μg epinecidin-1/fish treatments (Fig. 1b). Fish in the test tanks cultured at a constant 27 °C respectively exhibited 61%, 9%, 34%, 28%, 19%, and 17% survivals for PBS, NNV, and 10, 1, 0.1, and 0.01 μg hepcidin 1–5/fish treatments (Fig. 2a), while those which received an inoculation of virus again after 30 day had respective survivals of 61%, 13%, 53%, 54%, 48%, and 47% in the groups with PBS, NNV, and 10, 1, 0.1, and 0.01 μg hepcidin 1–5/fish treatments for a further 30 day (Fig. 2b). The mortality in the group receiving the peptide combined with the virus was significantly lower than that in the group receiving the virus alone ($p < 0.05$). These results show that epinecidin-1 and hepcidin 1–5 can reduce viral infection ability.

3.2. Pretreatment or posttreatment with the epinecidin-1 or hepcidin 1–5 peptide reduces fish mortality

In the study involving posttreatment with the epinecidin-1 or hepcidin 1–5 peptide at different times after injecting the virus, grouper respectively showed survivals of 64%, 13%, 25%, 17%, and 16% for PBS, NNV, and 8 h, 24 h, and 48 h treatments (Fig. 3a). However,

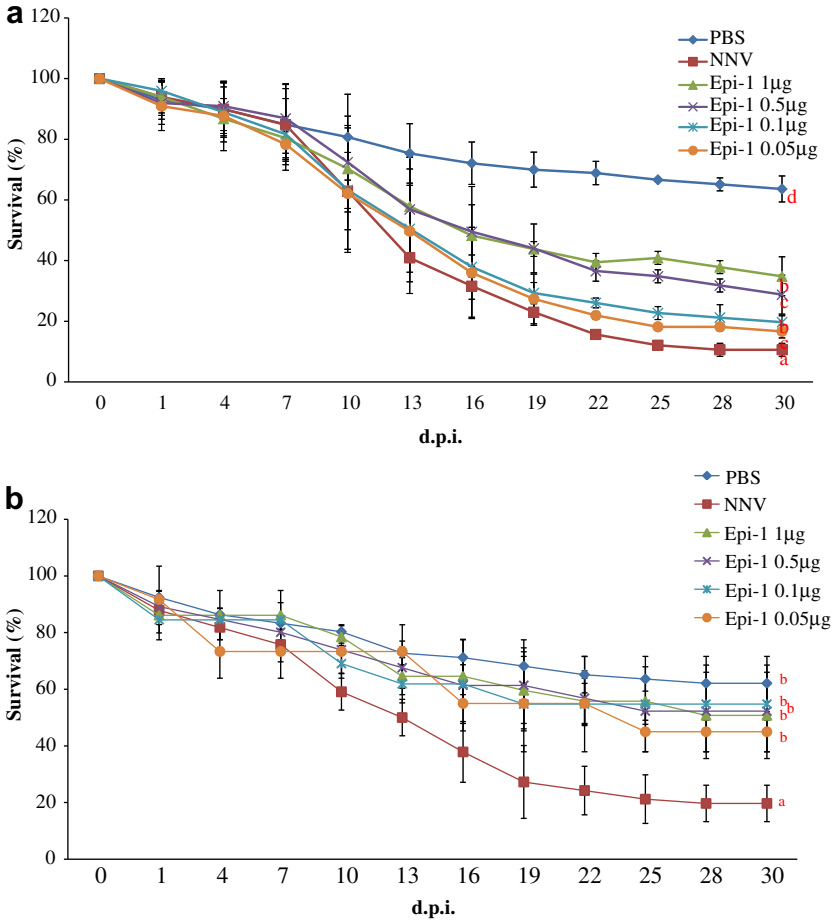

Fig. 1. The ability of the synthesized epinecidin-1 peptide to protect grouper from a lethal challenge of nervous necrosis virus (NNV; 1×10^7 TCID50/fish). (a) There were six groups in this experiment: PBS, inoculated with PBS only; NNV, inoculated with NNV only (1×10^7 TCID50/fish); Epi-1 1 μg, co-inoculated with 1 μg epinecidin-1/fish and NNV; Epi-1 0.5 μg, co-inoculated with 0.5 μg epinecidin-1/fish and NNV; Epi-1 0.1 μg, co-inoculated with 0.1 μg epinecidin-1/fish and NNV; and Epi-1 0.05 μg, co-inoculated with 0.05 μg epinecidin-1/fish and NNV. (b) Survivals after re-challenge with NNV (1×10^7 TCID50/fish) at 30 day for the same experimental groups described above. Each bar represents the mean value from three determinations with the standard error. Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among treatments.

