Antiviral activity of myticin C peptide from mussel: an ancient defence against herpesviruses

Running title: Myticin C: a novel antiherpetic agent from mussel

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Abstract

Little is known about the antiviral response in molluscs. As in other invertebrates, the interferon signalling pathways have not been identified, and in fact, there is a debate on whether invertebrates possess an antiviral immunity similar to that of vertebrates. In marine bivalves, due to their filtering activity, the interaction with putative pathogens, including viruses, is very high, suggesting that they should have mechanisms to address these infections. In this study, we confirmed that constitutively expressed molecules in naïve mussels confer resistance in oysters to Ostreid herpesvirus 1 (OsHV-1) when oyster haemocytes are incubated with mussel haemolymph. Using a proteomic approach, myticin C peptides were identified in both mussel haemolymph and haemocytes. Myticsins, antimicrobial peptides that have been previously characterized, were constitutively expressed in a fraction of mussel haemocytes and showed antiviral activity against OsHV-1, suggesting that these molecules could be responsible of the antiviral activity of mussel haemolymph. For the first time, a molecule from a bivalve has shown antiviral activity against a virus affecting molluscs. Moreover, modified myticin C peptides showed antiviral activity against human herpes simplex viruses types 1 (HSV-1) and 2 (HSV-2). In summary, our work sheds light on the invertebrate antiviral immune response with the identification of a molecule with potential biotechnological applications.

IMPORTANCE

Several bioactive molecules have been identified and isolated from marine invertebrates that have potential pharmaceutical or industrial applications. Myticin C, an antimicrobial peptide from the Mediterranean mussel (Mytilus galloprovincialis) that was identified by proteomic techniques in both mussel haemolymph and haemocytes,
showed potential as an antiviral agent against Ostreid herpesvirus 1 (OsHV-1), which represents a major threat to the oyster farming sector. Both haemolymph from mussels and a myticin C peptide inhibited OsHV-1 replication in oyster haemocytes. Additionally, a modified peptide derived from myticin C also showed antiviral activity against the human herpesviruses HSV-1 and HSV-2. Therefore, myticin C is an example of the biotechnological and therapeutic potential of molluscs.
Adaptive immunity, which is a characteristic of vertebrates, is based on the recognition of “non-self” structures by the major histocompatibility complex (MHC), T cell receptors and antibodies, among other effector molecules. However, the innate immune system is the only response present in the invertebrates, and it is able to recognize conserved pathogen-associated molecular patterns (PAMPs), common structures present in different microorganisms (1, 2) and even danger signals (3, 4). Furthermore, this “primitive” non-specific immune system has contributed to the survival of these animals and their evolution for more than 450 million years (5-7), which suggests that it must provide some biological advantages.

Aquatic animals in general, and bivalve molluscs in particular, are continuously exposed to different stress conditions due to the permanent and close contact with their natural environment, especially when feeding: the bivalves filter large amounts of water, and this activity results in permanent exposure to microorganisms, which can be concentrated in their bodies. The level of contact between the environment and bivalves is so close that these animals are also known as sentinels in toxicology because of the major role they play as biomarkers of contamination (8).

Since 2008, massive mortality associated with OsHV-1 outbreaks has been reported in Europe (mainly in France) among Crassostrea gigas spat and juveniles (9-15). Later, this virus was associated with major mortalities in Pacific oysters from Australia and Asia (16-18). Today, herpesviruses are considered an important threat to the worldwide production of Pacific oysters.

The family Herpesviridae comprises enveloped viruses with a large, linear, double-stranded DNA genome that cause several diseases in animals, including humans.
In particular, herpes simplex viruses types 1 (HSV-1) and 2 (HSV-2) are major human pathogens responsible for long-term latent infections, with periods of recurring viral replication (19). Due to the lack of effective vaccines, the moderate to high toxicity of the available anti-herpes compounds and the appearance of resistant viral strains, new inhibitors for these viruses have been extensively researched (20, 21).

The aims of this work were to confirm that mussels (*Mytilus galloprovincialis*) are more resistant than oysters to the Ostreid herpesvirus 1 microvariant (OsHV-1 µVar), one of the most important bivalve pathogens, and to determine the putative role of myticin C, a constitutively expressed molecule in mussels, in the resistance against herpesviruses.

**MATERIAL AND METHODS**

**Animals**

Adult *M. galloprovincialis* and *C. gigas*, 8–10 cm in shell length, were purchased from a commercial shellfish farm (Vigo, Galicia, Spain) and maintained in open-circuit filtered sea water tanks at 15 °C with aeration. The animals were fed daily with *Phaeodactylum tricornutum* and *Isochrysis galbana*. Prior to the experiments, the animals were acclimatized to aquarium conditions for at least one week.

**Viruses and cells**

The OsHV-1 µvar was kindly provided by Dr. Tristan Renault (IFREMER, France) as a viral suspension obtained from infected oyster haemocytes (22). An HSV-1 clinical isolate (>99% homology to isolate SK087 US4-6 genes; GenBank accession...
number: AY240815.1) and an HSV-2 clinical isolate (>99% homology to isolate 99-
62039 US4 gene; GenBank accession number: HM011430.1) were provided by the
Virology Service of Hospital Universitario Central de Asturias (Oviedo, Spain),
propagated in Vero cells (ECACC No. 84113001) and titrated by both the endpoint
dilution method (23) and by plaque assay.

