

## RESEARCH ARTICLE

# Oreoch-1: A broad-spectrum virus and host-targeting peptide against animal infections

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In recent decades, the global rise of viral emerging infectious diseases has posed a substantial threat to both human and animal health worldwide. The rapid spread and accumulation of mutations into viruses, and the limited availability of antiviral drugs and vaccines, stress the urgent need for alternative therapeutic strategies. Antimicrobial peptides (AMPs) derived from natural sources present a promising avenue due to their specificity and effectiveness against a broad spectrum of pathogens. The present study focuses on investigating the antiviral potential of oreochromicin-1 (oreoch-1), a fish-derived AMP obtained from Nile tilapia, against a wide panel of animal viruses including canine distemper virus (CDV), Schmallenberg virus (SBV), caprine herpesvirus 1 (CpHV-1), and bovine herpesvirus 1 (BoHV-1). Oreoch-1 exhibited a strong antiviral effect, demonstrating an inhibition of infection at concentrations in the micromolar range. The mechanism of action involves the interference with viral entry into host cells and a direct interaction between oreoch-1 and the viral envelope. In addition, we observed that the peptide could also interact with the cell during the CDV infection. These findings not only highlight the efficacy of oreoch-1 in inhibiting viral infection but also emphasize the potential of fish-derived peptides, specifically oreoch-1, as effective antiviral agents against viral infections affecting animals, whose potential to spill into humans is high. This research contributes valuable insights to the ongoing quest for novel antiviral drugs with the potential to mitigate the impact of infectious diseases on a global scale.

**KEYWORDS**

AMPs, antiviral, bunyavirus, emerging diseases, fish peptides, herpesvirus, oreochromicin-1, paramyxovirus, zoonotic diseases

## 1 | INTRODUCTION

In the last decades, several emerging infectious diseases occurred, threatening both animal and public health globally. Among them, viral infections are much more characterized by fatal outcomes and difficulties to limit the diffusion because viruses rapidly spread and accumulate mutations, and the arsenal of antiviral drugs and vaccines is very

restricted. The sources of many emerging viral diseases are animals, including domestic animals and wildlife, due to their close contact and the rising demand of animal products (egg, meat, milk, fish, and their derivatives).<sup>1,2</sup> This is intensified by globalization and trade, which can transport pathogens to distant parts of the world, countries and continents. The recent coronavirus disease 2019 (COVID-19) is only the last example of zoonotic infection, and now, there is a growing interest

in investigating the interface animal–human in order to prevent future epidemics/pandemics arising from cross-species transmission.<sup>3</sup>

Researchers have turned their attention to explore alternative therapeutic options beyond conventional antiviral drugs. Differently from the traditional antivirals, often associated with adverse effects and the risk of resistance, antimicrobial peptides (AMPs) derived from natural sources offer a promising option.<sup>4</sup> These peptides have evolved in organisms over millennia and are highly specific and effective against a wide range of pathogens, including bacteria, viruses, fungi and parasites.<sup>5</sup> In detail, fish peptides have been described since 1980, but they obtained a great success only around 15 years ago for their strong potential utilization in human health. In detail, the AMPs database (APD; <http://aps.unmc.edu/AP/main.php>) comprises over than 100 peptides recognized as fish-derived AMPs.<sup>6</sup>

In the aquatic environment, there is a recurring and significant level of exposure to a vast array of pathogens. For this reason, fish skin is covered by an extrinsic barrier consisting of mucus layer and AMPs which cooperate together as a protective chemical shield, by acting as the first line of defense against pathogens.<sup>7</sup> Therefore, AMPs, due to their small size and amphiphilic structure, rapidly move to the infection site upon microbial invasion. They target microorganism surface, altering membrane permeability and disrupting its structure. Their selectivity for pathogens over host cells arises from the different membrane targets. AMPs can induce pore formations, hinder cell-wall synthesis and impede crucial processes, leading to microbial death. Various models explain peptide mechanisms, including energy-dependent (barrel stave, carpet, and toroidal pore) and energy-independent (macropinocytosis) models.<sup>8–13</sup>

Furthermore, AMPs have the ability to eliminate pathogens that commonly exhibit resistance to numerous antimicrobial drugs, offering potential pathways for the advancement of future therapeutic molecules.<sup>14</sup> In fact, as intrinsic components of the innate immune system and due to their diverse structures, AMPs pose a significant obstacle to microorganisms in developing resistance. In addition, each AMP is characterized by a unique mechanism of action; thus, pathogens have to formulate an effective defense strategy every time.<sup>15</sup>

The antibacterial activity of fish AMPs is largely reported. For example, epinecidin-1 isolated from grouper acts by disrupting membrane of *Trichomonas vaginalis* at concentrations ranging from 200 to 25 µg/mL.<sup>16</sup> Hepcidin 1–5 is involved in the innate immunity response of tilapia and has an inhibitory effect against *Staphylococcus aureus* (*S. aureus*) at 50 µg/mL and *Enterococcus faecium* (*E. faecium*) at 100 µg/mL.<sup>17</sup>

Recently, the peptide type I interferon (IFN-1) from the grass carp has been discovered as a strong antimicrobial and anti-inflammatory agent.<sup>18</sup> In particular, authors identified a portion derived from the fifth helical region, rich in basic amino acids and highly cationic, with potent antibacterial effect both against Gram-positive and Gram-negative bacteria by disrupting and permeating membrane, respectively. The minimum bactericidal concentration (MBC<sub>90</sub>) ranges from 8 to 16 µg/mL for Gram-negative and from 2 to 4 µg/mL for Gram-positive bacteria.<sup>18</sup>

Conversely, the antiviral activity of fish peptides has been poorly elucidated, and mainly, they interfere with fish virus infections. In

detail, β-defensins 1<sup>19</sup> and the hepcidin 1–5 were demonstrated to block the viral hemorrhagic septicemia virus (VHSV) and nervous necrosis virus (NNV) infection, by inhibiting viral particles adsorption and penetration into host cells.<sup>20,21</sup> Piscidin 1 N, 1 H, 2, and 3, instead, were found to be active against the channel catfish virus (CCV) and frog virus 3 (FV3) by reducing the viral infectivity.<sup>22</sup>