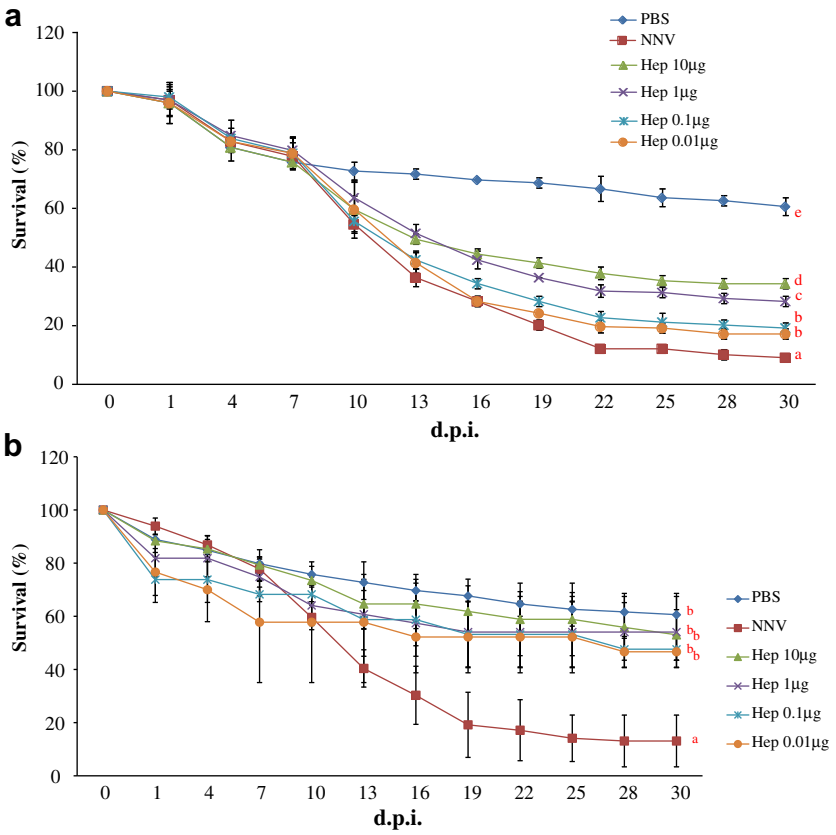

Fig. 2. The ability of the synthesized hepcidin 1–5 peptide to protect grouper from a lethal challenge of nervous necrosis virus (NNV; 1×10^7 TCID50/fish). (a) There were six groups in this experiment: PBS, inoculated with PBS only; NNV, inoculated with NNV only (1×10^7 TCID50/fish); hep 10 μg, co-inoculated with 10 μg hepcidin 1–5/fish and NNV; hep 1 μg, co-inoculated with 1 μg hepcidin 1–5/fish and NNV; hep 0.1 μg, co-inoculated with 0.1 μg hepcidin 1–5/fish and NNV; and hep 0.01 μg, co-inoculated with 0.01 μg hepcidin 1–5/fish and NNV. (b) Survivals after re-challenge with NNV (1×10^7 TCID50/fish) at 30 day in the same experimental groups as described above. Each bar represents the mean value from three determinations with the standard error. Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among treatments.

inoculation with the NNV then after 8 h inoculation of epinecidin-1 produced a significant difference (25% survival) (Fig. 3a), but no significant difference was found in the hepcidin 1–5 groups (Fig. 3b). These results suggest that posttreatment with epinecidin-1 may rescue infected fish from NNV infection.

To determine the optimal time for epinecidin-1 or hepcidin 1–5 pretreatment in grouper before inoculating the virus, grouper were inoculated with epinecidin-1 (100 μg ml^{-1}; 1 μg/fish) or hepcidin 1–5 (1000 μg ml^{-1}; 10 μg/fish) and then inoculated with the virus at different times. Survivals of fish in each group after virus challenge are shown in Fig. 4a for epinecidin-1 treatment and Fig. 4b for hepcidin 1–5 treatment. The survival of fish after epinecidin-1 treatment for 2 h which were then inoculated with the virus showed a significant difference (Fig. 4a). Hence, Fig. 4a shows that epinecidin-1 inhibited virus infection in grouper in a time-dependent manner, and survivals for PBS, NNV, and virus inoculated after 2 h, 4 h, and 8 h were 65%, 14%, 27%, 22%, and 16%, respectively. However, other groups with hepcidin 1–5 treatment (Fig. 4b) showed no significant differences for virus treatment only compared to AMP-treated groups with survivals for PBS, NNV, and virus inoculated after 2, 4, and 8 h of 63%, 14%, 27%, 25%, and 17%, respectively. Therefore, the optimal treatment condition was in the group treated with epinecidin-1 then after 2 h the virus was inoculated; the cumulative mortality of the group was significantly lower than that of the NNV group.