Vero cells were cultured in Dulbecco’s modified MEM (DMEM) (Gibco BRL)
supplemented with 10% foetal bovine serum (Sigma) and maintained in DMEM
without bovine serum.

In vivo and in vitro viral infections of bivalves

The in vivo effects of the OsHV-1 virus on mussels and oysters were
investigated using experimental infections. A total of 60 naïve mussels and 60 oysters
were inoculated intramuscularly (i.m.) in the posterior adductor muscle with 100 μl of
an OsHV-1 suspension (2.7×10⁴ copies of viral DNA/μl) at 15 ºC. Control groups were
inoculated with an equivalent volume of filter-sterilized sea water (FSW). All
individuals were maintained out of the water for 20-30 min before and after the
injection. Each treatment group was individually maintained in tanks with aeration.
Three experimental challenges were conducted. Cumulative mortalities were monitored
for 15 days.

The in vitro effects of OsHV-1 were assayed in mussel and oyster haemocytes
extracted from the adductor muscle in mussels and directly from the pericardial cavity
in oysters. For each experiment, four pools of haemolymph from five animals (mussels
or oysters) were used. The concentration of cells was adjusted to 3×10⁶ cells/mL in
FSW and 1 mL of the cell suspension was dispensed in each well of a 24-well plate.
The plates were incubated at 15 ºC for 1 h for settlement and further infected following
the procedure previously described by Renault et al. (22). All experiments were performed at 15 °C. Cells were sampled at 24 hours post-infection (h.p.i.) to determine the viral titre by quantitative polymerase chain reaction (qPCR). Each experiment was conducted four times.

Detection and quantification of OsHV-1 by qPCR

OsHV-1 detection and quantification were conducted using standard procedures (24, 25). Briefly, total DNA from the infected haemocytes was isolated using a LEV Blood DNA kit (Promega). Quantitative PCR was performed on an MX3000 Thermocycler (Stratagene) with the PCR conditions and primer sequences described in the IFREMER Standard Operating Procedures (25). For quantification, standard curves were obtained using six 10-fold dilutions of a plasmid carrying an OsHV-1 DNA target sequence.

Protein extraction

Mussel haemolymph was extracted and pooled from 5 mussels and subsequently centrifuged at 3,000 g for 10 min (4 °C) to separate the serum from the haemocytes. The haemocytes were resuspended in 1 mL of homogenization buffer (10 mM HEPES, 250 mM sucrose, 1 mM DTT, 1 mM EDTA, 1 mM PMSF) supplemented with 1% protease inhibitor cocktail (Sigma, Ref. P8340-1 ML) and lysed by passing vigorously through a 25G needle. The resulting lysate was centrifuged at 16,000 g for 1 h (4 °C), and the supernatant was stored at –80 °C. The serum was also treated with the protease inhibitor cocktail and stored at –80 °C until use.

Protein identification by LC–MS/MS analysis

The samples were methanol-chloroform precipitated, dissolved in 8 M urea/25
mM triethylammonium bicarbonate (TEAB) solution, reduced by 10 mM dithiothreitol (DTT) and alkylated with iodoacetamide. Samples were digested with trypsin at an enzyme-to-protein ratio of 20:1 (37 °C, overnight). All reagents were purchased from Sigma-Aldrich. The proteomic analysis was performed in the proteomics facility of Centro Nacional de Biotecnología that belongs to ProteoRed, PRB2-ISCIII.

The peptide samples were analysed on a nano-liquid chromatography system (Eksigent Technologies, NanoLC-Ultra 1D plus, AB SCIEX, Foster City, CA, USA) coupled to a TripleTOF 5600 mass spectrometer (AB SCIEX, Foster City, CA, USA) with a nano-electrospray ion source.

MS and MS/MS data for each sample were processed using Analyst TF 1.5.1 software (AB SCIEX, Foster City, CA, USA). Raw data were translated to a Mascot generic file (mgf) format and searched against the nrNCBI database with a taxonomic restriction to Metazoa, using an in-house Mascot Server v. 2.4 (Matrix Science, London, UK). Peptide mass tolerance was set to 50 ppm and 0.2 Da, in MS and MS/MS mode, respectively, and 1 missed cleavage was allowed. Typically, accuracy below 10 ppm was found for both MS and MS/MS spectra.

**Immunohistochemical analyses**

For *in vivo* immunohistochemical studies, mussels were injected with 200 µl of a solution containing zymosan (Zym) (1 mg/mL), lipopolysaccharide (LPS) (1 mg/mL), dead *Vibrio anguillarum* (7.5×10⁷ cells/mL) or FSW. Five mussels were used in each treatment, and after 24 h, haemolymph was extracted for further studies, including analyses of myticin expression in the haemocytes (both granulocytes and hyalinocytes).