Among the piscidin family, noteworthy peptides are oreochromicins, extracted from the epidermal mucus of *Oreochromis niloticus*, commonly known as Nile tilapia fish.<sup>23,24</sup> This species is characterized by a greater resistance against viral, bacterial and fungal infections compared with other species living in aquaculture. Moreover, its mucus secretion has the capability to impede the proliferation of *Vibrio* and other harmful bacteria, thus limiting their spread.<sup>25</sup>

In the last years, several studies have highlighted the significant antibacterial properties of different piscidins, evidencing their potential as novel and valuable weapons in the fight against microbial diseases.<sup>24–29</sup>

The present study aims to investigate for the first time the antiviral activity of oreochromicin-1 (oreoch-1), particularly against animal viruses such as canine distemper virus (CDV), Schmallenberg virus (SBV), and two animal herpesviruses (HVs), the caprine type 1 (CpHV-1) and the bovine type 1 (BoHV-1). By shedding light on the mechanisms of action and efficacy of oreoch-1, we aim to contribute valuable insights into the control of zoonotic diseases, ultimately improving both human and animal health care.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

Protected amino acids; coupling agents (HATU, Oxyma); Fmoc-Rink amide AM resin; solvents, such as acetonitrile (CH<sub>3</sub>CN) and dimethylformamide (DMF); and other products such as trifluoroacetic acid (TFA), sym-collidine, diisopropylethylamine (DIPEA), and piperidine were purchased from Merck (Milan, Italy). Cell culture medium, fetal bovine serum (FBS), and phosphate-buffered saline (PBS) were acquired by Microtech (Naples, Italy), while the antibiotic solution by Himedia (Naples, Italy). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit, carboxymethylcellulose (CMC), formaldehyde, and crystal violet were bought by Sigma-Aldrich (St. Louis, Missouri, USA).

### 2.2 | Peptide synthesis and characterization

Oreoch-1 (single-letter amino acid sequence: FIHHIIGLLFSVKGKIH-GLIHGH) was synthesized as a C-terminal amidated peptide following as reported elsewhere.<sup>29,30</sup> In detail, we used a fully automated and computer-controlled Syro I multiple peptide synthesizer from Multi-SynTech GmbH (Witten, Germany) equipped with a 24-position One U-Typ reactor block with 5 mL reactors. The peptide was synthesized using the solid-phase method at 50 µmol scale following the Fmoc

strategy, using Fmoc-derivatized standard amino acids and a Rink amide MBHA resin (0.35 mmol/g substitution) as the solid support. Amino acid activation was achieved using HATU/collidina (4 equiv.) and Oxyma/DIC (4 equiv.) in DMF, while Fmoc deprotections were performed using morpholine/DBU solutions in DMF. The peptide was removed from the resin by treatment with a TFA/TIS/H<sub>2</sub>O mixture (90:5:5, by volume) and was then precipitated in ice-cold diethyl ether and lyophilized. Purification was carried out on a WATERS 2545 HPLC preparative system (Waters, Milan, Italy) coupled with a WATERS 2489 UV/visible detector, using a linear gradient of CH<sub>3</sub>CN/0.05% TFA in H<sub>2</sub>O/0.05% TFA from 5 to 70% in 20 min, at a flow rate of 12 mL/min, using a Jupiter 5 μm C18 column (300 Å, 150 × 21.2 mm). LC/MS characterization of the purified peptide was performed using an Agilent 1290 Infinity ESI-TOF-MS LC/MS system coupled to an Agilent 6230 time-of-flight (TOF) LC/MS system (Agilent Technologies, Cernusco sul Naviglio, Italy), coupled with a photodiode array (PDA) detector and a 6230 TOF MS detector, along with a binary solvent pump degasser, a column heater, and an autosampler. The analysis was performed using a C18 Waters xBridge column (3 μm, 4.6 × 5.0 mm), applying a linear gradient of CH<sub>3</sub>CN/0.05% TFA in H<sub>2</sub>O/0.05% TFA from 5% to 70% in 20 min, at a flow rate of 0.2 mL/min. The relative purity of the peptide was calculated as the ratio of the peak area of the target peptide to the sum of the areas of all peaks detected by UV chromatograms at 210 nm. The purity of the peptide was estimated to be greater than 95%. The peptide was solubilized in sterile water and stored at −20°C until use.<sup>29,30</sup>

### 2.3 | Cell culture and viral strain

Vero/hSLAM cells (ECACC 04091501, based in Porton Down, United Kingdom), baby hamster kidney cells (BHK-21, ATCC CCL-10, located in Manassas, VA, USA), and Madin Darby bovine kidney cells (MDBK, ATCC CCL-22) were grown in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS.

For the propagation process, CDV (strain Onderstepoort) was grown on VERO/hSLAM cells, while BoHV-1 (Cooper strain) and CpHV-1 (reference Swiss strain E/CH) were cultured using the MDBK cell line. Instead, SBV was propagated on BHK-21 monolayers.<sup>31</sup>

All the cell lines were infected at a multiplicity of infection (MOI) of 0.01, and subsequent viral titers were assessed using plaque assays. The original titers of the viruses employed in this study were 10<sup>7</sup> plaque-forming units (PFU) per milliliter (mL) for CDV, BoHV-1, and CpHV-1. The SBV titer was determined using the tissue culture infectious dose (TCID<sub>50</sub>) method, following the endpoint titration approach of Reed and Muench.<sup>32</sup>

### 2.4 | Peptide cytotoxicity

Peptide cytotoxicity was evaluated via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay by measuring the

absorbance at 570 nm. Vero/hSLAM, BHK-21, and MDBK cells were seeded in 96-well microtiter tissue culture plates (2 × 10<sup>4</sup> cells) and incubated 24 h at 37°C in 5% CO<sub>2</sub>. Then, the cells were incubated with different concentrations of the peptide (ranging from 100 to 0.39 μM) for 24 h. The negative control (CTRL −) consisted of cells treated with pure DMSO, while the positive control (CTRL +) consisted of untreated cells. All experiments were performed in triplicate, and the mean standard deviations (SDs) were reported. To determine the 50% cytotoxic concentration (CC<sub>50</sub>), a nonlinear regression analysis was carried out using GraphPad Prism software (version 8.0.1) by GraphPad Software.

