Antimicrobial Peptides as Model Molecules for the Development of Novel Antiviral Agents in Aquaculture

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Abstract: Antimicrobial peptides (AMPs) are one of the components of the non-specific immune system that operate first lines of protection in many animal species including fish. They exert broad-spectrum antimicrobial activity, apart from many other potential roles in innate immunity, and represent a promising class of antiviral agents. Recent advances in understanding the mechanisms of their antiviral action(s) indicate that they have a dual role in antiviral defence, acting not only directly on the virion but also on the host cell. Despite the acute problems of viral diseases and restrictions in using chemicals in aquaculture, few but successful attempts to assess the antiviral activities of fish AMPs have been reported. This review focuses on the antiviral activities and mechanisms of action of some AMPs, and their potential relevance in the aquaculture industry, one of the most important sources of fishery products in the near future. It is a matter of notable concern to understand whether the AMPs can be used as model molecules for designing antiviral drugs that might help to solve the problems with viruses in the fish farming industry worldwide. In addition, because fish rely more heavily on their innate immune defences than mammals, they might constitute a potential rich source of antiviral compounds for fighting against mammalian viral infections.

Key Words: Antimicrobial peptides, fish virus, rhabdovirus, pleurocidin, cecropin, defensin, HNP1, VHSV, aquaculture, antivirals.

INTRODUCTION

An increasing number of antiviral agents are presently in various stages of development and testing for possible application, and an increasing number have been recently licensed for use in humans and animals. However, most of the available antiviral drugs often lead to the development of viral resistance coupled with the problem of side effects, recurrence and/or viral latency. In this regard, antiviral drug development focusing on the regulation of innate defense system molecules is an attractive approach [1].

The innate immune response is the first line of defence against viral infections since it is triggered immediately after an animal body encounter with a virus. This early immune response is characterized by the production of cytokines as well as other immune mediators and antiviral factors, such as the interferon (IFN)-inducible Mx proteins [2-4] and the antimicrobial peptides (AMPs). These early reactions try to delay viral replication thus providing time for the organism to elicit a more specific and strong adaptive immune response.

AMPs are gene-encoded small cationic peptides isolated from organisms spanning most of the phylogenetic spectrum [5, 6]. AMPs have a broad spectrum of actions against many microorganisms, including both enveloped and non-enveloped viruses [7-10, 11]. Selected by evolution, the diversified collection of sequences of the known AMPs, up to 1200 so far [12], fall into one of two structural groups: linear/α-helical and disulfide stabilized/β-sheet. Other remaining AMPs can be classified as extended helices and cyclized loops. For a list of some of these, see the Antimicrobial Peptide Database (APD: http://aps.unmc.edu/AP/main.php) [13]. In addition, most of AMPs share a common amphiphilic structure that contributes to a general, but not exclusive, mechanism of action based on their interaction with the lipid cell membranes of pathogens (pore forming model) such as bacteria and/or enveloped viruses [14, 15]. This interaction causes a fast destabilization/permeabilisation of the target pathogen lipid membranes. Besides these pore forming mechanisms, several observations suggest that the antimicrobial peptides can also inhibit the synthesis of the cell wall, nucleic acids, and proteins or even inhibit enzymatic activity [16, 17]

On the other hand, there is also an increasing evidence that AMPs also modulate some of the host early immune responses, including the induction of chemokine and cytokine production, alteration of gene expression in host cells, and inhibition of proinflammatory responses of host cells to bacterial components such as lipopolysaccharide (LPS) *in vitro* and *in vivo* [18-20]. Since AMPs act by multiple and complex mechanisms, AMP resistance is difficult to develop for the pathogens [9, 21, 22].

ACTUAL SITUATION OF FISH VIRAL INFECTIONS

Because biosecurity measures have increased in the past decades to maintain the health status of fish stocks and most bacteria- and parasite-caused diseases have been at least partially managed, viral diseases have emerged as the most serious infectious problems to the fish aquaculture industry. Contrary to what occurs in bacteria and parasites, viral spe-

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cific chemotherapies and in most cases efficient vaccines are not yet available. In fact, most of the fish infectious diseases notified to the OIE (Office International des Epizooties) belong to the viral-caused diseases, which indicates the actual worldwide importance of fish viruses.

