Anti-microbial activity and immunomodulation of recombinant hepcidin 2 and NK-lysin from flounder (*Paralichthys olivaceus*)

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Abstract:

Infections due to pathogens impact global aquaculture economy, where diseases caused by bacteria should be in particular focus due to antibiotic resistance. Hepcidin and NK-lysin are important innate immune factors having potential to be exploited as alternatives to antibiotics due to their antimicrobial activity and immunomodulatory capacity. In this study, the recombinant proteins of hepcidin 2 and NK-lysin (r*Po*Hep2 and r*Po*NKL) from flounder (*Paralichthys olivaceus*) were obtained via a prokaryotic expression system. The results exhibited that r*Po*Hep2 and r*Po*NKL killed both gram-negative and gram-positive bacteria mainly via attachment and disruption of the membrane. Interestingly, both peptides could bind to bacterial DNA. The antiviral assay showed that both peptides have antiviral activity against hirame nonvirhabdovirus. They exhibited no cytotoxicity to the mammalian and fish cell lines. *Po*Hep2 was found localized in G-CSFR-positive peritoneal cells. Moreover, r*Po*Hep2 significantly enhanced the phagocytosis of flounder leukocytes *in vitro*. These findings suggested that neutrophils contained r*Po*Hep2 and may respond to the immunoreaction of neutrophils. In summary, both r*Po*Hep2 and r*Po*NKL possess antimicrobial activities and may be exploited to replace traditional antibiotics. r*Po*Hep2 possess immune regulatory functions, that can be further investigated as an immunostimulant in aquaculture.

Keywords: Hepcidin; NK-lysin; Antimicrobial activity

1. Introduction

Pathogenic infections impact aquaculture globally, resulting in a significant constraint on animal welfare and economics [1]. The aquaculture industry has become the fastest-growing food production sector over the past few decades [2]. In the past, antibiotics were chosen to treat diseases, leading to the development of antibiotic resistance, which has become a threat to public health and economic development [3]. Therefore, there is a need to search for alternatives to antibiotics with high bactericidal effects and with low potential to induce drug resistance.

Antimicrobial peptides (AMPs) are a class of small molecule peptides with a potential to replace antibiotics. They are evolutionarily conserved innate defense components and are widely found in organisms of prokaryotes and in higher vertebrates. AMPs have broad−spectrum activities against pathogenic microorganisms such as bacteria, viruses, fungi, and parasites [4]. The antibacterial

mechanism of AMPs is mainly through interaction with bacterial membranes or intracellular target molecules, making it difficult for bacteria to develop resistance to AMPs [5]. This has led to increased focus for applications of antimicrobial peptides in food, medicines, and agriculture, and has to some extent alleviated the problem of drug resistance caused by the misuse of antibiotics. Fish have a wide range of antimicrobial peptides which constitute an important part of the innate immune system. The main types of AMPs identified in fish are like piscidins, defensins, hepcidins, cathelicidins, and histone-like peptides [6].

Hepcidin, a cysteine-rich cationic antimicrobial peptide, was first identified in human [7], it is mainly synthesized by liver cells also known as liver-expressed peptide (LEAP). The function of hepcidin as a master regulator of iron has been well studied. In addition, hepcidin acts as an antimicrobial peptide [8]. Hepcidin appears to maintain a high degree of sequence similarity between vertebrate species, however, unlike humans, fish have multiple variants of hepcidins [9]. Fish hepcidin genes can be grouped into two categories: HAMP1-like, with a single isoform similar to mammalian hepcidin possibly involved in the regulation of iron metabolism; HAMP2-like, with several isoforms that may often possess antimicrobial effects [10]. NK-lysin is a homolog of human granulysin belongs to cationic antimicrobial peptides and mainly synthesized by cytotoxic T lymphocytes cells and natural killer (NK) cells [11]. NK-lysin does not show significant similarity to cysteine-rich antimicrobial peptides such as hepcidin, defensins, protegrins, bactenecins, or tachyplesins [12,13]. NK-lysin has a unique saposin-B (SapB) structural domain, and nuclear magnetic resonance analysis (NMR) showed that the three-dimensional structure of this domain consists of three compact α-helix segments with six conserved cysteines and three disulfide bonds. This folding pattern is different from other cysteine-rich antimicrobial peptides, which are mostly β-sheet structures [14,15].

The activity of fish AMPs following bacterial infection is well documented, and attention is now being paid to the microbicidal of fish antimicrobial peptides against other pathogens (e.g., fungi, viruses) in the aquatic environment. In trout, a recombinant β-defensin-like peptide has an *in vitro* antiviral function against viral hemorrhagic septicemia virus (VHSV) infection [16]. Several fish-derived NK-lysin-like peptides exhibit *in vitro* antiviral activity against nervous necrosis virus (NNV), viral septicemia hemorrhagic virus (VHSV) and infectious pancreatic necrosis virus (IPNV) [17]. However, there is no study on the antiviral activity of antimicrobial peptides against hirame nonvirhabdovirus (HIRRV), which was first reported in flounder and has become a serious threat to the aquaculture industry of China [18]. As an important component of innate immunity, in addition to having a direct action on pathogens, some antimicrobial peptides possess, in fact, immunomodulatory activity. In fish, hepcidin and NK-lysin have shown that they can induce expression of cytokines, such as IL-1β, IL-8, and IL-10 [19,20].

In the present study, we have used a prokaryotic expression to produce recombinant hepcidin and NK-lysin peptides (r*Po*Hep2 and r*Po*NKL) isolated from flounder (*Paralichthys olivaceus*). The antibacterial and antiviral effect of these two recombinant peptides have been determined *in vitro*. After studying the bactericidal mechanism of r*Po*Hep2 and r*Po*NKL, their immunomodulatory effects were investigated.

2 Materials and methods

2.1 Experimental animals and cell line

Healthy flounders (*Paralichthys olivaceus*), with an average weight of 200 g, were purchased from a marine farm in Qingdao City of Shandong Province, China. The flounders were temporarily cultivated in the laboratory culture system for two weeks, and then used for subsequent experiments. Experimental mice were purchased from the Animal Experiment Center in Qingdao, Shandong Province, China, for antibody production. The animal experiments followed the procedures in the Guide for the Use of Experimental Animals of the Ocean University of China and all efforts were made to minimize suffering. The flounder embryonic cell line (HINAE) was kindly provided by Ikuo Hirono, a professor at the University of Tokyo, Japan. HEK293T cell line was purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China).