### 2.5 | Hemolytic activity

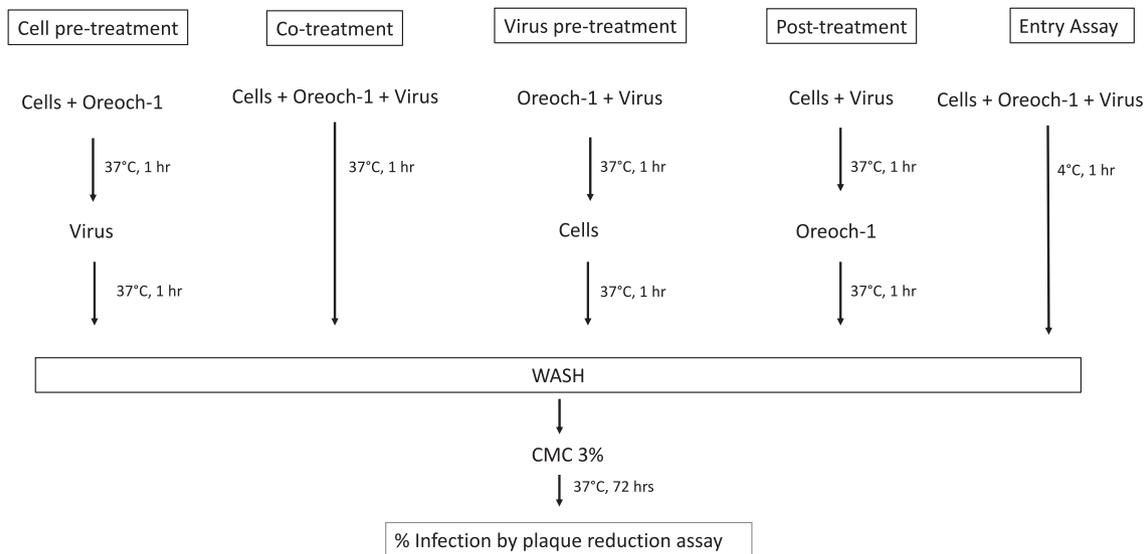
The hemolytic potential of oreoch-1 was assessed utilizing freshly human erythrocytes from a healthy volunteer. Initially, a 10 mL suspension of blood was subjected to centrifugation at 500 × g for 5 min. Following centrifugation, the plasma level was identified, gently aspirated, and discarded. The hematocrit tube was subsequently replenished with a solution containing 150 mM NaCl to restore it to the original plasma level and underwent three wash cycles. The erythrocytes were then diluted in PBS at a ratio of 1:50, and 190 μL of the resulting solution was dispensed into the wells of a U-shaped bottom 96-well plate. Then, 10 μL of oreoch-1, varying in concentrations from 25 to 0.7 μM, was added to the wells and incubated for 1 h at 37°C. Negative and positive controls were PBS and 1% (v/v) Triton X-100, respectively. Subsequently, the plate was subjected to centrifugation for 5 min at 500 g, and the supernatant was carefully transferred to a fresh plate. The absorbance of the released hemoglobin was then measured at 540 nm using the microplate reader TECAN SUNRISE (Tecan Group Ltd., Männedorf, Switzerland).<sup>32</sup> To determine the extent of hemolysis, the following formula was used:

$$\% \text{ of hemolysis} = [(\text{Abs sample}) / (\text{Abs Triton})] \times 100.$$

### 2.6 | Antiviral activity

The antiviral activity of the peptide against CDV, CpHV-1, and BoHV-1 was evaluated via plaque reduction assays (Figure 1). The tests conducted differ in the time at which the peptide was added to the cells<sup>33</sup>: during the infection (co-treatment), after incubation of the virus with the peptide (virus pre-treatment), before viral infection (cell pre-treatment), and finally, after the viral infection (post-treatment) as described in Figure 1.

In detail, Vero/hSLAM and MDBK cells were seeded into 24-well plates (1.2 × 10<sup>5</sup> cells) 24 h before the experiments. For the co-treatment assay, cells were simultaneously exposed to noncytotoxic peptide concentrations and virus at a MOI of 0.01 for 1 h at 37°C in an atmosphere containing 5% CO<sub>2</sub>. In the virus pre-treatment assay, the peptide was incubated with the virus at a MOI of 0.1 for 1 h at 37°C. Subsequently, the mixture was diluted and applied to the cells



**FIGURE 1** Schematic diagram of assays performed in this study. The conducted tests vary in the timing of peptide addition to the cells: prior to viral infection (cell pre-treatment), during infection (co-treatment), after the virus has been incubated with the peptide (virus pre-treatment), or ultimately after viral infection (post-treatment). In the entry assay, cells were infected with the virus together with oreoch-1 for 1 h at 4°C.

for another hour at 37°C with 5% CO<sub>2</sub>, so that the peptide reached a nonactive concentration and the virus was at the final MOI of 0.01. In the cell pre-treatment assay, cells were initially exposed to the peptide and then infected with the virus (MOI 0.01) at 37°C with 5% CO<sub>2</sub>. In the post-treatment assay, cells were first infected with the virus (MOI 0.01) for 1 h, followed by the addition of peptide for another hour at 37°C with 5% CO<sub>2</sub>.