For *in vitro* analyses, haemocyte cultures were treated with fluorescent Zym A-TRED particles (Molecular Probes) at a particle-to-cell ratio of 10:1 for 1.5 h at 15 °C. Control cells were incubated with FSW. The immunohistochemistry assay of mussel
haemocytes was performed using routine procedures: haemocytes were attached to a
glass coverslip and incubated overnight (4 °C) with anti-myticin C primary antibody
(26). Alexa Fluor-488 conjugated anti-rabbit secondary antibody (Life Technologies)
was used at a dilution of 1:1000. The cells were stained with DAPI for nuclear
localization. The samples were mounted using ProLong Antifade Reagents for Fixed
Cells (Life Technologies). The images were captured using a TSC SPE confocal
microscope (Leica) and processed using the LAS-AF (Leica) and ImageJ software.

Flow cytometry analyses

The distribution of myticin C in different cell populations was assayed in mussel
haemocytes by flow cytometry (FACSCalibur, BD). The cell suspensions were adjusted
to 3×10^6 cells/mL in FSW, dispensed in 24-well plates (1 mL per well) and incubated
for 30 min at 15 ºC to allow for cell settlement. Supernatants were removed, and the
cells were washed and fixed with 2% PFA. An anti-myticin C antibody was used to
label cells expressing this protein. The density plots were generated using Cell Quest
Pro software (BD).

Synthetic myticin C peptides

A synthetic myticin C mature peptide (Myt-C) (27) and a modified myticin C
peptide (Myt-Tat) with a purity > 95 % were purchased from New England Peptide
(Gardner, MA, USA). The Myt-Tat amino acid sequence contains 40 amino acid
residues belonging to the Myt-C peptide plus 13 additional C-terminal amino acid
residues corresponding to the cell-penetrating peptide (CPP) of the human
immunodeficiency virus type 1 (HIV-1) Tat protein. Myt-C aliquots were diluted in
dH_2O supplemented with a protease inhibitor cocktail.
Antiviral activity of myticin peptides against OsHV-1 in oyster haemocytes

Primary cultures of oyster haemocytes were infected with the OsHV-1 preparation for 1 h at 22 °C, as previously described. After infection, the inoculum was removed, and the cells were incubated at 15 °C with filtered mussel haemolymph, oyster haemolymph or myticin peptides at a final concentration of 1 µg/mL. At 24 h.p.i., samples were taken to evaluate the viral load by qPCR. Two pools of haemolymph from five oysters were used for each assay. The experiment was conducted four times.

Cytotoxicity and antiviral activity of synthetic myticin peptides against human herpesviruses in Vero cells

First, the cytotoxic effects of Myt-C and Myt-Tat on Vero cells were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as previously described (28). Briefly, increasing concentrations of the test samples were added to confluent Vero cells in 96-well plates, with six replicates per concentration. After a 72 h incubation, the MTT solution (0.5 mg/mL) was added, and the plates were incubated for 3 h. The formazan precipitate was dissolved with 2-propanol, and the OD 570nm was measured using a µQuant spectrophotometer (Biotek Instruments) with a reference wavelength of 620 nm. The resulting cell viabilities after treatment with the test samples were calculated as described by Mosmann (29).

For testing the antiviral activity of Myt-C and Myt-Tat against HSV-1 and HSV-2, 96-well plates containing confluent Vero cell monolayers were pre-incubated (1 h) with 100 µL/well of increasing non-cytotoxic concentrations of the peptides (0-45 ) µM, with a replicate number of six wells per test concentration) prior to HSV-1 or HSV-2 infection (10 TCID 50). The plates were incubated at 37 °C in a 5% CO 2 humidified atmosphere and observed daily for cytopathic effects (CPEs) using a light microscope.
When CPEs were observed in all virus control wells (approximately 36 h.p.i.), the percentage of wells with CPEs was determined for each treatment concentration, as previously described (30). The plates were further incubated for 72 h, and then freeze-thawed. The content of replicated wells treated with the same concentration of peptides as well as those used as controls were pooled and titrated by plaque assay, and the viral titres were expressed as plaque-forming units per millilitre (pfu/mL). Acyclovir (ACV) at concentrations ranging from 0.1 to 10 µg/mL served as a positive control.

The concentration of the sample reducing cell viability by 50% (mean cytotoxic concentration, CC50) and that reducing viral-induced CPEs by 50% (mean effective concentration, EC50) were calculated by regression analysis using the dose-response curves generated from the experimental data. A selectivity index (SI) was calculated for each myticin peptide and virus by dividing the CC50 value by the EC50 value.

Preparation of nanovesicles carrying the Myt-C peptide and antiviral activity of Myt-C-nanosomes