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2.2 Bacteria and virus

Vibrio anguillarum, Vibrio harveyi, Edwardsiella tarda, Staphylococcus aureus, and HIRRV CNPo2015 were previously isolated from diseased fish and stored in our laboratory [21,22]. HIRRV suspensions were inoculated into HINAE cells maintained in L-15 medium (Gibco, Grand Island, USA) supplemented with 5% (V/V) fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin G and 100 mg/mL streptomycin (Gibco) at 20 °C. After observing the appearance of cytopathological effects, the supernatant of the cell cultures was collected by centrifugation and stored at -80 °C for subsequent experiments.

2.3 Sequence analysis, RNA extraction, cDNA synthesis, and gene cloning

The physical and chemical features of *Po*Hep2 and *Po*NKL were predicted using the ExPASy online software (https://www.expasy.org/). The structural domains of these two peptides were predicted using the SMART online software (http://smart.embl-heidelberg.de/). Their tertiary structures were predicted using SWISS-MODEL online software (https://www.swissmodel.expasy.org/) and modified by PyMOL software. Multiple sequence alignment of hepcidin 2 and NK-lysin proteins was performed by the ClustalX 2.0 program (http://www.clustal.org/clustal2/) and the results were edited by GeneDoc software. MEGA X was used to structure a phylogenetic tree using the neighbor-joining method with 1,000 bootstrap trials.

The total RNA was extracted from the liver and head kidney of flounder with the TRIzol method mentioned previously (Vazyme, Nanjing, China) and quantified using a NanodropND-8000 spectrophotometer (Thermo, Waltham, MA, USA) [23]. cDNA was synthesized using SuperScript II reverse transcriptase (Vazyme) according to the instructions provided by the manufacturer.

The primers *Po*Hep2-F/*Po*Hep2-R, and *Po*NKL-F/*Po*NKL-R (Table 1) were designed to amplify the cDNA of *Po*Hep2 and *Po*NKL according to the gene sequences published on GenBank (accession No. AB198061.1 and No. AU260449.1). The PCR products were checked by 1% agarose gel electrophoresis and confirmed by gene sequencing.

2.4 Sampling and gene expression assay

To study the constitutive expression of *Po*Hep2 and *Po*NKL, three healthy flounders were euthanized by using MS-222 (Sigma, St. Louis, MO, USA), and eight different tissues (liver, blood, spleen, head kidney, trunk kidney, gill, intestine, muscle) were sampled and stored at -80 °C for subsequent experiments. Total RNA extraction and cDNA synthesis from different tissues was carried out according to the method described in 2.3. qRT-PCR was accomplished in a LightCycler® 480 II Real-Time System by using SYBR Green I Master Mix (Roche, Basel, Switzerland) and the primers listed in Table 1. The test was performed in triplicate and analyzed by the $2^{-\Delta\Delta Ct}$ method with β-actin as an internal control.

2.5 Preparation of recombinant peptides, production of polyclonal antibody and indirect immunofluorescence assay

Based on the sequences of pET-32a plasmid and *Po*Hep2 and *Po*NKL genes, the primers Ex-*Po*Hep2-F/Ex-*Po*Hep2-R, and Ex-*Po*NKL-F/Ex-*Po*NKL-R (Table 1) were designed with endonuclease site *B*am HI. The PCR products of Ex-*Po*Hep2 and Ex-*Po*NKL were purified using a DNA extraction kit (TIANGEN, Beijing, China) and inserted into the *B*am HI-digested pET-32a with Trelief SoSoo Cloning Kit (Tsingke, Qingdao, China). The recombinant plasmids pET32-*Po*Hep2and pET32-*Po*NKLwere transformed into *E. coli* BL21 (DE3, Tsingke), and then spread on Luria Bertani (LB) solid medium (LAND BRIDGE, Beijing, China) containing ampicillin (Sangon, Shanghai, China). Monoclonal colonies were picked after incubation overnight at 37 °C and sent to a sequencing company (Tsingke) for sequencing. The positive clone correctly identified by sequencing was expanded in 500 mL LB broth medium with 50 μg/mL ampicillin in a 220-rpm shaking incubator at 37 °C. The isopropyl-β-D-thiogalactoside (IPTG) (Beyotime, Shanghai, China) was added to the medium until the OD_{600} values reached 0.6, and then incubated overnight in a 180-rpm shaking incubator at 16 °C. Bacteria were crushed using an Ultrasonic cell pulverizer, and the recombinant proteins were affinity purified using His Trap™ HP Ni-Agarose (GE Healthcare China, Beijing, China). The proteins were then refolded as described

previously [4]. In brief, the proteins were dialyzed in serials reconstitution buffers (50mM Tris-HCl, 100mM NaCl, 1 mM EDTA, 20% glycerol, 1 mM L-Glutathione oxidized, 1 mM L-Glutathione reduced, 5% Glycerin, and decreasing concentrations of urea (4 M, 2 M, 1M, 0.5M, 0.25M, and 0 M)). Refolded proteins were then dialyzed three times in phosphate buffer saline (PBS) to remove imidazole. The purified recombinant proteins were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue R-250 (Beyotime).

The concentrations of r*Po*Hep2 and r*Po*NKL were determined using the BCA Protein Assay Kit (Solarbio, Beijing, China) and adjusted to 2 mg/mL for immunization of BALB/c mice. Anti-r*Po*Hep2 and anti-r*Po*NKL polyclonal antibodies were produced according to the previous method [24]. In brief, BALB/c mice were immunized four times with adjusted r*Po*Hep2 and r*Po*NKL at weekly intervals. Serum samples were collected after four immunizations. The polyclonal antibodies were purified by protein G agarose affinity chromatography (Thermo, Waltham, MA, USA).

To obtain peritoneal cells from flounder (~500 g), fish were intraperitoneally injected with sterilized phosphate buffer saline (PBS), and the peritoneal cells were withdrawn by a sterile syringe. After washing three times at 480 *g* for 10 minutes, the peritoneal cells (5×10^6 cells/mL) were dropped onto the adherent slides (CITOTEST, Jiangsu, China) and settled at room temperature for 2 hours followed by fixing with 4% paraformaldehyde (Thermo, Waltham, MA, USA) for 20 minutes. After washing three times with PBS, five percent BSA in PBS was added to slides for 1 hour for blocking, and then the peritoneal cells were incubated with anti-r*Po*Hep2 or anti-r*Po*NKL at 37 °C for 1.5 hours. After washing three times with PBS, the peritoneal cells were incubated with Alexa Fluor 649-conjugated goat anti-mouse IgG (1:1000, Sigma, St. Louis, MO, USA) for 45 minutes. The cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Bio-Legend, Santiago, Chile) followed by observation with a fluorescence microscope (Zeiss, Jena, Germany).