3.3. Virus, Mx2, and Mx3 detection by RT-PCR

We next determined whether epinecidin-1 or hepcidin 1–5 suppresses the virus, and examined Mx2 and Mx3 mRNA expressions in grouper. Co-treatment with epinecidin-1 or hepcidin 1–5 and the virus caused significant repression of NNV gene expression levels 7 day after the inoculation (Fig. 5a). However, a statistically significant repressive phenomenon was also observed in the 8 h-Hep and Hep-2 h groups (Fig. 5a). Inhibition of virus infectivity when using a pre-inoculation of epinecidin-1 or hepcidin 1–5 before virus infection suggested that the Mx2 and Mx3 genes might have been induced by both epinecidin-1 and hepcidin 1–5 in grouper after 14 day (Fig. 5b, c). The Mx2 and Mx3 genes were selected to represent interferon (IFN) response genes. At least two Mx proteins occur in vertebrates, and these proteins are key components of the innate defence against viral infection. The Mx2 and Mx3 genes were both downregulated in grouper treated with epinecidin-1 or hepcidin 1–5 although induction was higher in response to viral treatment after 14–21 day (Fig. 5b,c).

4. Discussion

This study elucidates how epinecidin-1 and hepcidin 1–5 were able to decrease the *in vivo* production of a fish virus strain and exerted a therapeutic effect, increasing survival levels after the virus was again inoculated in a grouper model of infection. Therefore, increasing data support AMPs not only having a role as antibacterial effectors of the innate immune response but also as antiviral peptides [13]. Epinecidin-1 has antibacterial, antitumor, and anti-parasitic functions [18,26,27], but there are still no descriptions of epinecidin-1 or hepcidin 1–5 having any direct effects on virions or any effects on innate and adaptive immunity in fish. However, there is a paucity of information regarding the antiviral effects of fish AMPs compared to mammalian defensin. Epithelial cell-derived beta-defensins were shown to inhibit

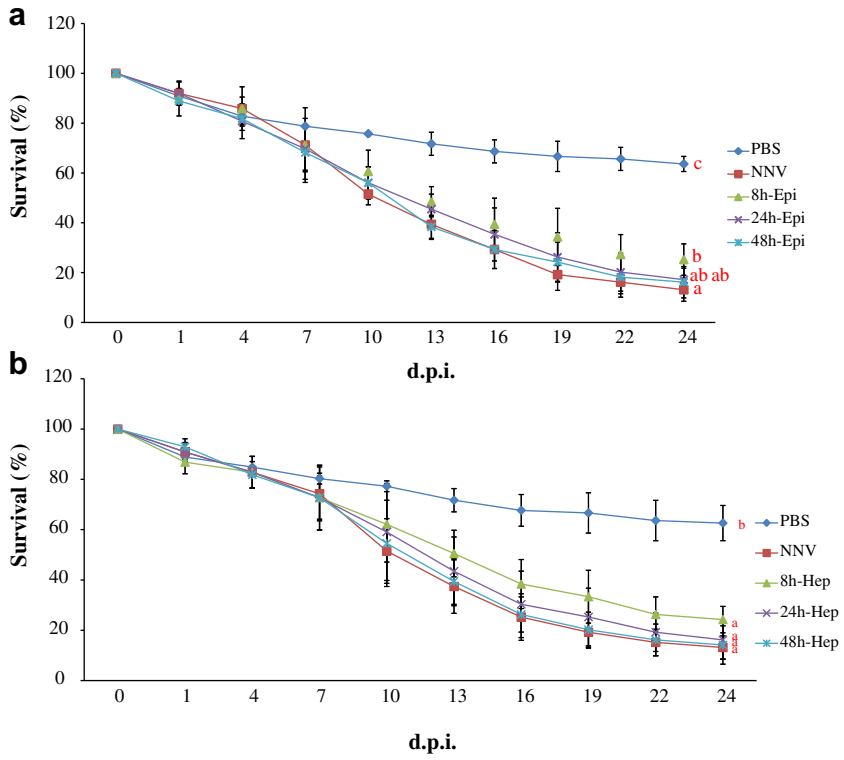

Fig. 3. Time-dependent effects of the pretreatment with nervous necrosis virus (NNV; 1×10^7 TCID50/fish) and then an inoculation of epinecidin-1 (a) or hepcidin 1–5 (b) after different time lapses. Each bar represents the mean value from three determinations with the standard error. Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among treatments.