At the end of each treatment, the cell monolayer was first washed with PBS 1× and then overlaid with 3% CMC in the presence of the culture medium at 37°C in 5% CO<sub>2</sub>. After 72 h, cells were fixed and stained with 4% formaldehyde and 0.5% crystal violet, respectively, and viral plaques were counted. The calculation of the percentage of viral inhibition (%) was carried out by comparing it with the infected control (CTR –), using the following formula:

$$\% \text{viral inhibition} = \left[ 100 - \frac{\text{(plaques counted in the test sample)}}{\text{(plaques estimated in the negative control)}} \right] \times 100.$$

The evaluation of peptide antiviral efficacy against SBV was conducted through the TCID<sub>50</sub> assay. BHK-21 cells were seeded into 96-well microtiter tissue culture plates at a density of  $2 \times 10^4$  cells and incubated for 24 h. The next day, different concentrations of peptide and SBV were incubated together for 1 h at 37°C in an environment with 5% CO<sub>2</sub>. Subsequently, a diluted mixture totaling 100 μL was applied to the cell monolayer. The wells were monitored for any signs of cytopathic effect (CPE) over a 72 h period, and the Reed and Muench method was employed to calculate the outcomes as  $10 \times$  TCID<sub>50</sub> per milliliter.

## 2.7 | Entry assay

In the entry assay (Figure 1), Vero/hSLAM and MDBK cells were initially seeded in 24-well plates as specified above. Cells were infected with the virus (MOI 0.01) together with oreoch-1 at a given concentration for 1 h at 4°C. Finally, the cells were washed with PBS 1× and covered with complete medium supplemented with 3% CMC and incubated at 37°C in an environment with 5% CO<sub>2</sub>. As in the other antiviral assays, viral plaques were counted. The percentage of infectivity inhibition was determined by comparing the plaques of cells treated with oreoch-1 and those of the negative control (infected cells).

As in antiviral assays, BHK-21 cells were seeded into 96-well microtiter tissue culture plates at a density of  $2 \times 10^4$  cells and incubated for 24 h. The next day, cells were infected with SBV virus (MOI 0.01) together with the different dilution of oreoch-1 for 1 h at 4°C. Then, the cells were washed with PBS 1× and covered with complete medium and incubated at 37°C in an environment with 5% CO<sub>2</sub>. Wells were monitored for any signs of CPE over a 72 h period, and the Reed and Muench method was used to calculate the outcomes as  $10 \times$  TCID<sub>50</sub> per milliliter.

## 2.8 | Statistical analysis

All tests were performed in triplicate and expressed as mean ± SD calculated by GraphPad Prism (version 8.0.1). One-way ANOVA followed Dunnett's multiple comparisons test was performed; a value of  $p \leq 0.05$  was considered significant.

### 3 | RESULTS

#### 3.1 | Peptide synthesis and characterization

Oreoch-1 was chemically synthesized and purified to >95%. The identity and quality of the synthetic peptide by RP-HPLC and mass spectroscopy were validated (Table 1).<sup>29</sup>

#### 3.2 | Cytotoxicity

Prior to assess the potential antiviral activity of oreoch-1, an in vitro cytotoxicity analysis was conducted by MTT dye reduction. The cell lines BHK-21, Vero/hSLAM, and MDBK used in this paper were incubated with the peptide at concentrations from 0.75 to 100  $\mu\text{M}$ , for 24 h. Cell viability was quantified as a percentage relative to untreated cells (CTRL +). As shown in Figure 2, oreoch-1 has no cytotoxic activity at concentrations lower than 50  $\mu\text{M}$  in all cell lines tested.

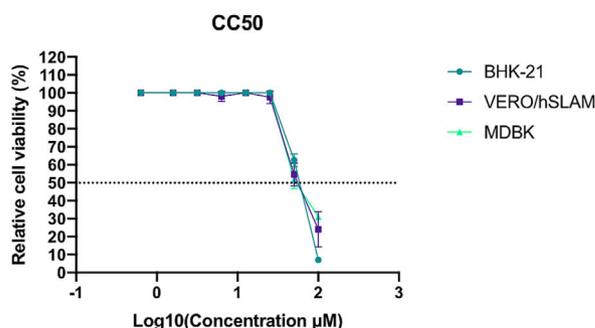
#### 3.3 | Hemolytic activity

To better evaluate the potential lytic effect of oreoch-1 on cellular membranes, the hemolysis assay was conducted. The assay revealed erythrocyte lysis rates of 12% and 5.5% at concentrations of 25 and 12.5  $\mu\text{M}$ , respectively (Figure 3). Very low levels of hemolysis were observed at the remaining concentrations. Triton X-100 at the concentration of 1%, used as positive control, exhibited complete lysis (100%).

**TABLE 1** Schematic table of oreoch-1 characterization.

Entry	Sequence (single-letter)	Theoretical MW (Da)	Experimental MW (Da)	Net charge
Oreoch-1	FIHHIIGGLFVSGKHHIHLGH	2525.39	2525.36	1.5

Note: Amino acid sequence of oreoch-1, theoretical and experimental peptide masses, and net peptide charge at pH 7.0. Oreoch-1 was prepared as a C-terminal amidated peptide. The MWs were reported as monoisotopic masses.



**FIGURE 2** Dose–response curves for the compound against each cell line and  $\text{CC}_{50}$  (reported in  $\mu\text{M}$ ), HillSlope, and  $R^2$  values obtained by the fitting of the curves. The viability of BHK-21, Vero/hSLAM, and MDBK cells was analyzed 24 h after exposure to different concentrations of oreoch-1. HillSlope: measure of the steepness of the dose–response curve. A positive value indicates a cooperative response, while a negative value suggests an inhibitory effect.  $R^2$  (coefficient of determination): indicates how well the model fits the data. Ranges from 0 to 1, with 1 representing a perfect fit. Higher  $R^2$  values suggest a more accurate prediction of the biological response to varying concentrations.