2.6 Antimicrobial activity assay

The minimum inhibitory concentrations (MICs) of r*Po*Hep2 and r*Po*NKL against four pathogenic bacteria (*Vibrio anguillarum*, *Vibrio harveyi*, *Edwardsiella tarda*, and *Staphylococcus aureus*) were determined according to previous reports [25]. In brief, r*Po*Hep2 or r*Po*NKL were two-fold serially diluted with Mueller-Hinton (MH) broth medium (LAND BRIDG) and added to sterile 96-well microtiter plates (Corning, New York, NY, USA) at 100 μL per well. The cultured bacteria were diluted to 1×10^7 CFU/mL and added to each well. Thioredoxin (rTrx) and antibiotic (kanamycin sulfate) (Kana)-treated wells were used as control groups. After incubation at 28 °C for 24 hours, The OD_{600} values of the wells in 96-well microtiter plates were measured with a microplate reader (TECAN, Canton of Zurich, Switzerland). The MIC was defined as a range between the lowest peptide concentration that causes 100% inhibition of bacterial growth and the highest peptide concentration at which bacterial growth can be observed.

The antiviral effects of r*Po*Hep2 and r*Po*NKL were measured according to the method described previously with some modifications [26]. HIRRV was first co-incubated with different concentrations (125-500 μg/mL) of r*Po*Hep2 or r*Po*NKL for one hour at 20 °C. Meanwhile, rTrx (500 μg/mL) and Kana (500 μg/mL) were incubated with HIRRV as a parallel control group. The 50% tissue culture infectious dose ($TCID_{50}$) of all groups was measured after incubation, then residual infectivity was calculated following the formula, residual infectivity = x/y × 100% ("x" represent $TCID_{50}$ of the residual virus after treated by AMPs, "y" represents $TCID_{50}$ of the residual virus after treated by rTrx for control)

2.7 Localization of r*Po*Hep2 and r*Po*NKL in bacteria

The localization of r*Po*Hep2 and r*Po*NKL studies were based on the previous method with some modifications [27]. In brief, two-fold MIC concentrations of r*Po*Hep2 or r*Po*NKL were co-incubated with bacteria for 15 minutes. After washing thrice, microbial cells were applied on adhesive slides (CITOTEST) for 1 hour, then fixed with 4% paraformaldehyde for 30 minutes. The slides were blocked with 5% BSA (Sangon) for 1 hour at 37 °C, then incubated with mouse anti-r*Po*Hep2 or anti-r*Po*NKL for 1.5 hours at 37 °C. Later, the bacteria incubated with Alexa Fluor 649-

conjugated goat anti-mouse IgG (1:1000) (Beyotime) for 45 minutes and finally stained with DAPI (Bio-Legend), followed by observation with a fluorescence microscope (Zeiss).

2.8 Bacterial membrane integrity assay

Bacterial membrane integrity of *V. harveyi* and *S. aureus* after r*Po*Hep2 or r*Po*NKL treatment were detected with the SYTOX Green uptake assay described previously [28]. In brief, *V. harveyi* and *S. aureus* were cultured to the exponential phase in LB medium and adjusted to a concentration of 5×10^6 CFU/mL in PBS. SYTOX Green (Thermo) was added to the bacteria suspensions and incubate for 10 minutes in the dark. Thereafter, r*Po*Hep2 or r*Po*NKL was added at 2 × MIC concentration, and the fluorescence intensity (excitation $\lambda = 485$ nm, emission $\lambda = 530$ nm) was measured at 1-minute intervals for 15 minutes by using a fluorescence spectrophotometer (TECAN). At the same time, rTrx (100 μg/mL) was added and its autofluorescence intensity was measured as a control.

2.9 Bacterial DNA binding assay

The ability of the antimicrobial peptides to bind bacterial genomic DNA was detected by a gel shift assay. The genomic DNA (gDNA) of *V. harveyi* and *S. aureus* were extracted using Bacteria Genomic DNA Kit (TIANGEN, Beijing, China). The concentrations of 200 μg/mL r*Po*Hep2, r*Po*NKL, and recombinant flounder β-defensin (r*Po*β-defensin) (an AMP prepared in our previous study) were incubated with 100 ng bacterial gDNA [23]. The mixture was incubated for 6 hours at 28 °C and then examined by 1% agarose gel electrophoresis. Meanwhile, rTrx was mixed with bacterial gDNA as a control group for parallel experiments.

2.10 Cytotoxicity Assay

Cytotoxicity of r*Po*Hep2 or r*Po*NKL to mammalian or fish cells was measured by Cell Counting Kit-8 (CCK-8) assay. HEK293T and HINAE cells were seeded in 96-well microtiter plates at 10^4 cells/well and cultured overnight. The culture medium was replaced by serial dilutions of r*Po*Hep2 or r*Po*NKL. PBS treated cells were used as controls. After incubation for 24 hours, cell viability was determined by the Cell Counting Kit-8 (CCK-8, Vazyme). The absorbance at 450 nm was measured by a microplate reader. Each treatment was performed in triplicate.

2.11 Protease and salt resistance

Protease resistance of r*Po*Hep2 or r*Po*NKL were assessed by protease digestion assay described previously with some modifications [29,30]. In brief, r*Po*Hep2 or r*Po*NKL (10 μg) were incubated with elastase (Yuanye, Shanghai, China) or α-chymotrypsin (Yuanye) at different concentrations. Considering the concentration in host or bacterial culture, 0.2, 2, 20, and 200 μg/mL elastase or 0.12, 0.6, 1.2, and 12 μg/mL α-chymotrypsin were incubated together with the peptides for one hour at 37 °C. The mixtures were then diluted to 1 × MIC of peptides and the bacteriostatic effect were tested according to the method described in section 2.6. The buffer containing proteases without peptides and buffer containing untreated peptides were used as control groups. Each treatment was performed in triplicate.

Salt resistance of r*Po*Hep2 or r*Po*NKL were assessed under various concentrations of NaCl at 1×MIC of peptides. The antimicrobial activity of the two peptides were tested as described before. Briefly, *S. aureus* was cultured and diluted to 1×10^7 CFU/mL, then incubated with 1 × MIC r*Po*Hep2 or r*Po*NKL in 96-well microtiter plates. Different concentrations of NaCl (0, 20, 40, 80 and 160 mM) were added to wells. Bacterial suspension treated without peptides and peptides suspension treated without bacterial were control groups, and each treatment was performed in triplicate.

2.12 Phagocytosis assay

Spleen and head kidney leukocytes of flounders were obtained according to a previously described method [31]. The extracted leukocytes were resuspended in L-15 medium (1×10^7 cells/mL) and seeded in a 24-well cell culture plate (Corning) at 600 μL per well. Two hours were given for cell attachment. Then, different concentrations of r*Po*Hep2 or r*Po*NKL (10 μg/mL or 20 μg/mL) were added to each well and incubated for 4 hours at 25 °C. Green-fluorescent microspheres (Polysciences, Warrington, PA, USA) were added into each well at a ratio to cells of 20:1 followed by incubation for 3 hours in the dark. After being collected and washed three times with PBS, the leukocytes were harvested and analyzed via a flow cytometer (Accuri

C6, BD, New York, NY, USA).