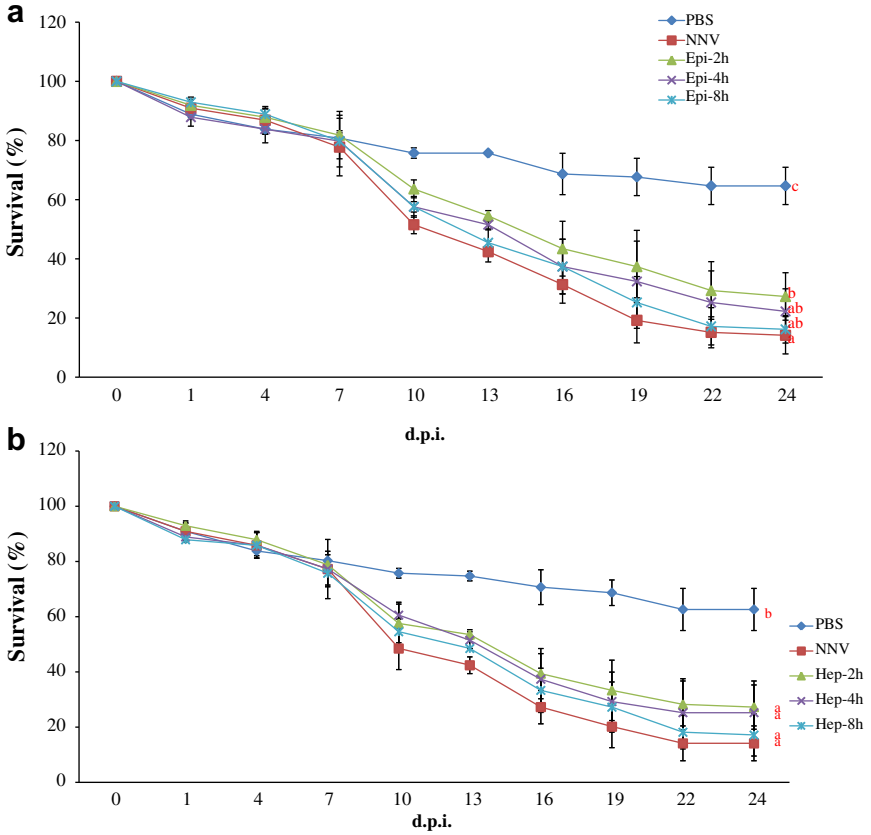

Fig. 4. Each grouper was inoculated with epinecidin-1 (a) or hepcidin 1–5 (b), and then each was inoculated with nervous necrosis virus (NNV; 1×10^7 TCID50/fish) at different time intervals. Survival was determined at 0, 1, 4, 7, 10, 13, 16, 19, 22, and 24 day. Each bar represents the mean value from three determinations with the standard error. Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among treatments.