In order to promote the internalization of Myt-C into the cells, a commercial kit for nanovesicles preparation (Pronanosomes Nio-N Reagent, NanoVex® Biotechnologies SL, Spain) was used to encapsulate the synthetic Myt-C peptide into non-ionic surfactant nanosomes, following the manufacturer’s indications. Briefly, 250 mg of pronanosomes were mixed by vortexing with 1.5 mL of a 2 mg/mL Myt-C stock solution, pre-heated at 50 °C, or with an equivalent amount of pre-heated PBS (negative control). The resulting nanosomes solutions were used as stock solutions for preparing the test concentrations of both Myt-C-containing nanosomes (1.01-45.0 μM Myt-C plus 0.52-16.6 mg/mL of nanosomes, respectively) and peptide-free nanosomes (0.52-16.6 mg/mL of nanosomes) (negative control).
After 1 h pre-incubation with nanosomes (containing or lacking Myt-C) the cells were infected with 10 TCID$_{50}$/well of either HSV-1 or HSV-2 and further incubated for 36 h at 37 °C in a 5 % CO$_2$ atmosphere. Due to the turbidity of nanosomes preparations, a minor modification to the antiviral assay was performed: the supernatants from each treatment concentration were completely collected and pooled for virus titration. The cells were washed gently three times with PBS, left in PBS after the last wash, and microscopically examined for virus-induced CPE. In parallel, the cytotoxic effects of Myt-C-containing and free nanosomes were assessed in Vero cells using the MTT assay in a 96-well plate format, as described elsewhere. After a 72 h incubation, the supernatants were removed and the cells were washed three times with PBS prior to the addition of MTT reagent.

Statistics

Data from different treatment conditions were compared using unpaired Student’s $t$-tests or ANOVAs when possible or the Kruskal-Wallis nonparametric test when necessary, using IBM SPSS-22 Statistics software. The differing means from multiple means comparisons were investigated using the Student-Newman-Keul’s test and contrasted with Tukey’s HSD test or with Dunnett’s T3 test.

RESULTS

Mussels are less susceptible to OsHv-1 than oysters

Differential susceptibility to OsHV-1 infection was observed between mussels and oysters after inoculation. Bivalve mortalities were recorded in the 3 experimental challenges, and differences were also observed. The mortalities among infected oysters
started as soon as 4 days post-infection and increased until the end of the experiment, reaching high mortality rates (84 ± 7 %). In contrast, the mortality of mussels inoculated using the same viral doses and conditions was similar to the control animals injected with FSW (Fig. 1A).

Next, we determined whether these differences in mortality were related to different abilities of mollusc cells to support OsHV-1 replication. For this purpose, we infected primary cultures of mussel and oyster haemocytes with the same OsHV-1 viral suspension. Significant differences in viral yields were found between oyster and mussel haemocytes (Fig. 1B). The oyster haemocytes cultures had higher viral loads, with values ranging from $2 \times 10^4$ to $2 \times 10^5$ viral DNA copies/µL at the end of the experiment. Viral load in the mussel haemocytes was significantly lower and did not significantly change during the experiment ($10^3$ viral DNA copies/µL) (Fig. 1B).

**Mussel haemolymph contains components with antiviral activity**

The differences in mortality and viral replication rates between mussels and oysters suggested that mussel haemolymph could contain compounds conferring resistance to OsHV-1 infection. To evaluate this hypothesis, we determined viral DNA yields by qPCR in oyster haemocytes incubated with mussel haemolymph and compared these values to the viral DNA yield of infected oysters incubated with their own haemolymph at 24 h.p.i. We observed a significant reduction in the viral DNA yield of oyster haemocytes treated with mussel haemolymph compared to oyster haemocytes incubated with their own haemolymph. There was an approximately 100-fold reduction from $2.9 \times 10^5$ DNA copies/µl in mock-treated oyster haemocytes to $1.5 \times 10^3$ DNA copies/µl in oyster haemocytes incubated with mussel haemolymph (Fig. 2).
To study the composition of mussel haemolymph and to identify the putative compound with antiviral activity, we conducted a proteomic study. Reverse-phase HPLC followed by high-resolution (triple TOF) ESI-MS/MS was carried out. Metabolic and structural proteins, such as actin, were detected in both the serum and haemocytes. Proteins involved in the recognition of foreign particles, such as fibrinogen-related proteins and C1q domain-containing proteins, were also present in these samples. Notably, several peptides derived from myticin, a class of previously reported antimicrobial peptides with known activity against fish viruses, were detected in mussel serum and haemocytes (26) (Table 1).

**Myticin C has antiviral activity against OsHV-1**

To confirm that myticsins are constitutively expressed in mussels, we detected these molecules using immunocytochemistry and flow cytometry experiments. We confirmed that myticin C is present in the cytoplasm of normal, unstimulated haemocytes (Fig. 3A). However, not all haemocytes could be labelled using the anti-myticin C primary antibody, suggesting the heterogeneity of these cells (Fig. 3A and 3B). Moreover, the expression of myticin C was not related to external stimuli. After *in vitro* stimulation of haemocytes with Zym, the number of cells expressing myticin C did not significantly change compared to unstimulated haemocytes (42.4% vs 41.4%) (Fig. 3B). Furthermore, the *in vivo* treatment of mussels with LPS, Zym or dead bacteria did not significantly modify the number of granulocytes or hyalinocytes expressing myticin C (Fig. 3C).

Another interesting finding during the flow cytometry analysis was the lack of correlation between phagocytosis and myticin C expression: not all the phagocytic cells were labelled with the anti-myticin C antibody. On the other hand, other cells in
addition to phagocytes expressed myticin C. Therefore, myticin C does not appear to be involved in phagocytosis. Our results also suggest that there is a much more complex functional division in the haemocytes than the classical division into granulocytes and hyalinocytes (Fig. 3D).