2.13 Co-localization of *Po*Hep2 or *Po*NKL with G-CSFR positive peritoneal cells

The peritoneal cells were obtained from flounder according to the method described in 2.6. Peritoneal cells were added to the adherent slides and left to settle for 2 hours, then 4% paraformaldehyde was added to fix cells (15 minutes fixation). After using 5% BSA for unspecific blocking (1 hour), the cells were co-incubated with rabbit anti-G-CSFR and mouse anti-r*Po*Hep2 or mouse anti-r*Po*NKL for 1 hour at 37 °C. After washing three times with PBS, the peritoneal cells were incubated with Alexa Fluor 649-conjugated goat anti-mouse IgG (1:1000) and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000) for 45 minutes. Finally, the slides were counterstained with DAPI (Bio-Legen) and observed with a fluorescent microscope (Zeiss).

2.14 Statistical analysis

Statistical analysis of all data in this study was performed by using IBM SPSS Statistics 25. Significant differences between groups were tested by independent-samples t-test and Duncan's multiple polar difference test (DMRT). Data were represented as mean ± standard deviation, differences were considered significant when $p < 0.05$ and highly significant at $p < 0.01$.

3. Results and discussions

3.1 Molecular characterization of *Po*Hep2 and *Po*NKL

The open reading frame (ORF) of *Po*Hep2 was 270 bp encoding 89 amino acids with a molecular weight of 9.77 kDa, and its theoretical isoelectric point (PI) is 7.96. The ORF of *Po*NKL was 444 bp encoding 147 amino acids with a molecular weight of 16.43 kDa, and a theoretical PI of 8.75. The secondary structure of *Po*Hep2 includes a signal peptide (1 to 24 aa), a propeptide (25 to 63 aa), and a mature peptide (64 to 89 aa), the *Po*NKL protein includes a signal peptide (1 to 22 aa) and a maturation peptide (23 to 147 aa) which has a surfactant-associated protein B domain (48 to 122 aa).

Antimicrobial peptides can be broadly classified into four major categories according to their secondary structures namely α-helical peptides, β-sheet peptides, loop-shaped structure peptides, and linear flexible peptides rich in amino acids such as Gly, Trp, Arg, Pro, and His [32]. In this study, the mature peptides of *Po*Hep2 and *Po*NKL were predicted to possess five α-helical structures (Fig. 1B) and two β-sheet structures (Fig. 1A), respectively, which tally with the typical characteristics of antimicrobial peptides. Previous studies have shown that human hepcidin has a four-disulfide bond structure formed by eight cysteine residues [7]. *Po*Hep2 possesses the same eight cysteine residue structures, suggesting that this structure of hepcidin is evolutionarily conserved in vertebrates. Similarly, *Po*NKL also had structural features which are conserved in human granulysin and teleost NK-lysins, including the SaposinB domain and six cysteine residues. In addition, the results of the phylogenetic tree showed that the amino acid sequence of both *Po*Hep2 and the *Po*NKL clustered those of the *Hippoglossus hippoglossus* at a branch and they clustered with teleost orthologs, that branches away from mammalian hepcidin and NK-lysin (Fig. 1E, F).

3.2 Tissue distribution of *Po*Hep2 and *Po*NKL

The mRNA expression of *Po*Hep2 and *Po*NKL in eight different tissues of healthy flounder were analyzed by qRT-PCR. The results showed that *Po*Hep2 and *Po*NKL were expressed in all the tissues examined. In *Dissostichus mawsoni* and *Danio rerio*, hepcidin mRNA expression was detected in ten different tissues where the hepcidin mRNA in the liver often was highest [33]. In this study, the highest expression of *Po*Hep2 was also found in the liver (Fig. 2A). This is consistent across most species, from mammals to teleost, as hepcidin acts as a key regulator of iron uptake and their release is mainly from hepatocytes [34]. In addition, the expression of *Po*Hep2 was relatively high in the flounder spleen which may suggest a function in the immune system, in line with the suggestion made from results obtained in *Danio rerio* [35].

Flounder NK-lysin basically was constitutively expressed in all tissues. From other reports, the highest expression of NK-lysin mRNA has been found in different tissues from various species. In golden pompano, the expression level of NK-lysin mRNA was highest in the spleen, followed by the intestine stomach, and gill [35],

while in the yellow catfish the highest expression was observed in gill, followed by head kidney and skin [36]. In this study, *Po*NKL mRNA was expressed in all tissues examined with the highest expression in peripheral blood, followed by gills and spleen, whereas the expression in muscle was relatively low (Fig. 2B). Taken together, this suggests that the expression of NK-lysin is dependent on the species under study.

3.3 Production of r*Po*Hep2 and r*Po*NKL and its antimicrobial activities

The CDS (coding sequences) of *Po*Hep2 (270 bp) and *Po*NKL (444 bp) genes were successfully cloned from the cDNA library of head kidney by PCR (Fig. 3A, C). The purified PCR products of *Po*Hep2 and *Po*NKL were effectively inserted into the expression vector pET-32a. The recombinant *Po*Hep2 fused with Trx (molecular weight approximately 30.17 kDa) and *Po*NKL fused with Trx (36.83 kDa) were successfully produced and purified (Fig. 3B, D). After reconstitution, the yield was determined by the BCA protein assay kit, it was concluded that *E. coli* (BL21) cultured in 1 liter of LB medium was able to acquire 14.72 mg of r*Po*Hep2 and 11.65 mg of r*Po*NKL. Peritoneal cells were used for specificity testing of the *Po*Hep2 and *Po*NKL mice polyclonal antibodies, using immunofluorescence. The results suggested that the polyclonal antibodies recognized the *Po*Hep2 and *Po*NKL cell proteins (Fig. 3E).