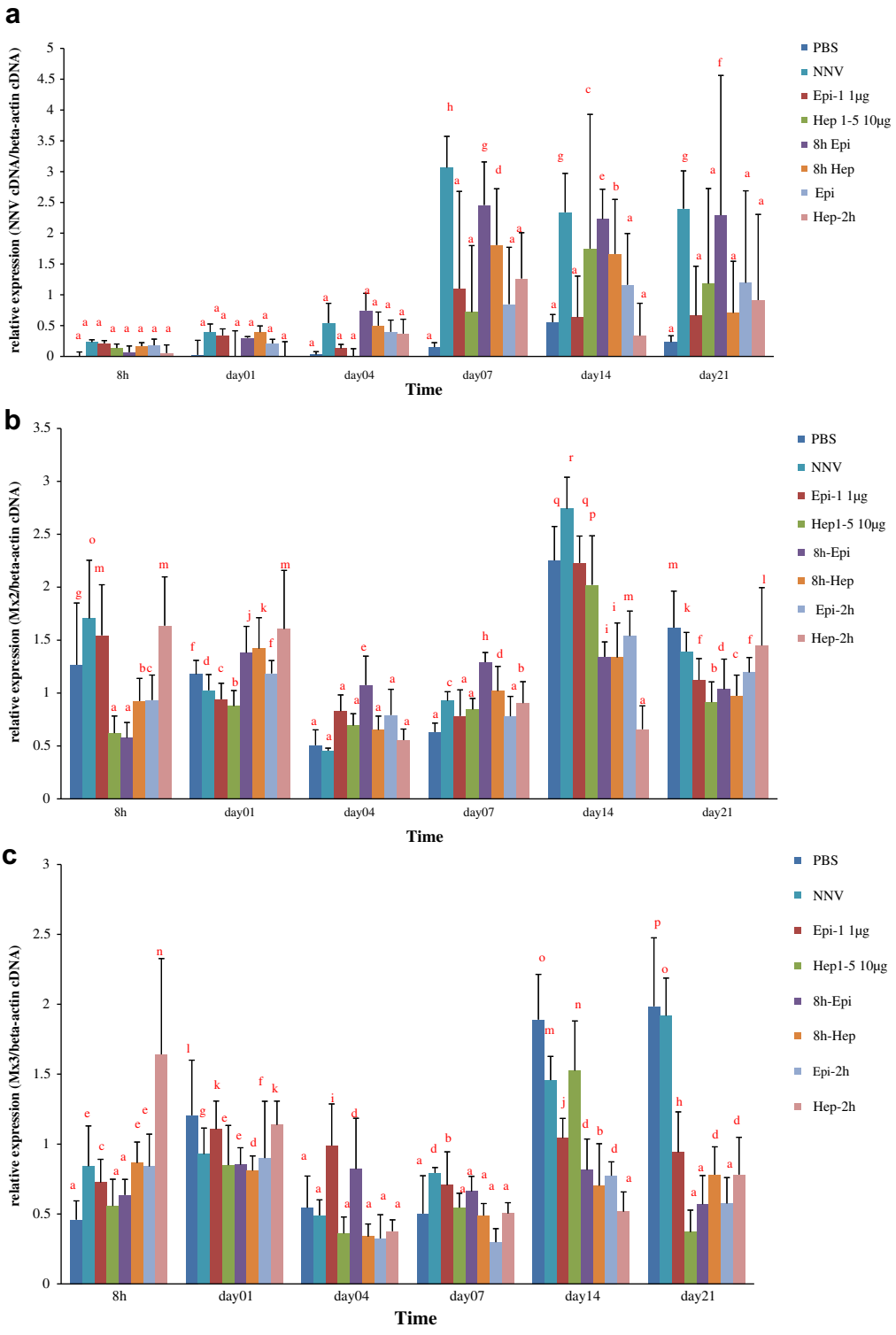

Fig. 5. Comparative RT-PCR analysis of the nervous necrosis virus (NNV) (a), and Mx2 (b) and Mx3 (c) mRNA gene expression levels after different experimental conditions as described in "Materials and methods". Each bar represents the mean value from three determinations with the standard error. Data (mean ± S.E.) with different letters significantly differ ($p < 0.05$) among treatments.

adenoviral infections [28]. It was found in human neutrophils that alpha-defensins inhibit adenoviral infection and HIV replication *in vitro* [29]. Recently published results suggest that the activity of defensins as antivirals involves mechanical membrane disruption [30]. But more work is needed to substantiate this assumption for the actions of epinecidin-1 and hepcidin 1–5 against NNV.

Of the AMPs described above, although *in vitro* testing can provide evidence of an AMP's potential antiviral properties, with or without associated toxicity, it does not necessarily reflect how epinecidin-1 or hepcidin 1–5 will perform *in vivo*. In the present study, the experiment in which the virus was mixed with epinecidin-1 or hepcidin 1–5 and then inoculated into grouper and after 30 day the