Among fish viruses, rhabdoviruses such as viral haemorrhagic septicaemia (VHSV) and infectious necrosis haematopoietic (IHNV) virus, are responsible for the greatest losses in aquaculture production not only because of their worldwide distribution but also because they not only affect fish at the early stages of development as most other viruses that infect fish, but can also produce a high percentage of mortality in adult fish of higher economic value. Despite extensive research carried over throughout many years, the development of cheap, effective and safe vaccines for the prevention of the diseases caused by these viruses has proven to be a difficult task. For example, the use of the DNA vaccine based on the IHN rhabdovirus glycoprotein G gene licensed in Canada [23] has been not authorised by the European Union due to safety concerns, even though the economic costs of rhabdovirus-caused diseases are high. For example, the viral haemorrhagic septicaemia (VHS)-disease costs to the European salmonid aquaculture industry about 40-50 million euros per year [24].

Due to the above-mentioned facts, and in order to find safe and effective natural antiviral agents against fish rhabdoviruses as well as against other fish viruses, the effects of some AMPs on the infectivity of some fish virus and on fish immune system have been studied [1, 6, 25]. The results obtained indicated that AMPs might be a potential source of antiviral agents for aquaculture, and fish AMPs could lead to a new generation of chemicals with negligible environmental impact and capable to be safely administered to the aquatic media. This review intends to provide a comprehensive view of the current knowledge on this subject, including the mode of action, specificity and efficacy of these antiviral alternatives in aquaculture.

INHIBITORY EFFECTS OF AMPS ON FISH VIRAL INFECTIONS

Antiviral activity against fish viral infections (Table **1**) has been reported for only a few AMPs of fish origin: piscidins and defensins [6, 26]. The activity of non-fish AMPs against fish viral infections has been only studied for cecropins and defensins (Table **1**) [1, 27]. In addition, with the exception of defensins, no AMPs from fish have been described with both antiviral and immunoenhancing capabilities.

Moreover, due to the fact that the proven effectiveness of DNA vaccination against fish rhabdoviruses, at least at the laboratory level [28], opens a whole new field to be explored: the understanding of how AMPs work in fish might help to design new DNA constructions incorporating molecular adjuvants in the form of AMP sequences. Those newly-incorporated AMP sequences would be not only immunostimulatory but also antiviral [1].

i) Direct Effect on Viral Particles

Several studies have demonstrated that the direct effect of AMPs on most pathogens is predominantly carried out by disrupting the lipidic cell membranes [15, 29-31] through different mechanisms. Among them the barrel-stave, carpet-like, toroidal pore formation and detergent-type micellization models [32-34]). In all these models, over a certain concentration threshold, peptides finally form transient pores or ion channels [21, 35, 36] leading to membrane permeabilization, leakage of cell content and osmotic instability, and/or peptide diffusion to intracellular targets [34]. The final consequence is the inevitable death of the microorganisms. Although these models are based on specific cationic antimicrobial peptides and are not applicable to all antimicrobial peptides, the structure-function models of the Shai-Matsuzaki-Huang (SMH) model [14] provide a reasonable explanation for the antimicrobial activity of most of these compounds [9]. Briefly, SMH model suggests that the AMPs should initially gain access to the pathogen membrane and interact then with the lipid bilayer causing the formation of transient pores. Transport of lipids and peptides into the inner membrane leaflet would then take place, with the eventual diffusion of peptides onto intracellular targets. At this stage, the membrane would collapse into fragments [9, 12, 34].