Hepcidin and NK-lysin peptides are known to be broad-spectrum bactericidal. In this study, the MIC values of r*Po*Hep2 and r*Po*NKL against four fish pathogenic bacteria were analyzed by the gradient dilution method. Both antimicrobial peptides showed a concentration-dependent inhibitory activity against both gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*V. anguillarum*, *V. harveyi*, *E. tarda*) (Fig. 4A to D). Their MICs values against four pathogenic bacteria are found in Table 2. Also, antibiotic kanamycin was used as a control group and its MIC values were determined and presented in Table 2. The inhibitory effect of kanamycin on *E. tarda* was significantly higher compared to the two peptides, with MIC values of 1.95-3.9 μg/mL (Fig. 4A). Its inhibitory ability against *V. anguillarum* and *V. harveyi* was similar to that of r*Po*Hep2, and the inhibitory ability against *S. aureus* was similar to r*Po*NKL and higher than r*Po*Hep2. The antibacterial effects of the two recombinant proteins were also different. The MIC values of r*Po*Hep2 against four bacteria ranged from 31.25 to 62.5 μg/mL, and the MIC values of r*Po*NKL ranged from 3.9 to 31.25 μg/mL. Especially, the MIC value of r*Po*NKL against gram-positive bacteria *S. aureus* was relatively low (3.9-7.81 μg/mL) (Fig. 4D). Previous studies have shown that most of the positively charged antimicrobial peptides bind to negatively charged bacterial surface by electrostatic interaction. When hydrophilic and hydrophobic amino acid residues of AMPs are located on each side of the molecule, this would allow AMPs to be incorporated into the lipid bilayer of the bacterial membrane [37,38]. Thus, the positive charge, as one of the main parameters of AMPs, can significantly influence their antibacterial effect [25]. AMP's positive charge depends on the amount of positively charged residues (arginine, lysine, and histidine) in the sequence [39]. According to ExPASy software analysis, the number of positively charged residues of *Po*NKL peptide (18) is higher than that of *Po*Hep2 peptide (8). Besides, the net charge of mature peptide *Po*NKL (+ 4???) also higher than mature peptide *Po*Hep2 (+ 3???). These positively charged residues may be superior to enhance the interaction of the peptide with negatively charged bacterial membranes, possibly leading to the better bactericidal efficacy of r*Po*NKL.

AMPs are commonly proposed as alternatives to antibiotics due to their antimicrobial properties, but the research on AMPs' killing effects against other pathogens is limited [15]. In addition to bacteria, there are viruses, fungi, parasites, etc. in the aqueous environment. Thus, the study of AMPs which can prevent or treat viral diseases is a novel area of research. In previous research reports, zebrafish β-defensin 2 was shown to reduce spring carp virus (SVCV) infection by 90% in the ZF4 cell line [40]; β-defensin from the orange-spotted grouper reduced infection of Singapore grouper iridovirus and viral nervous necrosis virus [41]. Further, Spotted scat hepcidin 2 was shown to exhibits antiviral activity against siniperca chuatsi rhabdovirus (SCRV) and largemouth bass micropterus salmoides reovirus (MsReV) [42]. In our study, the antiviral activity of r*Po*Hep2 and r*Po*NKL was assayed by using the $TCID_{50}$ method, and the results were expressed as the viral residual

infectivity. The residual infection rate of HIRRV was 0.2%, 0.6%, and 5% at r*Po*Hep2 addition concentrations of 500-125 μg/mL, respectively (Fig. 4E). The residual infection rate of HIRRV was less than 50% at r*Po*NKL addition concentrations of 500 and 250 μg/mL, while at the concentration of 125 μg/mL, the HIRRV residual infectivity was 56% (Fig. 4F). Meanwhile, kanamycin showed no antiviral effect on HIRRV. HIRRV is an RNA virus that is a serious menace to aquaculture, yet there are no reports on the antiviral activity of AMPs against HIRRV. The results demonstrate the antiviral activity of hepcidin and NK-lysin against HIRRV *in vitro.* The primary antiviral mechanism of some human AMPs is thought to be a direct action on the viral envelope, or a prevention of viral binding to target cell receptors [43]. For fish AMPs, the mechanism of antiviral is still unclear, but it is noteworthy that hepcidin from tilapia (*Oreochromis mossambicus*) could induce agglutination of NNV and thus likely inhibited cell contact [44]. For r*Po*Hep2 and r*Po*NKL, their antiviral mechanisms are yet to be investigated in future experiments. In conclusion, r*Po*hep2 and r*Po*NKL have broad-spectrum antibacterial and antiviral activities.

3.4 The antibacterial mechanism of r*Po*Hep2 and r*Po*NKL

Many AMPs have been demonstrated to directly target and disrupt bacterial membranes. Spampcin from *Scylla paramamosain* permeabilize *S. aureus* and *P. aeruginosa* outer and inner membranes [45]; A type I interferon (named gcIFN-20) from grass carp was shown to adsorb to the surface of *S. aureus,* and had a disrupting effect on the integrity of the bacterial cell membrane [28]**.** In the current study, localization of r*Po*Hep2 and r*Po*NKL in bacteria were determined by indirect immunofluorescence. As shown in Fig. 5, r*Po*Hep2 and r*Po*NKL bind to bacteria targeting the bacterial membrane. During exposure, there an aggregation of bacteria was observed and the morphology of bacteria was partially deteriorated. Following on, the integrity of bacterial membranes after r*Po*Hep2 and r*Po*NKL treatment was examined by SYTOX Green uptake assay. The results showed that both antimicrobial peptides induced a rapid and significant disruption of bacterial (G^+ and G^-) cell membranes. The disruption of membranes occurred after treatment with the antimicrobial peptides for one minute, and significant disruption of bacterial membrane integrity was shown after 10 minutes of exposure (Fig. 6 A-D). As with most cationic AMPs, *Po*Hep2 and *Po*NKL may kill bacteria by a selective binding to negatively charged hydroxylated phospholipids and LPS on the bacterial membrane, rapidly disrupting the membrane lipids, resulting in bacterial burst.

In recent years, it has been shown that some AMPs are able to target various intracellular organelles (such as proteins and DNA) which may cause bacterial death [46]. In this study, both r*Po*Hep2 and r*Po*NKL bound directly to bacterial genomic DNA in vitro, and was more effective in binding to *V. harveyi* gDNA than *S. aureus* gDNA (Fig. 6 E, F). However, it seems that the ability of r*Po*β-defensin binding to bacterial gDNA [23] was more effective compared to our study using r*Po*Hep2 or r*Po*NKL. The general assumption is that AMPs binds electrostatically to bacterial DNA and RNA [47].

It is well known that one mechanism of antibiotics is to inhibit the production of bacterial cell wall constituents, a mechanism that may be counter effected by a development of drug-resistance [5,47]. The antimicrobial peptides r*Po*Hep2 and r*Po*NKL act mainly by disrupting bacterial cell membranes. In addition, they have the ability to bind bacterial genomic DNA *in vitro*. Thus, *Po*Hep2 and *Po*NKL seem to inhibit bacterial growth through different mechanisms. Such mechanisms, in synergy, is less likely to induce drug resistance, therefore, *Po*Hep2 and *Po*NKL possess a potential to be exploited as new bacteriocidic or bacteriostatic agents.