Because myticin was constitutively expressed in mussel haemolymph and because we previously reported the antiviral effects of this class of peptides against a fish virus (26), we hypothesized that the naturally occurring resistance of mussels to OsHV-1 infection and the protective effects conferred on oysters by mussel haemolymph to this herpesvirus could be attributed to myticin.

The viral titre in infected oyster haemocytes treated with synthetic myticin decreased by 99.95 % at 24 h.p.i. This reduction was similar to that recorded for oyster haemocytes incubated with mussel haemolymph (reduction of 99.39 %). However, the combined treatment of myticin peptide plus mussel haemocytes did not synergistically decrease the viral titre. This suggests that the antiviral activity in mussel haemolymph is predominantly due to myticin C, although we cannot rule out the involvement of other molecules in the mollusc antiviral response (Fig. 4).

A myticin C-derived peptide inhibits HSV-1 and HSV-2 replication in vitro

The antiviral effect of myticin C on oyster herpesvirus prompted us to investigate whether this class of molecules has a general anti-herpetic activity. For this purpose, we used human herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) replicating in Vero cells as a surrogate model.

The effects of Myt-C on the viability of Vero cells were evaluated using the MTT colorimetric assay, which is based on the production of formazan by mitochondrial
enzymes in viable cells. In the range of concentrations tested, Myt-C was not toxic to
the cells because no morphological alterations were detected in the monolayers under a
light microscope during the experiment (data not shown). In fact, the highest
concentration tested (200 µg/mL, equivalent to 45 µM) resulted in 80% cell viability
(Fig. 5A). However, up to this concentration, Myt-C was unable to prevent the
appearance of virus-induced cytopathic effects (CPEs) in HSV-infected monolayers
(Fig. 5A). The lack of protection against HSV-1 and HSV-2 in these conditions was
confirmed by the high viral titres obtained from these cells (in the order of 10^7-10^8
pfu/mL) (Fig. 5A).

Then, we used a modified version of the myticin C peptide (referred to as Myt-
Tat) (see Disclaimer section) which possesses improved uptake properties by cells and
analysed its effects against both human herpesviruses. The cytotoxicity (MTT) assay
revealed that the modified peptide Myt-Tat was slightly more toxic to Vero cells than
Myt-C, which is in agreement with the microscopic observations during the antiviral
assay. The main morphological alterations consisted of cell rounding, the appearance of
dark nuclear and cytoplasmic inclusion bodies, refringence and moderate cell
detachment (data not shown). These alterations were recorded at the highest
concentration tested (32.6 µM, equivalent to 200 µg/mL), which resulted in a cell
viability of 69.7% using the MTT assay (Fig. 5B).

A significant reduction of virus-induced CPE was observed at relatively low
doses of Myt-Tat against both HSV-1 and HSV-2 (Fig. 5B). The mean effective
concentrations (EC_{50}) for Myt-Tat were 3.97 ± 0.94 µM (24.35 ± 5.74 µg/mL) against
HSV-1 (R^2=0.9871) and 3.09 ± 1.02 µM (18.98 ± 6.27 µg/mL) against HSV-2
(R^2=0.9688). Accordingly, the titres of virus recovered from cells treated with Myt-Tat
decreased with increasing concentrations of the peptide, ranging from \( \sim 10^7 \) pfu/mL (treatment with 1.01 µM Myt-Tat) to \( \sim 10^2-10^3 \) pfu/mL (treatment with 4.07 µM Myt-Tat). No virus was recovered after the treatment with this peptide at a concentration of 8.15 µM or above (Fig. 5B).

One of the best approaches for measuring the antiviral activity is the selective index (SI), which is calculated as the ratio of CC_{50}/EC_{50}. An SI value greater than 1 indicates specific antiviral activity (31). However, higher SI values are desirable as they indicate a higher safety margin in antiviral therapy. Because the highest concentration of Myt-Tat used in these assays did not allow the calculation of CC_{50} value, it can only be suggested that CC_{50} will be higher than 32.6 µM. Therefore, the putative SI values for Myt-Tat will be >8.21 against HSV-1 and >10.5 against HSV-2.

The EC_{50} values obtained for ACV, which was used as a positive control in the antiviral activity assay, (EC_{50}=0.9 µg/mL for HSV-1; EC_{50}=1.1 µg/mL for HSV-2) are in agreement with previous studies (32, 33) and suggested that both the HSV-1 and HSV-2 isolates used throughout this study are sensitive to acyclovir (EC_{50}<2 µg/mL) (34).

The cellular uptake of myticin is required for its anti-HSV effects

Taking into consideration the reported cell-penetrating activity of the Tat-derived peptide that is C-terminally fused to myticin C in the primary sequence of Myt-Tat, and the striking differences between Myt-C and Myt-Tat regarding their anti-HSV activities, we reasoned that the antiviral effects of myticin probably depend on an efficient internalization of the peptide, perhaps because the inhibition takes place through the arrest of some intracellular viral replication event. The Myt-C peptide, lacking the Tat-
derived cell-penetrating peptide, is not active against HSVs probably because it cannot be efficiently intaken.