Although little is known about the antiviral effect of AMPs, cecropins, pleurocidins, defensins, and piscidins have been studied. In general, AMPs are more effective on envel-

Table 1. Activity of Fish and Non-Fish AMPs Against Fish Viruses

Family	Name	Origin	Virus	Swiss-Prot AC* / Reference
Cecropin	CecB CF17	*Hyalophora cecropia* Synthetic analogue	IHNV, SHRV, VHSV, IPNV	P01508** / [27]
Defensin	HNP1 omBD1	*Homo sapiens* *Oncorhynchus mykiss*	VHSV	P59665 / [1] A6YT28 / [6]
Pleurocidin	MDPle	*Limanda limanda*	VHSV	Q0H3B3 / [26]
Piscidin	Piscidin1, 2 and 3	*Oncorhynchus mykiss*	CCV, FV3	P0C006*** / [26]

*AC, accession number.

**CecB.

***Piscidin3.

oped viruses than non-enveloped viruses. Two mechanisms have been proposed to explain their direct antiviral action: (i) inactivation of the viral particles by perturbing lipid components of their membranes [37] and (ii) prevention of viral dissemination into the host cell by inhibiting viral-cellular membrane fusion [38, 39]. In addition, further studies have shown that the same AMP may act on different viruses through different mechanisms [37-41].

Cecropins are a family of AMPs initially discovered in giant silk moth (*Hyalophora cecropia*) [42]. The secondary structure of cecropin B consists of an amphipathic amino-terminal helix joined to a largely hydrophobic carboxy-terminal helix by a hinge region [43, 44]. No members of this family have been found in fish so far. The antiviral effect of insect cecropin B (CecB) and a synthetic analogue (CF17) were first investigated *in vitro* against infectious necrosis haematopoietic virus (IHNV), an enveloped rhabdovirus affecting many salmonid species [27]. Both of them inhibited the infection of IHNV in a dose-dependent manner when chinook salmon embryonic (CHSE) cell monolayers were treated with CecB peptides prior to infection. However, co-incubation of IHNV with the CecB peptides was more effective on reducing the viral titre. In this way, the synthetic analogue CF17 achieved almost complete inhibition at 500μM, the highest concentration tested. This effect was less important when IHNV and CF17 were co-incubated at 4ºC. These data and a dose-dependent binding between IHNV and the peptides suggested that direct disruption of the viral envelope may be involved in the inhibition of viral [27]. Peptides CecB and CF17 also showed a significant inhibitory activity against other viral pathogens of fish, such as snakehead rhabdovirus (SHRV) and haemorrhagic septicaemia virus (VHSV) (also from the *Rhabdoviridae* family). Unexpectedly, CecB and CF17 were also active against infectious pancreatic necrosis virus (IPNV) a non-enveloped virus from the *Birnaviridae* family. To date, the cause of the observed anti-IPNV effect remains unknown. It might be explained by either destabilizing/disintegrating the viral capsids and/or another mechanisms such as binding to and masking viral peptides that interacts with host cellular receptors [27]. Future studies are required to determine the antiviral mechanism of action of CecB on non-enveloped virus.

Pleurocidins (Ple) are linear cationic peptides belonging to a large family of flatfish AMPs [45, 46] that was initially isolated from the mucus of Winter flounder (*Pseudopleuronectes americanus*). Winter flounder pleurocidin (Swiss-Prot accession number P81941) is predicted to form amphipathic α-helixes and has a broad range of action since it is active against Gram-positive and Gram-negative bacteria [47, 48]. From NMR structural studies [48] a structure of Winter flounder pleurocidin was determined to be in a random coil conformation in aqueous solution whereas it assumes a rigid alpha-helical structure in TFE and in dodecylphosphocholine (DPC) micelles. This rigid alpha-helical structure perfectly accounts of pleurocidin activity, the cell lysis through the formation of holes in the pathogen membranes according to the toroidal pore formation model [49]. Mud dab (*Limanda limanda*) pleurocidin (MDPle) [50] (Fig. (**1**)) has also antiviral effects against VHSV like cecropins, showing inhibition of VHSV infection by means of the pre-

Fig. (1). Ribbon diagram of MDPle. The location of the basic (black) and acid (light grey) amino acids are displayed. Winter flounder pleurocidin (PDB file code: 1z64) [51] was used as template structures. Representation obtained using Swiss-PdbViewer v3.7.

incubation of the virus with the peptide prior to infection of the cells. Since VHSV is an enveloped virus, the disruption of the viral membrane could be underlying the antiviral activity of MDPle.