#### 3.4 | Antiviral activity

##### 3.4.1 | Antiviral activity of oreoch-1 against CDV

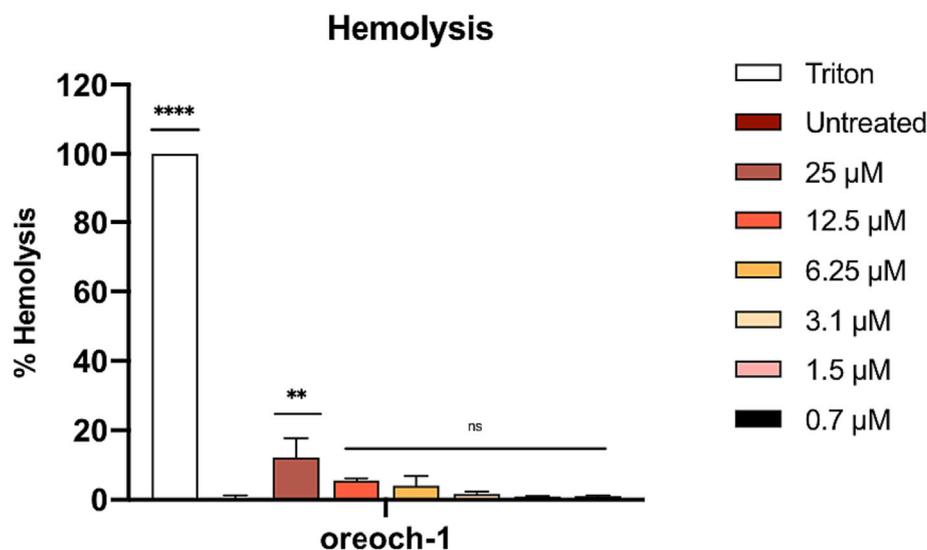
The potential antiviral activity of oreoch-1 was evaluated in vitro by plaque assay against CDV in Vero/hSLAM cell model, using a serially diluted peptide ranging from 25 to 0.75  $\mu\text{M}$ . Furthermore, a series of experiments were conducted using four distinct treatment schemes, as depicted in the Figure 1, to evaluate at which phase of the viral infection, whether extracellular or intracellular, the peptide can exert its antiviral effect.

As shown in Figure 4, oreoch-1 exerts significant dose-dependent antiviral activity in virus pre-treatment and co-treatment experiments. The peptide, in fact, completely blocks the infection at the maximum concentration tested (25  $\mu\text{M}$ ) and shows, in both cases, an  $\text{IC}_{50}$  of 7.5  $\mu\text{M}$ . In contrast, in cell pre-treatment experiments, the peptide blocks infection by 60% at 25  $\mu\text{M}$ , while it is totally ineffective in post-treatment tests. These results showed that oreoch-1 provides significant protection against CDV and suggests that it not only targets the virus but also has effects on cell membranes.

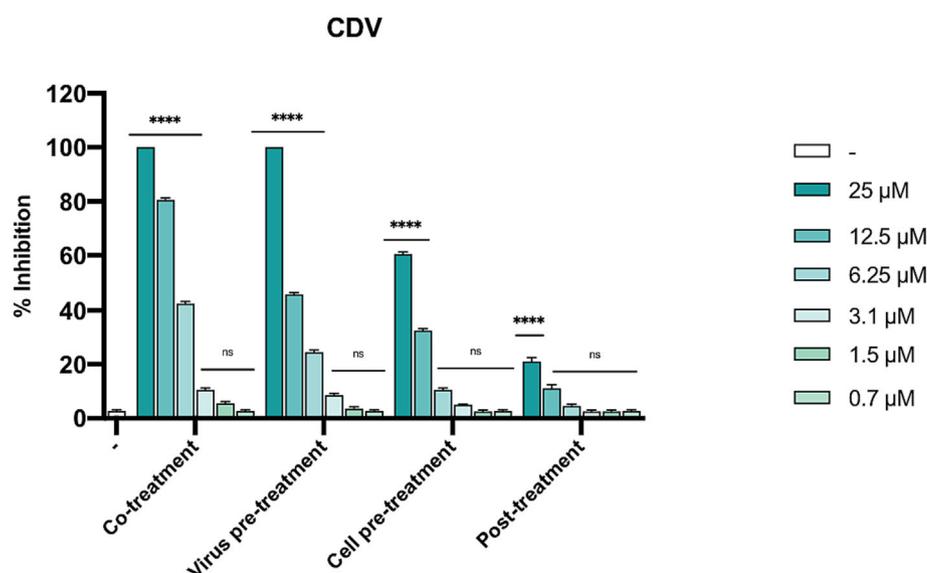
##### 3.4.2 | Antiviral activity of oreoch-1 against CpHV-1 and BoHV-1

The efficacy of oreoch-1 against animal HVs, namely, CpHV-1 and BoHV-1, was evaluated using the same experiments performed against CDV (Figure 5) but in MDBK cells. Similar to what was observed for CDV, the peptide significantly and dose dependently inhibits viral infection in co-treatment and virus pre-treatment assays,

Cell Line	$\text{CC}_{50}$	HillSlope	$R^2$
BHK-21	56.07	-4.709	0.9985
VERO/hSLAM	58.58	-2.629	0.9684
MDBK	61.40	-2.325	0.9466



**FIGURE 3** The ability of oreoch-1 to interfere with eukaryotic red cells. Thus, the cytotoxicity was assessed by hemolysis assay. Statistical analyses were determined by ANOVA with Dunnett's test for multiple comparisons. Significances are referred to the untreated red cells. \*\*\*\*  $p < 0.0001$ ; \*\*  $p = 0.0067$ ; ns: non-significant.