fish were challenged again with the virus showed that AMPs enhance the survival ability to protect fish during a second viral infection (Fig. 1). Our co-treatment results are the first clinical evaluation of an aquatic virus with fish AMPs that induced $a > 2.60$-fold increase in the grouper survival with epinecidin-1 and $a > 3.08$-fold increase with hepcidin 1–5 compared to viral infection only. These data suggest that using AMP for co-treatment with the virus could increase fish survival. However, using vaccines is another solution and a common method in the aquaculture industry for effectively controlling a number of economically important diseases. The most common methods for therapy of fish diseases are traditional inactivated vaccines [4]. Currently, all fish virus vaccines for sale are based on inactivated viruses or recombinant proteins [31]. There are no live attenuated or DNA vaccines currently licensed, but one DNA vaccine against IHN is being tested in controlled field trials in Canada [32]. NNV is one of the most significant viral pathogens that causes disease early in the lifecycle of marine fish, and there are no miracle drugs or vaccine for this problematic disease. Although developing live attenuated influenza vaccines for humans against avian influenza strains with pandemic potential is an important public-health strategy, this method is not commonly used to develop vaccines for fish [33]. Our findings add an aquatic virus that is sensitive to epinecidin-1 and hepcidin 1–5 and propose a novel mechanism for epinecidin-1 and hepcidin 1–5 in viral inhibition. Concerning the antiviral activity of epinecidin-1 and hepcidin 1–5, we could not conclude that the AMP present in the fish is responsible for the viral inhibition but observed the survival of fish increase after viral inoculation again. The lack of specific antibodies against immune-related molecules in grouper makes it difficult to determine the molecules responsible for this inhibition. More research work should be performed to characterize these immune-related molecules and the actual roles of epinecidin-1 and hepcidin 1–5 in fish viral infections by a co-treatment method.

There were several unexpected findings in the present study. The inhibition of the infectivity of NNV in our experiments was separated into pretreatment and posttreatment with AMPs. The antiviral effects of both epinecidin-1 and hepcidin 1–5 were most prominent when the virus was directly treated with the peptides, whereas no or a less-significant effect was observed when epinecidin-1 was applied to grouper prior to or after viral infection (Figs. 3 and 4). The difference in susceptibility of the virus to the two different AMPs raises a question as to the roles of AMPs in the different rescue abilities in grouper, suggesting that epinecidin-1 and hepcidin 1–5 possibly induce innate immunity in grouper through different signal transduction pathways. Recently published data suggest that alpha defensins induce interleukin (IL)-8 release *in vitro* [34,35] and enhance the synthesis and secretion of IL-8 and IL-1 through the P2Y6 signal pathway [13,36]. However, no reference to the relative expressions of these chemokines was indicated in those studies. On the other hand, Mx1, Mx2, and Mx3 transcripts were expressed in RTG-2 cells when induced by poly I:C, with Mx3 transcripts being predominantly expressed [37]. Other studies reported that IHNV was not a good Mx inducer in RTG-2 cells [38], while we found that NNV-infected grouper efficiently expressed Mx2 transcripts after 14 day and Mx3 transcripts after 7 day (Fig. 5). In contrast, Mx2 and Mx3 transcripts were downregulated after epinecidin-1 or hepcidin 1–5 pretreatment or posttreatment. These alternative patterns of expression of Mx2, Mx3, and NNV suggest that the relative expressions of the different Mx isoforms depend on the treatment methods of epinecidin-1 or hepcidin 1–5 by inducing different immune responses. Furthermore, inoculation of VHSV not inducing Mx3 in the fish liver is interesting since it was in contrast to inoculation of pMCV1.4-G as a DNA vaccine which produced Mx3 expression and was correlated with protection [39], suggesting that epinecidin-1 or hepcidin 1–5 might be as effective in controlling infectious pathogens (viruses) in fish as they are in controlling bacterial pathogens [27], but other immune-related factors might also be involved. In contrast to amphibian and piscine AMPs, our results extend our knowledge of epinecidin-1 or hepcidin 1–5 from our previous findings and predicted that the function of epinecidin-1 or hepcidin 1–5 resembles that of piscidins in reducing the infectivity of FV3 due to interactions with lipid membranes [40], but the mechanism by which these pathways reduce virus infectivity remains to be determined.

The effect of epinecidin-1 or hepcidin 1–5 with pretreatment, co-treatment, and posttreatment *in vivo* on NNV infection was unanticipated, as it tends to be proinflammatory and reduces a fish's overall clinical situation and mortality. In this study, series of concentrations of epinecidin-1 and hepcidin 1–5 were used for therapy, which accentuated the survival against a high-concentration NNV infection. Another limitation of this study was the small number of grouper used in each group, a common problem associated with work in the field such as on grouper culture farms, which may make the statistical findings less reliable due to sample size variations. However, studies with large numbers of grouper can provide novel insights into disease prophylaxis or disease mechanisms and should be tested in fry culture aquariums. Our results are the first to show that epinecidin-1 and hepcidin 1–5 have *in vivo* antiviral activities, and protect grouper against a repeated viral challenge and reduce aggravation by nervous necrosis virus infection.

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