The human neutrophil peptide 1 (HNP1) a defensin, is a non-fish AMP like the CecB/CF17 peptides, that has demonstrated antiviral effects against a fish viral pathogen, the rhabdovirus VHSV [1]. HNP1 belongs to the family of defensins which are cysteine-rich cationic AMPs with β-pleated sheet structures stabilized by intramolecular disulphide bonds [52-55]. HNP1 has shown inhibitory effects against both non-enveloped [56] and enveloped viruses [11] including human immunodeficiency virus 1 (HIV1) [57, 58], type 1 and type 2 herpes simplex viruses (HSV1 and 2, respectively), influenza virus [59] as well as vesicular stomatitis virus (VSV), the prototype virus of the *Rhabdoviridae* family [37]. HNP1 inhibited the infectivity of VHSV *in vitro* in a dose-dependent manner in both epithelioma papulosum cyprini (EPC) and rainbow trout gonad (RTG) fish cell lines when the peptide was co-incubated with the virus prior to infection [1]. Similarly to CecB and CF17, direct binding between VHSV and HNP1 was observed by solid phase binding and ELISA assays. Recent studies have demonstrated that defensins, including HNP1, can inactivate enveloped virus by interacting with *N*-linked or *O*-linked glycans present on the viral surface glycoproteins in a lectin-dependent manner and altering the ability of these glycoproteins to bind to their receptors at the target cells [11, 60-64]. Inactivation of VHSV particles by HNP1 was also involved in the interactions with VHSV-G protein preventing the fusion with the host cellular membranes [1].

The ability of piscidins 1-3, amphipatic cationic AMPs from fish origin [65-67], to inhibit fish viral infections *in vitro* has been also shown [26]. Piscidins are able to inactivate viral pathogens regardless of the presence of a lipid membrane envelope. They inactivated both, channel catfish

virus (CCV), which is a major enveloped virus pathogen of fingerling channel catfish, and frog virus 3 (FV3), an emerging non-enveloped virus pathogen of frogs. Inactivation of those viruses was rapid and occurred, in contrast to mammalian AMPs, over a wide temperature range. As in many AMPs, both the number of positively charged residues (two arginines, one lysine, and four histidines) and the ability to form an amphipathic helical structure in membrane-mimicking environments seem to be the two main features responsible for the antimicrobial activity of piscidin by means of the formation of toroidal pores in the bacterial membrane [17].

All the above mentioned results suggest that AMP-mediated viral inactivation may play an important part in protection from viral disease in fish.

ii) Immunostimulatory Role of AMPs

In the above mentioned studies [1, 27], cecropins CecB/CF17 and HNP1 inhibited the infection of IHNV and VHSV, respectively, also when the fish cell line monolayers were treated with the peptides prior to infection. These results suggested that some antiviral mechanisms acting on host cells other than those acting on viral particles could be operating. This will make fish cells resistant and/or protected from viral infections in a different way.

Since cellular defense mechanisms of salmonid embryo-derived CHSE cell lines against viral infection were supposed to be triggered by CecB/CF17 [27], it was not surprising that the expression profiles of some representative immune genes were also modulated in a rainbow trout spleen-derived macrophage RTS cell line in response to CecB/CF17 and Ple [25]. Briefly, those AMPs altered the expression of interleukin 1β (*il1β*) and, to a lesser degree, of *cox2*. It has been also reported that MDPle produces *in vivo* a significant up-regulation of the levels of expression of some pro-inflammatory cytokines in rainbow trout [6].