**FIGURE 4** Antiviral activity of oreoch-1 against CDV infection. Different assays were performed in Vero/hSLAM cells to explore at which stage of the infection the peptide was active: co-treatment, virus pre-treatment, cell pre-treatment, and post-treatment (see Figure 1). Infected cells used as negative control are indicated as CTRL -. \*\*\*\*  $p < 0.0001$ ; ns: non-significant.

suggesting the same operating mode. However, compared with CDV, against the HVs, the peptide provides a more pronounced antiviral effect by completely blocking viral infection up to 6.25 and 12.5  $\mu\text{M}$ , respectively, against CphV-1 and BoHV-1. Furthermore, against HV, oreoch-1 is ineffective not only in post-treatment assays but also in cell pre-treatment assays. The results obtained once again suggest the action of oreoch-1 against the very early stages of the infectious process and clearly indicate that against HVs, the peptide targets the viral membranes and not that of host cells.

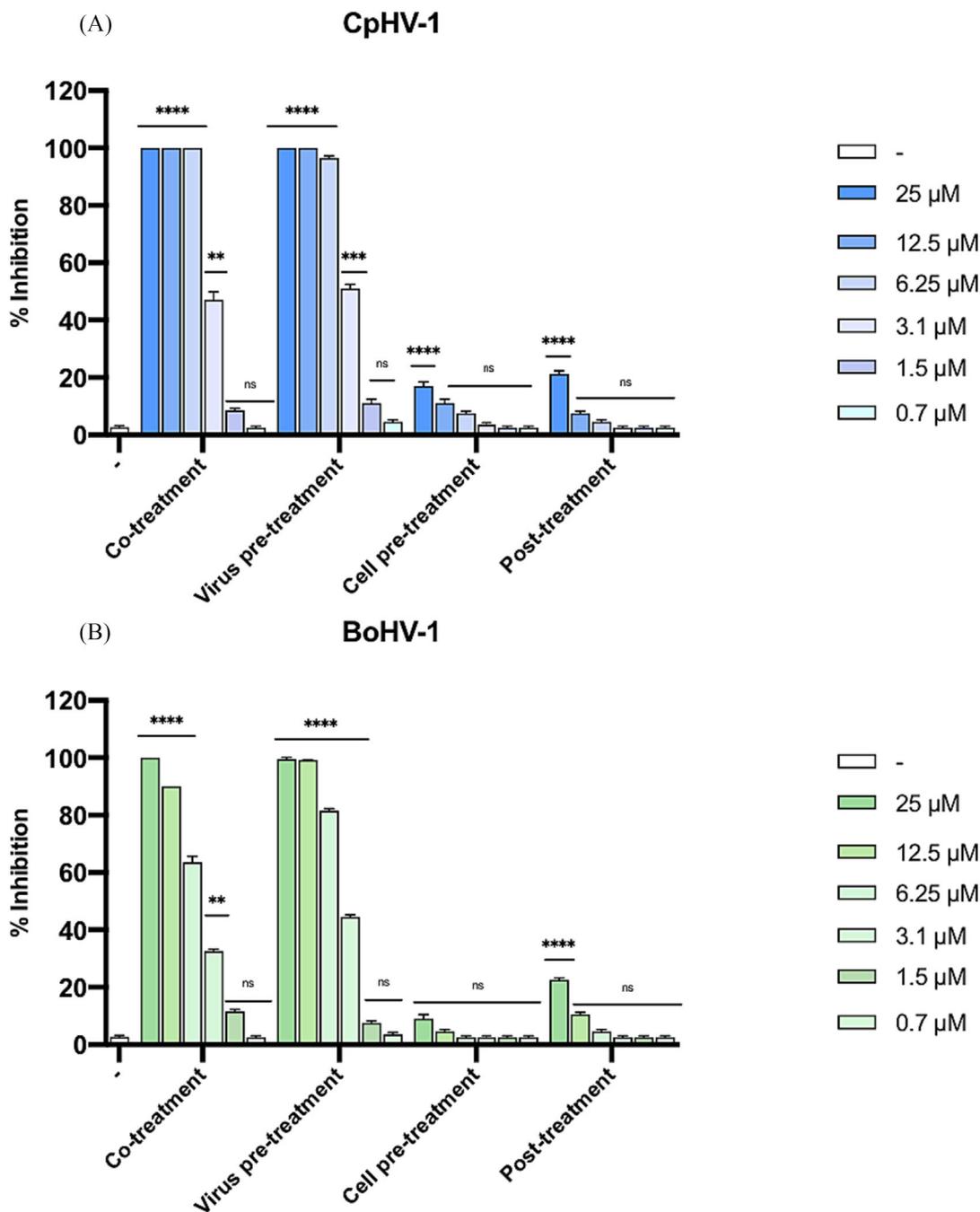
### 3.4.3 | Antiviral activity of oreoch-1 against SBV

The impact of oreoch-1 on SBV infection was evaluated on the BHK-21 cell line via the TCID<sub>50</sub> method. Briefly, the same treatment schemes were performed on cells at noncytotoxic concentrations of oreoch-1. The CPE was monitored, and the data were analyzed using a colorimetric assay (Figure 6). Again, infection was reduced when the

peptide was incubated at the same time as the virus, suggesting a direct inhibitory effect on viral particles, as observed with other animal viruses. Moreover, the peptide is more active against SBV than the other viruses tested, as indicated by the IC<sub>50</sub> of 5 and 4  $\mu\text{M}$  in the virus co-treatment and pre-treatment tests, respectively.

### 3.5 | Entry assay

The next question investigated was if oreoch-1 could act in the entry phase of viral infection. Therefore, cells were first infected with each animal virus at 4°C, and then, the peptide was added to the cell monolayer at 37°C (Figures 1 and 7). The low temperature blocks the virus to penetrate into the cell, and the subsequent shift in temperature allows the virus and peptide to compete for the entry. Oreoch-1 is able to interfere with the entry stage of each virus used in the present study, as demonstrated by the low IC<sub>50</sub> at 12.5  $\mu\text{M}$  for CDV, 4.8  $\mu\text{M}$  for CphV-1, 5.5  $\mu\text{M}$  for BoHV-1, and 6.2  $\mu\text{M}$  for SBV. These data



**FIGURE 5** Antiviral activity of oreoch-1 against (A) CpHV-1 and (B) BoHV-1 infections in MDBK cells, by performing different schemes of treatment, namely, co-treatment, virus pre-treatment, cell pre-treatment, and post-treatment (see Figure 1). Infected cells were used as negative control (CTRL -). Dunnet's multiple comparison test: (A) \*\*\*\* $p < 0.0001$ ; \*\*\* $p = 0.0002$ ; \*\* $p = 0.0011$ ; ns: non-significant. (B) \*\*\*\* $p < 0.0001$ ; \*\* $p = 0.0011$ ; ns: non-significant.