In order to probe this hypothesis, we attempted to potentiate the cell uptake of Myt-C by encapsulating this peptide into commercially available nanovesicles. In these conditions, the Myt-C peptide recovered its antiviral activity and inhibited both HSV-1 and HSV-2 replication in Vero cells with EC$_{50}$ values of 5.85 ± 1.24 µM (25.98 ± 5.49 µg/mL) and 5.41 ± 0.95 µM (24.03 ± 4.21 µg/mL), respectively (Fig. 5C). Since the highest concentration of Myt-C conjugated to nanosomes used in the cytotoxicity assays (45 µM Myt-C plus 16.6 mg/mL nanosomes) resulted in cell viability values greater than 50 %, it can only be inferred that CC$_{50}$ value is higher than 45 µM, thus the inferred SI values for encapsulated Myt-C were SI>7.69 and SI>8.32 for HSV-1 and HSV-2, respectively. The inhibitory effects of Myt-C-containing nanosomes were confirmed by the virus plaque titres obtained from the supernatants of treated cells, which decreased with increasing concentrations of the peptide, from $\sim 10^7$ to $\sim 10^2$ pfu/mL. No virus was recovered when the infected cells were treated with the Myt-C-nanosomes complexes at a concentration of 22.5 µM Myt-C or above, indicating a complete suppression of virus replication (Fig. 5C).

The nanosomes themselves did not inhibit virus replication, since 100 % of virus-induced CPE was observed among HSV-1 and HSV-2-infected cells incubated with concentrations of mock-encapsulated nanovesicles at concentrations equivalent to those used in Myt-C-containing nanosomes experiments (0.52-16.6 mg/mL of nanosomes) (Fig. 5D). In agreement with this, the viral titres recovered from the supernatants of these cells were similar to those of untreated virus controls ($\sim 10^7$ pfu/mL) (Fig. 5D).
It is worth to mention that the combination of Myt-C with nanosomes during the antiviral evaluation (Fig. 5C) resulted in the most pronounced cytotoxic effect and the viability of Vero cells treated with the highest concentrations of Myt-C and nanosomes (45 µM and 16.6 mg/mL, respectively) was as low as 62.5 %, near the CC50 value. This toxic effect was characterized mainly by a noticeable drop in monolayer confluency from 100 % to near 70 %, although this loss of confluency did not prevent the evaluation of occurrence of virus-induced CPE (data not shown).

DISCUSSION

In vertebrates, the interferon (IFN) response is the major defence mechanism against virus infections. In invertebrates, however, IFN signalling pathways have not been identified, and there is a debate on whether invertebrates possess antiviral immunity similar to the IFN system of vertebrates. Other mechanisms, such as RNA interference or even apoptosis, are considered the main antiviral responses in these animals. However, recent studies have shown that viral infections can trigger inducible and transcriptional responses that still have yet to be characterized (35).

In the case of molluscs, the knowledge of their antiviral response is even more limited than that of other invertebrates, such as insects. The lack of established mollusc cell lines constitutes a major bottleneck in working with mollusc viruses. Due to their filtering activity, marine bivalves are in contact with a high number of viruses, which commonly reach $10^7$ particles per millilitre in the ocean, including putative pathogens (36). Due to this fact, they should have a constitutive “ready-to-use” repertoire of antimicrobial molecules that can act in dangerous situations. This could be the case of myticins in mussel. Interestingly, this species, with the exception of some infrequent
pathologies, does not appear susceptible to diseases (37), in contrast to other bivalves, such as oysters and clams (38-40). In this paper, we have shown that they are resistant to one of the most dangerous bivalves pathogens worldwide, OsHV-1, which does not appear to be a threat for this species.

Although it is possible that the resistance of mussels to OsHV-1 could be due to the lack of specific receptors for the virus, in this study, we confirmed that molecules that are constitutively expressed in naïve mussels confer resistance to oysters when their haemocytes are incubated with mussel haemolymph. Although there may be other compounds with potential antiviral activity in the mussel serum, in this work, we focused on myticins because they were identified in the proteomic analysis in both serum and haemocytes. Mussel myticins A and B were first described by Mitta et al. (41, 42) as molecules with antibacterial activity against gram-positive bacteria, and with activity against the fungus Fusarium oxysporum and the gram-negative bacteria Escherichia coli. Myticin C was next identified as an extremely variable antimicrobial peptide with antiviral and antibacterial activities being also the first chemokine/cytokine-like molecule identified in bivalves and one of the few examples among all invertebrates (26, 27).

It is well known that mussel myticins show high variability at the transcriptional level, but the reasons and the mechanisms underlying this variability are still poorly understood (43-45). The lack of information is even more pronounced at the proteomic level. This study constitutes the first attempt to identify proteins in the haemolymph and immune cells from these animals. The presence of myticin in serum suggests that this molecule is a secreted antiviral compound.