3.5 *In vitro* determination of r*Po*Hep2 and r*Po*NKL characteristics

Despite the innate activity of AMPs, studies have reported that some synthetic or natural antimicrobial peptides produce toxicity to eukaryotic cells such as mammal cells, which would be detrimental to the development of clinical drugs and feed additives [48-50]. Various characteristics of AMPs, including cytotoxicity, protease resistance, and salt tolerance were determined in this study. Both r*Po*Hep2 and r*Po*NKL showed no cytotoxic effect on mammalian (HEK293T) and fish (HINAE) cells, even at a concentration of 500 μg/mL (Fig. 7).

Although more than 3,000 AMPs have been discovered and many AMPs analogs have been designed, only a few have been used in clinical and manufacturing

applications [51]. One of the most critical obstacles is their poor stabilization against the host body's inhibitory factors, such as saline, acidic pH, anionic proteins, and proteases [52,53]. This has been shown especially for linear AMPs, which are more susceptible to proteases such as neutrophil elastase, bacterial metalloprotease [29]. In our study, the antimicrobial activity of r*Po*Hep2 and r*Po*NKL against *S. aureus* after protease and salt treatment were tested. After a high concentration (200 and 20 μg/mL) of elastase digestion, the antimicrobial activity of r*Po*NKL against *S. aureus* was significantly decreased, whereas chymotrypsin did not have a significant effect on its antimicrobial activity even at the highest concentration of 12 μg/mL treatment (Fig. 8 C, D). In contrast, the anti-*S. aureus* activity was mildly affected on r*Po*Hep2 only when it was treated with the highest concentration (12 μg/ml) of α-chymotrypsin (Fig. 8 A, B). As shown in Fig. 8 E and F, high concentrations (160 and 80 mM) of NaCl decreased the anti-*S. aureus* activity of r*Po*Hep2 and r*Po*NKL. However, even with the addition of the highest concentration of NaCl, neither peptide completely lost their antimicrobial activities, and the concentrations of NaCl below 40 mM had no effect on the antimicrobial activity of the peptides. r*Po*NKL was, however, mildly sensitive to elastase. Overall, *Po*Hep2 and *Po*NKL may have the potential to be used in the clinical or food industry in the right setting – as it has been shown that protease-treated AMPs have different effects on the killing activity against different bacteria [29]. As such, further studies on the peptides´ protease stability are needed.

3.6 Effects of r*Po*Hep2 and r*Po*NKL on the phagocytosis of leukocytes

Recently, it has become clear that the main function of AMPs is not only to directly destroy the pathogens, but also the ability to perform immunomodulatory activities [38]. It involves the influence on cells in the microenvironment, to recruit and activate other cells such as phagocytic and cytotoxic cells, supporting the process to clear the infection [38]. Human AMPs β-defensin 2 and 3 have been shown to promote phagocytosis of *Pseudomonas aeruginosa* and Zymosan particles by macrophages [54]. In fish, flounder β-defensin has been reported to significantly attract leukocytes and to enhance their phagocytic activity *in vitro* [23]. In our study, the leukocytes that were exposed to r*Po*Hep2 showed higher phagocytic activity of green fluorescent microspheres with a concentration-dependent trend. The increase in phagocytosis of spleen leukocytes was not significant at 10 μg/mL r*Po*Hep2 ($p > 0.05$), but significant at 20 μg/mL r*Po*Hep2 addition ($p < 0.05$) (Fig. 9). The phagocytic activity by leukocytes exposed to r*Po*NKL was not significantly changed compared with control cells (Fig. S1). Taken together, the results show that r*Po*Hep2 has both bactericidal and antiviral effect, and possess a bioactivity that increase phagocytic activity.

3.7 The co-localization of *Po*Hep2 and *Po*NKL with G-CSFR positive cells

Neutrophils act as the first line of defense against invading pathogens with two classical strategies: firstly, by the secretion of AMPs (degranulation) and secondly, phagocytosis of bacteria [55]. In addition, neutrophils have been shown to release chromatin and granule proteins into the extracellular space to form DNA traps to defend against invading pathogens [56]. In mammals, several antimicrobial peptides have been identified from neutrophils and their DNA traps, such as cathelicidins, defensins and histone-like peptides. In human, LL-37, a member of the cathelicidin family, is the main AMPs component secreted by neutrophils and is present in neutrophil extracellular traps [57]. However, there are only a few reports on AMPs in neutrophils from teleosts. In one of our previous study, flounder β-defensin was shown to be co-localized in the extracellular traps released from the leukocytes (neutrophils) and contributed to a bacterial inhibition effect. In the current study, the indirect immunofluorescence assay showed that the *Po*Hep2 was confined to G-CSFR positive cells. As shown in Fig. 10, *Po*Hep2, *Po*NKL (red), and G-CSFR (green) proteins were distributed on the cytosol. In addition, the *Po*Hep2 and G-CSFR proteins were co-localized at the plasma membrane showing a composite yellow color, but any co-localization of *Po*NKL and G-CSFR proteins was not observed. We conclude that *Po*Hep2 proteins are associated with G-CSFR positive cell structures/organelles and may be secreted by granulocytes as an effector. *Po*NKL was not found co-localized with G-CSFR-positive cells or organelles. NK-lysin is generally considered to be synthesized mainly by cytotoxic T lymphocytes cells and by natural killer cells. As effectors of different immune cells, we speculate that there

may be synergistic effects between *Po*Hep2 and *Po*NKL in a given circumstance where cytotoxic T cells, natural killer cells and neutrophils cooperate.

4. Conclusion

In this study, the recombinant proteins of the flounder antimicrobial peptides hepcidin 2 and NK-lysin were produced by a prokaryotic expression system. The results revealed that both r*Po*Hep2 and r*Po*NKL possessed antibacterial and antiviral activities. SYTOX Green uptake assay, gel retardation assay, and indirect immunofluorescence studies showed that r*Po*Hep2 and r*Po*NKL could killed bacteria by disrupting cell membrane, and also have ability to bind with bacterial gDNA in vitro, may inhibit bacterial growth through different bactericidal mechanisms. r*Po*Hep2 and r*Po*NKL possessed no cytotoxicity to mammalian and fish cells and were quite resistant to salt. r*Po*Hep2 possessed resistance to proteases (α-chymotrypsin and elastase), whereas r*Po*NKL was moderately sensitive to high concentrations of elastase. In addition to its direct antimicrobial activities, r*Po*Hep2 can also acted on phagocytes to increase phagocytosis. Our studies provide an important basis for the development and application of these two antimicrobial peptides products as antimicrobial agents.