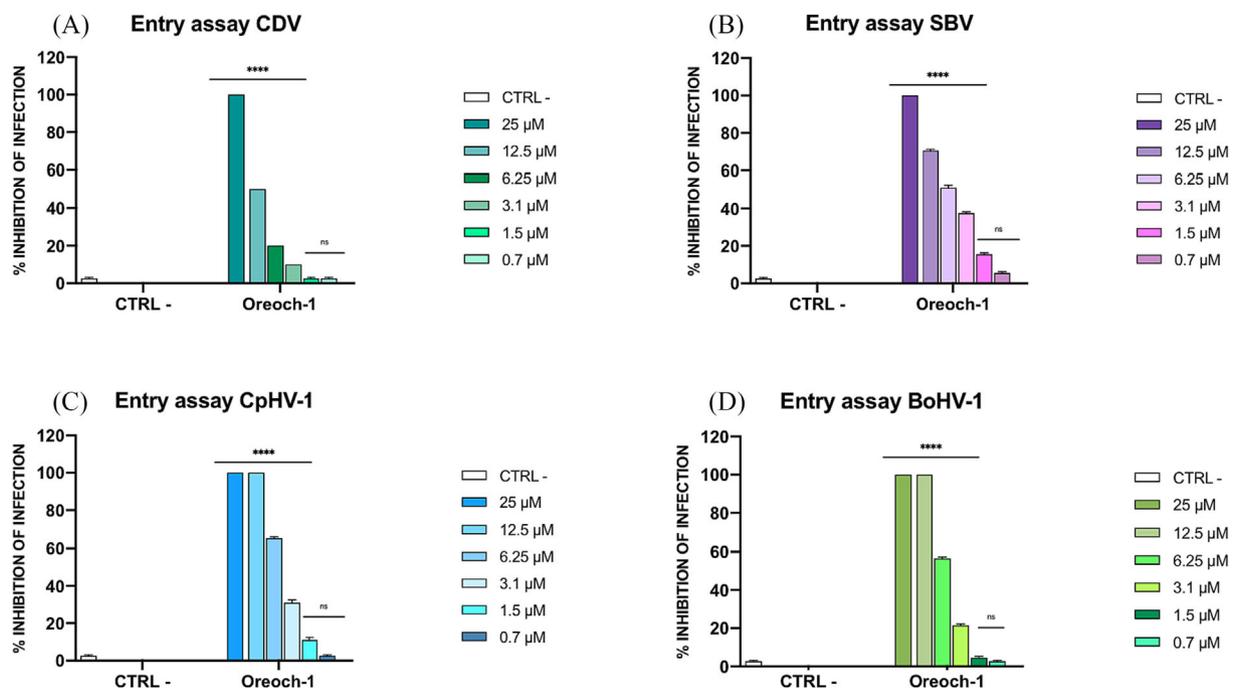
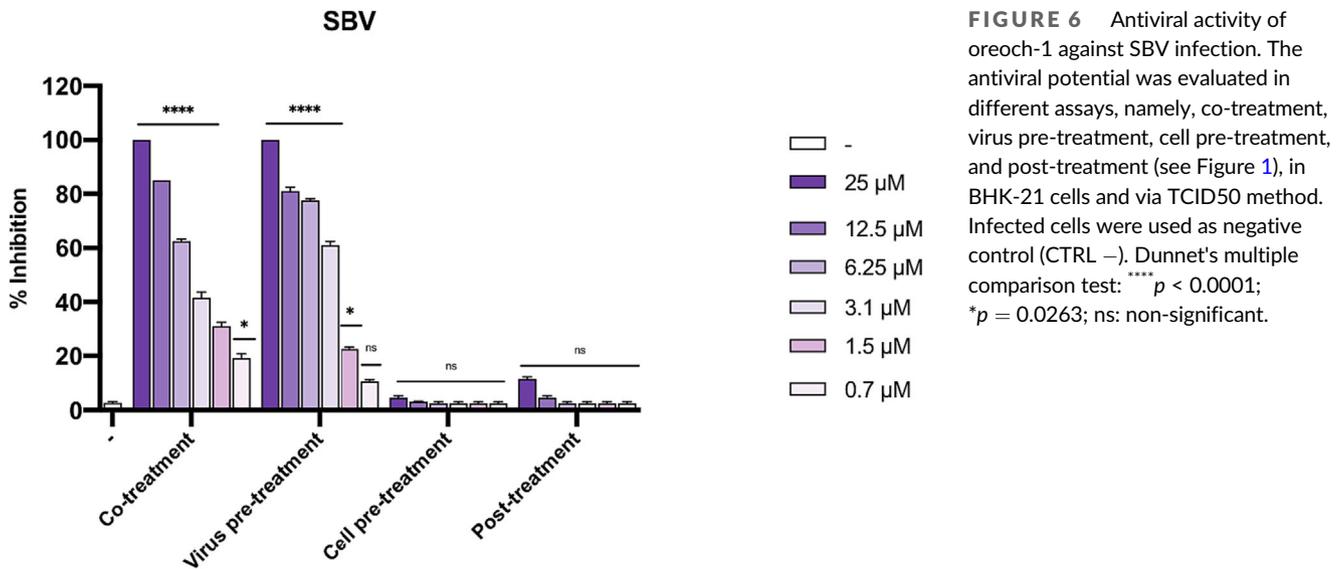
indicate that the peptide is able to block the penetration of virus inside the cells, thus inhibiting all the subsequent steps of viral lifecycle.