Not all haemocytes express myticins, and the expression of this peptide remained
unchanged after immune challenge and was not associated with phagocytosis. It is, therefore, a molecule that is constitutively expressed in mussel cells in normal conditions and is present in the haemocytes but also secreted to the serum. Until now, non-secreted compounds with antiviral effects have been identified in mollusc haemolymph in experiments involving hybrid abalone, *Haliotis rubra × laevigata*, which was infected with abalone herpesvirus (AbHV-1) (46). Prior to this study, the only reported antiviral activity for molluscs was found in the Pacific oyster fresh filtered haemolymph against HSV-1 (47). The EC$_{50}$ value found in that study was 425 µg/mL, which in light of the current knowledge on antiviral compounds is too high for the inhibitory activity claimed: EC$_{50}$ values beyond 100 µg/mL are of limited practical value. Therefore, in other molluscs, antiviral activity against the herpes simplex virus has been observed, but it did not correlate with higher resistance to bivalve herpesviruses (46-48). In this study, for the first time, a molecule from a bivalve has shown antiviral activity against a virus affecting molluscs.

The inhibitory activity of myticin against OsHV-1 prompted us to investigate whether it could also inhibit human herpesviruses. The lack of antiviral activity of the unmodified myticin C peptide against human herpesviruses could be related to intrinsic availability properties of this peptide for the cell system employed, rather than to specific differences between both ostreid and human herpesviruses, for which a common viral ancestor has been suggested (49). An interesting finding of this study was the observation of anti-HSV activity of Myt-C when the cell-penetrating motif derived from the HIV-1 Tat protein was fused to its C-terminus. This modification of myticin C appeared to improve its internalization in Vero cells without significantly increasing its cytotoxicity.

In the absence of Tat-derived cell-penetrating motif, a similar enhancement in
anti-HSV activity of Myt-C was recorded when this peptide was encapsulated into non-ionic surfactant nanovesicles. These nanovesicles (or nanosomes) are able to fuse with the cell plasma membrane releasing the peptide into the cytoplasm. Taken together, these results suggest that myticin peptides selectively inhibit an intracellular step of the virus replication cycle, instead of directly inactivating free virus particles in the extracellular environment (virucidal activity). The findings presented here highlight a potential use of these antimicrobial peptides from mussels in antiviral therapy not only for veterinary purposes but also for human medicine. Further investigation is needed to elucidate the precise mechanisms of antiviral action of myticins.

In conclusion, we still do not know much about the antiviral immune response of invertebrates, and the case of marine bivalves is especially important due to the enormous impact that viral diseases have on shellfish production. Molluscs have the potential to be a source of antimicrobial and antiviral compounds, but most members of the phylum Mollusca have not yet been examined for the presence of these bioactive molecules (36). Myticins are examples of the biotechnological and therapeutic potential of bivalves, which are currently limited to serving as a food supply, and may represent an added value for their culture.

ACKNOWLEDGEMENTS

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Antonio Figueras and Beatriz Novoa especially want to acknowledge Prof. Amparo Estepa, one of the co-authors of this article, who unfortunately passed away at a very young age before it was published. We want to remember her intelligence,
vitality and charm and above all we thank her for being our friend: we miss you, Amparo.

**FUNDING INFORMATION**

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Table 1. Triple TOF Mascot search results of *M. galloprovincialis* hemocytes and serum samples. Matched peptides are highlighted in the complete myticin sequences. Acc. nº; accession number; % Cov.: % of coverage; DH: dehydrogenase

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MKATILLAVLVAV
FVAGTEAHSHACT
SYWCGKF**FCGTAS**
CTHYLCRVLHPG
KMCACVHCSRVRN
NPFRVNQVAKSIN
DLDYTPIMKSME
NLNGMDML

MKATILLAVVVVV
IVGVQEAEQSIPCTS
YYCSKF**FGSAGCS**
LYGCLYLHPGKIC
YCLHCRRAESPLA
LSGARNVNEQNK
EMVNSPVNMVE
NLNGEMNMF

MKATILLAVLVA
FVAGTEAHSHACT
SYWCGKF**FCGTAS**
CTHYLCRVLHPG
KMCACVHCSRVRN
NPFRVNQVAKSIN
DLDYTPIMKSME
NLNGMDML
Figure legends