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Table 1. Primer sequences

Primer Name	**Sequence (5′→3′)**	**Used**
*Po*Hep2-F	ATGAAGGCATTCAGCATTGC	Gene clone
*Po*Hep2-R	TCAGAACTTGCAGCAGGGG	Gene clone
*Po*NKL-F	ATGGGAACGTCTTCAATCCTC	Gene clone
*Po*NKL-R	TTAGGGATATTCGATGATTTCAGTT	Gene clone
Ex-*Po*Hep2-F	CAAGGCCATGGCTGATATCGGATCCATGAAGGCATTCAGCATTGC	Protein expression
Ex-*Po*Hep2-R	GCTTGTCGACGGAGCTCGAATTTCAGAACTTGCAGCAGGGG	Protein expression
Ex-*Po*NKL-F	CAAGGCCATGGCTGATATCGGATCCATGGGAACGTCTTCAATCCTC	Protein expression
Ex-*Po*NKL-R	GCTTGTCGACGGAGCTCGAATTTTAGGGATATTCGATGATTTCAGTT	Protein expression
qβ-actin-F	GAGGGAAATCGTTCGTGACAT	qRT-PCR
qβ-actin-R	ATTGCCGATGGTGATGACCTG	qRT-PCR
q*Po*Hep2-F	GCCTTTGTTTGCATTCAGGAC	qRT-PCR
q*Po*Hep2-R	ACTCTCCATCCACGATTCCATT	qRT-PCR
q*Po*NKL-F	AACGTCTTCAATCCTCCTGCT	qRT-PCR
q*Po*NKL-R	CACAGAGGGTTCCACATCCA	qRT-PCR

Note: The endonuclease site *B*am HI recognition sequence is underlined.

Table 2. The minimum inhibitory concentrations (MICs) of r*Po*Hep2 and r*Po*NKL

Microorganisms	**r*Po*Hep2 MIC (μg/mL)**	**r*Po*NKL MIC (μg/mL)**	**Kanamycin (μg/mL)**
Gram-negative			

Gram-negative			
E. tarda	31.25-62.5	15.63-31.25	1.95-3.9
V. harveyi	31.25-62.5	15.63-31.25	31.25-62.5
V. anguillarum	31.25-62.5	7.81-15.63	31.25-62.5
Gram-positive			
S. aureus	31.25-62.5	3.9-7.81	7.81-15.63

Figure legend

Fig. 1. Amino acid sequence analysis of *Po*Hep2 and *Po*NKL. (A) The predicted three-dimensional structure of mature sequences of *Po*Hep2. The model shows β-sheet structures containing four disulfide bridges. (B) The predicted three-dimensional structure of mature sequences of *Po*NKL. The model also shows α-helix structures containing three disulfide bridges. Multiple alignment of the amino acid sequences of hepcidin (C) and NK-lysin (D) among different species were analyzed by ClustalX 2.0 program, the positions of cysteine residues were labeled with arrows. The nonrooted neighbor-joining trees of hepcidin (E) and NK-lysin (F) were generated by MEGA X program.

Fig. 2. The relative expression of *Po*Hep2 (A) and NK-lysin (B) in different tissues. Tissues distribution of *Po*Hep2 and *Po*NKL was measured by quantitative real-time PCR (qRT-PCR). The expression of β-actin was used as the internal control.

Fig. 3. The preparation of recombinant *Po*Hep2 and *Po*NKL and the localization of *Po*Hep2 and *Po*NKL in peritoneal cells. The agarose gel electrophoresis of *Po*Hep2 gene (A) and *Po*NKL gene (C) amplified by PCR. Lane M, DNA marker; lane 1, PCR products of *Po*Hep2 or *Po*NKL. r*Po*Hep2 (B) and r*Po*NKL (D) analyzed by SDS-PAGE. Lane M, protein marker; lane 1, uninduced whole-bacterial lysate; lane 2, induces whole-bacterial lysate; lane 3, purification of r*Po*Hep2 or r*Po*NKL peptides. (E) The peritoneal cells were detected by Alexa Flour 649-labeled secondary antibody. The nucleus was visualized by DAPI. Bar = 10 μm.

Fig. 4. Antibacterial and antiviral activities of r*Po*Hep2 and r*Po*NKL. The inhibitory effects of different concentrations of r*Po*Hep2, r*Po*NKL, and Kanamycin on the four pathogenic bacteria (A to D) were examined in vitro using a two-fold dilution method. The results were expressed as absorbance values of the culture solution at 600 nm. $TCID_{50}$ was measured after treatment of HIRRV with different concentrations of r*Po*Hep2 (E) and r*Po*NKL (F), and the results were expressed as residual infectivity.

Fig. 5. Localization of r*Po*Hep2 (A) and r*Po*NKL (B) in *V. harveyi* and *S. aureus*. Bacterial suspensions at a concentration of 1×10^7 CFU/mL were incubated with 2 × MIC concentrations of r*Po*Hep2 or r*Po*NKL for 30 minutes, meanwhile incubated with 200 μg/mL rTrx as a control group. All cells in treatment groups were subjected to immunofluorescence analysis.

Fig. 6. Detection of membrane integrity by r*Po*Hep2 (A and B) or r*Po*NKL (C and D) of *V. harveyi* or *S. aureus* using SYTOX Green absorption assay. rTrx was added to the bacteria as a control. The fluorescence intensity of each group was measured every minute. Gel retardation assay was used to detect the binding of antimicrobial peptides to the bacterial genomic DNA of *V. harveyi* (E) and *S. aureus* (F). 200 μg/mL AMPs were incubated with 100 ng DNA for 6 hours, and rTrx was used as the negative control.

Fig. 7. Cytotoxicity of r*Po*Hep2 and r*Po*NKL to HEK293T and HINAE cells. Effect of r*Po*Hep2 and r*Po*NKL on the viability of HEK293T cells (A) and HINAE cells (B) at 24h of incubation with peptides at the concentration of 15.63 to 500 μg/mL.

Fig. 8. Protease and salt tolerance of r*Po*Hep2 and r*Po*NKL against *S. aureus*. The antimicrobial activity of r*Po*Hep2 or r*Po*NKL post-digestion by elastase at the concentrations of 0.2 to 200 μg/mL (A and C). The antimicrobial activity of r*Po*Hep2 or r*Po*NKL post-digestion by α-chymotrypsin at the concentrations of 0.12 to 12 μg/mL (B and D). The antimicrobial activity of r*Po*Hep2 or r*Po*NKL against *S. aureus* under NaCl at the concentrations of 20 to 160 mM (E and F). Treated with *S. aureus* only was set as a control group.