On the other hand, the cell-mediated antiviral effects induced by the incubation of cells with HNP1 before viral infection led to a 70-80% inhibition of VHSV infectivity in both EPC and RTG cell line monolayers [1] and it was demonstrated that some interferon (IFN)-related mechanisms were operating in those fish cells lines as shown by the up-regulation of *mx3*, a well known marker of IFN-induction in fish. Further studies are needed to clarify the mechanism underlying this activity. Possibilities include the binding of HNP1 to cellular receptor/s, receptor-mediated or independent endocytosis, transport across channels, etc. Trout head kidney leucocytes were also used to investigate the HNP1 ability to modulate fish immune responses. Four IFN-related genes (*mx1*, *mx2*, *mx3* and *vig1*) were significantly modulated in head kidney leucocytes incubated with HNP1, thus confirming that HNP1 might trigger an antiviral response dependent of IFN induction. Moreover, *il1β* and inducible nitric oxide synthase (*inos*) genes were also up-regulated, indicating the immunomodulatory role of HNP1 on the leucocytes-regulated immune responses in fish.

HNP1 was also able to modulate *in vivo* the expression profiles of some genes related to the innate immune response in rainbow trout [68]. HNP1 strongly increased pro-inflammatory cytokines *il1β*, tumour necrosis factor α1 (*tnfα1*) and interleukin 8 (*il8*) in muscle tissue, and also in blood in the case of *il1β*, and in head kidney in the case of *il8*. The levels of expression of *il8* induced by HNP1 were, however, remarkably higher. Because IL8, a CXC chemokine, is characteristic of the early immune response [69], and HNP1 was also capable of increasing the levels of expression of two of the CC chemokines *ck5b* and *ck7a*, (homologues of the mammalian RANTES, regulated on activation, normal T cells expressed and secreted, and MCP, monocyte chemotactic protein, respectively), chemokine-mediated attraction of leukocytes might be implicated on their effects. Moreover, it was found that HNP1 considerably attracted trout blood leukocytes. All these results demonstrate that AMPs play a major role in chemotaxis, in part indirectly, by the activation of other chemokines, and in part directly by being themselves chemotactic.

The expression of some IFN-related genes such as the different *mx* isoforms found in rainbow trout and *irf3* were also modulated by AMPs. For instance, in head kidney, all three *mx* isoforms were induced, while only *mx3* was significantly induced in muscle [1].

There are some evidences that defensins from fish origin could act in a similar way than the above mentioned AMPs. Thus, transfected fish cells expressing omBD1, a β-defensin-like peptide from rainbow trout (*Oncorhynchus mykiss*), (Fig. (**2**)) were protected against infection with VHSV. Culture medium from EPC cells transfected with the cDNA sequence of omBD1 has been shown to contain acid and heat-stable antiviral activity. omBD1 transfected cells also showed up-regulation of carp *mx1* gene, suggesting that a type I IFN-related antiviral response could also be operating in the fish cells transfected with omBD1 [6].

Fig. (2). Ribbon diagram of OmBD1. The location of the basic (black) and acid (light grey) amino acids are displayed. Human β-defensin 1 (PDB file code: 1e4s) was used as template structure. Representation obtained using Swiss-PdbViewer v3.7.

NOVEL APPROACHES AND PERSPECTIVES

Up to now the identification and characterization of AMPs has been carried out one-by-one mainly by applying biochemical methods. However, new advances in fish ge-

nomics might now help to the discovery of new members of currently known families of fish AMPs.

AMPs display many activities that make them promising candidates for therapeutants. They are active against a large variety of pathogens, including bacteria, fungi and viruses [7], and even some cancer cells [33, 70, 71]. AMPs exhibit many advantageous properties over conventional antibiotics [21, 22], among them, there are increasing evidences of their potential roles as effectors of immune responses, clearly of the greatest interest from an immunotherapeutic perspective.

The large natural stock of fish AMPs to be discovered would probably provide a large amount of potential therapeutants for testing [72] and selecting the most effective and safer compounds to discover new viral disease treatments.

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