**Figure 1.** Experimental *in vivo* and *in vitro* infections of *M. galloprovincialis* and *C. gigas* with OsHV-1. (A) Naïve mussels and oysters were injected in the adductor muscle with an OsHV-1 suspension at 15 ºC. Control groups were inoculated with an equivalent volume of FSW. Cumulative mortality was determined in mussels and oysters infected with OsHV-1 for 15 days. The data are presented as the mean value from three independent infections with standard deviations. Infected oysters reached high mortality rates (84 ± 7 %), whereas the mortality of infected mussels was similar to the control animals. (B) Time course of OsHV-1 replication in primary cultures of mussels and oysters haemocytes. The cells were seeded in a 24-well plate adding 1 mL/well of a 3×10^6 cells/mL haemocyte suspension and incubated at 15 ºC for 1 h for adhesion. After this incubation the supernatants were removed and the cells were infected with an OsHV-1 suspension diluted 1:5 in FSW for 1 h at 22 ºC. After infection, the supernatants were removed and cells were washed with FSW. Additional haemolymph from oysters and mussels was extracted, centrifuged to eliminate cell components and 0.22 mm-filtrated, and it was added to the corresponding wells for incubation at 15ºC. Cultures were sampled at 1, 6 and 24 h.p.i. to evaluate viral load by qPCR. This experiment was conducted using duplicated cell cultures. The data are presented as the mean value from four independent infections with standard deviations. The viral load in oyster haemocytes was higher than in mussel, and it showed an increasing tendency during the experiment, whereas it did not significantly change in mussel haemocytes.
Figure 2. Viral loads determined by qPCR in oyster haemocytes infected with OsHV-1 and incubated for 24 h in the presence of oyster haemolymph or mussel haemolymph. Oyster haemocytes were seeded in a 24-well plate adding 1 mL/well of a $3 \times 10^6$ cells/mL haemocyte suspension in FSW and incubated at 15 ºC for 1 h before the infection with OsHV-1. After this incubation the supernatants were removed and the cells were infected with an OsHv-1 suspension diluted 1:5 in FSW for 1h at 22 ºC. After infection, the supernatants were removed and cells were washed with FSW. New haemolymph from oysters and mussels was extracted, centrifuged to eliminate cell components and 0.22 mm-filtrated. Half of the cell cultures were incubated in oyster haemolymph and the others received mussel haemolymph. The haemocytes were incubated at 15ºC and sampled after 24 h. to evaluate viral load by qPCR. Two replicates were used in each of the four independent experiments conducted. The data represent the mean value of a representative experiment with the standard deviation. The asterisk represents statistically significant differences between the treatments (p<0.05). Oyster haemocytes incubated with mussel haemolymph showed a significant reduction in the viral titer compared with those incubated with their own haemolymph.

Figure 3. (A) Immunohistochemical localization of myticin C in mussel haemocytes. Mussel haemocytes were then incubated with an anti-myticin C primary antibody and stained with a fluorophore-conjugated secondary antibody (green fluorescence) and DAPI (blue, cell nuclei). It was observed that myticin C is constitutively expressed in the cytoplasm of mussel haemocytes, although not all haemocytes were positives for myticin C labelling, suggesting the heterogeneity of these cells. (B) Distribution of myticin C-positive cells in non-stimulated samples, as determined by flow cytometry. An anti-myticin C antibody and a fluorophore-conjugated secondary antibody were used for labelling cells expressing this protein. The cut-off used in the X parameter
FL1-H (log scale) was 37.81. (C) Percentage of Myt-C-positive cells after in vivo stimulation with LPS, Zym or dead bacteria compared with control individuals (FSW). Haemolymph was extracted after 24 h and the percentage of granulocytes and hyalinocytes expressing myticin C was determined by flow cytometry using the anti-myticin C antibody and a fluorophore-conjugated secondary antibody. The cut-off used in the X parameter FL1-H (log scale) was 37.81. The number of myticin C-positive cells was not significantly affected by the treatments. (D) Confocal image of Myt-C-positive haemocytes engulfing Zym A-TRED particles. The percentage of cells positive and negative for Myt-C expression and/or Zym A-TRED signals is shown in the table. Scale bar=10 µm.

**Figure 4.** Viral load determined by qPCR in oyster haemocytes infected with OsHV-1 and incubated with oyster haemolymph (OH), mussel haemolymph (MH) or with the synthetic myticin C peptide diluted in oyster haemolymph (OH+peptide) or mussel haemolymph (MH+peptide) 24 h after infection. Oyster haemocytes were seeded in a 24-well plate adding 1 mL/well of a 3×10^6 cells/mL haemocyte suspension in FSW and incubated at 15 ºC for 1 h before the infection with OsHV-1. After this incubation the supernatants were removed and the cells were infected with an OsHv-1 suspension diluted 1:5 in FSW for 1h at 22 ºC. Then, the supernatants were removed and cells were washed with FSW. New haemolymph from oysters and mussels was extracted, centrifuged to eliminate cell components and 0.22 mm-filtrated. Cell cultures were incubated with OH, MH, OH+peptide or MH+peptide. The haemocytes were incubated at 15ºC and sampled after 24 h to evaluate viral load by qPCR. Two replicates were used in each of the four trials conducted. The data are presented as the mean value from four independent experiments with the standard deviations. An important reduction of
the viral titer was observed both in oyster haemocytes incubated with MH and in those treated with the peptide.

**Figure 5.** Cytotoxic effects on Vero cells as determined by the MTT assay (solid lines) and antiviral effects of myticin peptides against HSV-1 (dark bars) and HSV-2 (light grey bars), as determined by the decrease in virus-induced CPE on Vero cells: (A) cytotoxic and antiviral activities of unmodified Myt-C peptide; (B) cytotoxic and antiviral activities of Myt-Tat peptide; (C) cytotoxic and antiviral activities of unmodified Myt-C peptide encapsulated into nanosomes; (D) cytotoxic and antiviral activities of empty nanosomes. Data are presented as the mean value from 3 independent experiments with their standard deviations. The percentages of CPE associated with different treatment concentrations within each virus were compared using the Bonferroni post-hoc test. The asterisks represent the level of significance of the differences found for each concentration compared to the untreated (negative) controls. Significant differences are displayed as ***(0.0001<p<0.001),**(0.001<p<0.01) or *(0.01<p<0.05). The scientific notation numbers above each bar represent the virus titres obtained by plaque assay from each pool of replicate treatments within the corresponding concentration (pfu/mL).