#### 4 | DISCUSSION

Throughout history, viruses were responsible for devastating pandemics and epidemics, causing widespread illness and death.

Furthermore, these infections have produced a collapse in the global economy, overwhelming healthcare systems and affecting global trade and travel. Despite the availability of vaccines for numerous viral diseases, complete eradication remains elusive and the ability of viruses to rapidly change and adapt strongly contributes to their persistence.<sup>34</sup>

Approximately 60% of infectious diseases affecting humans and 75% of newly emerging infections are zoonotic, with two thirds



**FIGURE 7** Entry assay. Oreoch-1 was tested to assess its ability to block the entry phase of (A) CDV, (B) SBV, (C) CpHV-1, and (D) BoHV-1 infections (see Figure 1). The data indicated that oreoch-1 inhibited each viral entry into the cells until 25  $\mu\text{M}$ . Infected cells were used as negative control (CTRL -). Dunnet's multiple comparison test: (A) \*\*\*\*  $p < 0.0001$ ; ns: non-significant. (B) \*\*\*\*  $p < 0.0001$ ; ns: non-significant. (C) \*\*\*\*  $p < 0.0001$ ; ns: non-significant; (D) \*\*\*\*  $p < 0.0001$ ; ns: non-significant.

originating in wildlife. As the human population expands and urbanization accelerates, the ongoing risk of viral diseases persists and their emergence and re-emergence are shaped by various virologic and environmental factors.<sup>35</sup> Moreover, the extensive use of antiviral drugs exerts selective pressure especially on animal reservoirs, including livestock, bats, and pangolins, which may lead to the development of drug resistance. This poses a risk of transmission to humans through newly emerged viral strains with acquired resistance from these reservoirs.<sup>36</sup>

For this reason, the request for new antiviral drugs is stringent and involves diverse strategies, such as identifying new targets, unraveling viral lifecycles, guiding immune responses, modifying existing drugs, and rediscovering latent properties in old ones.

In this context, AMPs have gained relevance due to their broad-spectrum antimicrobial activity.<sup>37-39</sup> Fishes, in particular, have been identified as a rich source of these short peptides, documented for their antimicrobial and immunomodulatory activities.<sup>7</sup> In the present study, we investigated for the first time the antiviral effect of a fish

peptide, namely, oreoch-1, little explored for its antimicrobial properties.

We demonstrated that oreoch-1 was active in inhibiting the infection of all enveloped animal viruses used in the present study. In detail, oreoch-1 exhibited a strong activity with an  $IC_{50}$  ranging from 2.5  $\mu$ M to 12.5  $\mu$ M when incubated prior with the virus (virus pre-treatment, Figure 3–5). The greatest antiviral effect has been observed against SBV (Figure 5), while the lowest is that against CDV (Figure 3). On the other hand, once cells were pre-treated with the peptide and then infected with CDV (cell pre-treatment), we observed a significant inhibitory activity (Figure 3) indicating that oreoch-1 exerted a double effect by targeting viral and cell membranes. The fact that oreoch-1 had an action on the cell surface during CDV infection could be explained by the presence of specific receptors on the infected cells. For instance, CDV recognizes and enters in the host cells through the binding to the SLAM and nectin-4 occurring thanks to H and F proteins, respectively.<sup>40,41</sup> We hypothesized that oreoch-1 could interact with these receptors, absent on cells infected with the other viruses used in the present study, that is, SBV, CpHV-1, and BoHV-1, thus reducing CDV entry and infection.

On the contrary, oreoch-1 does not show the same effect against the other animal viruses, neither it is able to affect intracellular pathways. Consequently, our findings propose that oreoch-1 manifests its antiviral effect by inhibiting the initial stages of viral life cycle, with a specific focus on impeding the entry process into the host cell. The observed reduction in infectivity among these enveloped viruses strongly implies a direct interaction between the peptide and viral particles. The mechanism of action may, to some extent, involve hindering the extracellular and early phases of infection. This inhibition has been confirmed in the entry assay for all the viruses tested, demonstrating the ability to impede viral entry at very low concentrations, except for CDV where oreoch-1 showed the  $IC_{50}$  at 12.5  $\mu$ M.

Only a few members of the piscidin family isolated from the mast cells of fishes have reported an antiviral effect. For instance, piscidin-1 was able to reduce the swine-origin pseudorabies virus (PRV) infection by direct interaction with the virus particles in a dose-dependent manner, protecting also the cells from PRV-induced apoptosis.<sup>42</sup> Also, the peptide TH1–5 hepcidin exhibited a virucidal activity against the Japanese encephalitis virus (JEV) both in vivo and in vitro.<sup>43</sup>

These peptides shared a common cationic nature, providing a plausible explanation for their antiviral activity against enveloped viruses. Their interaction with the negatively charged viral membrane likely leads to the physical disruption of the lipid bilayer in the viral envelope, explaining why these peptides are particularly effective in inhibiting the initial phases of viral infection, notably the entry process.

Specifically, the higher cationic character of oreoch-1, likely achieved also through the amidation of its C-terminal end, offers insight into its potent antiviral potential. The remarkable potency of oreoch-1 antiviral activity, in stark contrast to the limited data available in current literature, could pave the way for new and interesting studies in the search for promising antiviral agents that can effectively

prevent or combat emergent and re-emergent zoonotic viral diseases on a global scale.

## 5 | CONCLUSIONS

In light of the urgent need for new antiviral drugs to combat emerging and re-emerging viruses, particularly in the face of increasing drug resistance, entry inhibitors constitute a promising opportunity. Our study shows for the first time the potential of a fish-derived peptide, oreoch-1, as an entry inhibitor against a broad spectrum of enveloped animal viruses, including CDV, BoHV-1 and CpHV-1, and SBV. This inhibitory effect likely occurs through a physical interaction with the hydrophobic surfaces of the viral envelope. While our findings are encouraging, further in vitro and in vivo studies are necessary for a comprehensive understanding and eventual clinical application into humans.

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