Fig. 9. The phagocytosis of leukocytes from spleen (A and B) or head kidney (C and D) with the treatment of r*Po*Hep2 protein at of 10 and 20 μg/mL doses. Fluorescence

histograms were used to represent the phagocytosis rate of leukocytes from the spleen or head kidney (A and C). Three biological replicates were performed and the data were analyzed (B and D). * indicates significant ($p \leq 0.05$), ** indicates highly significant ($p \leq 0.01$).

Fig. 10. Indirect immunofluorescence assay showed co-localization of *Po*Hep2 and *Po*NKL with G-CSFR positive peritoneal cells. The red color indicates *Po*Hep2 or *Po*NKL, the green indicates G-CSFR (A signature molecular protein of neutrophils in fish), and the blue indicates the cell nuclei. Bar = 10 μm.

Fig. 1

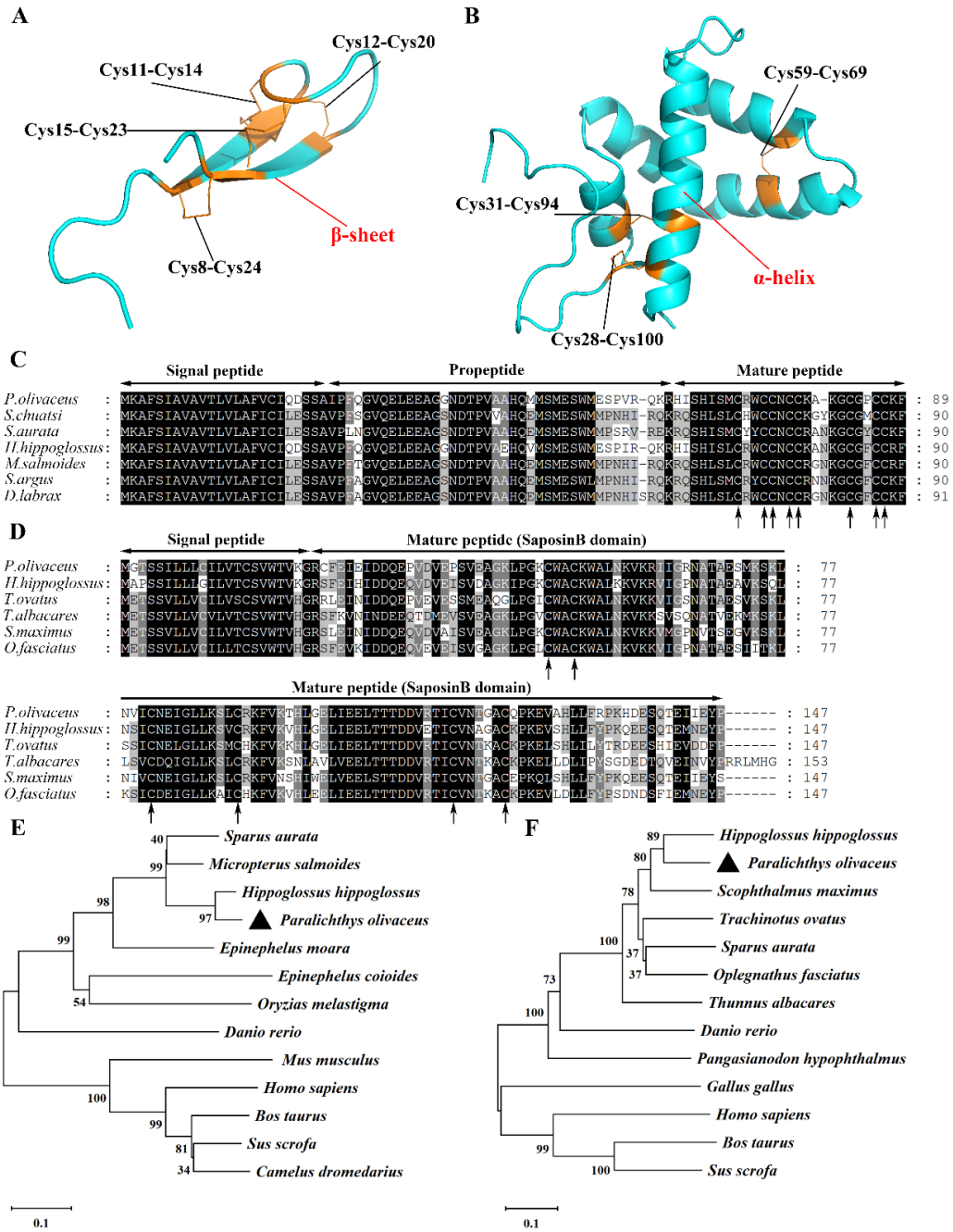


Fig. 2

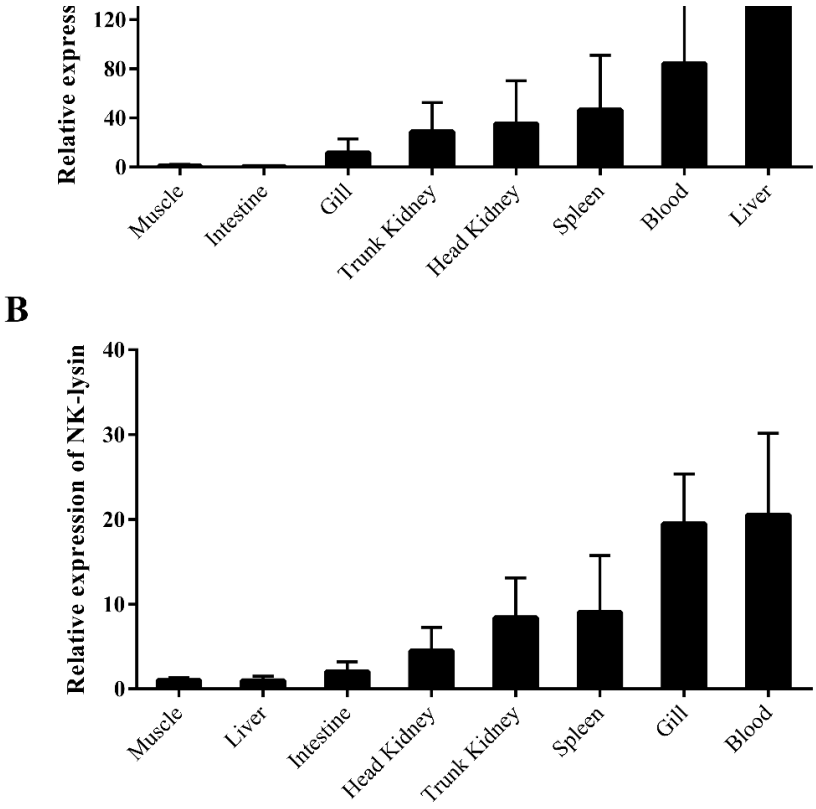
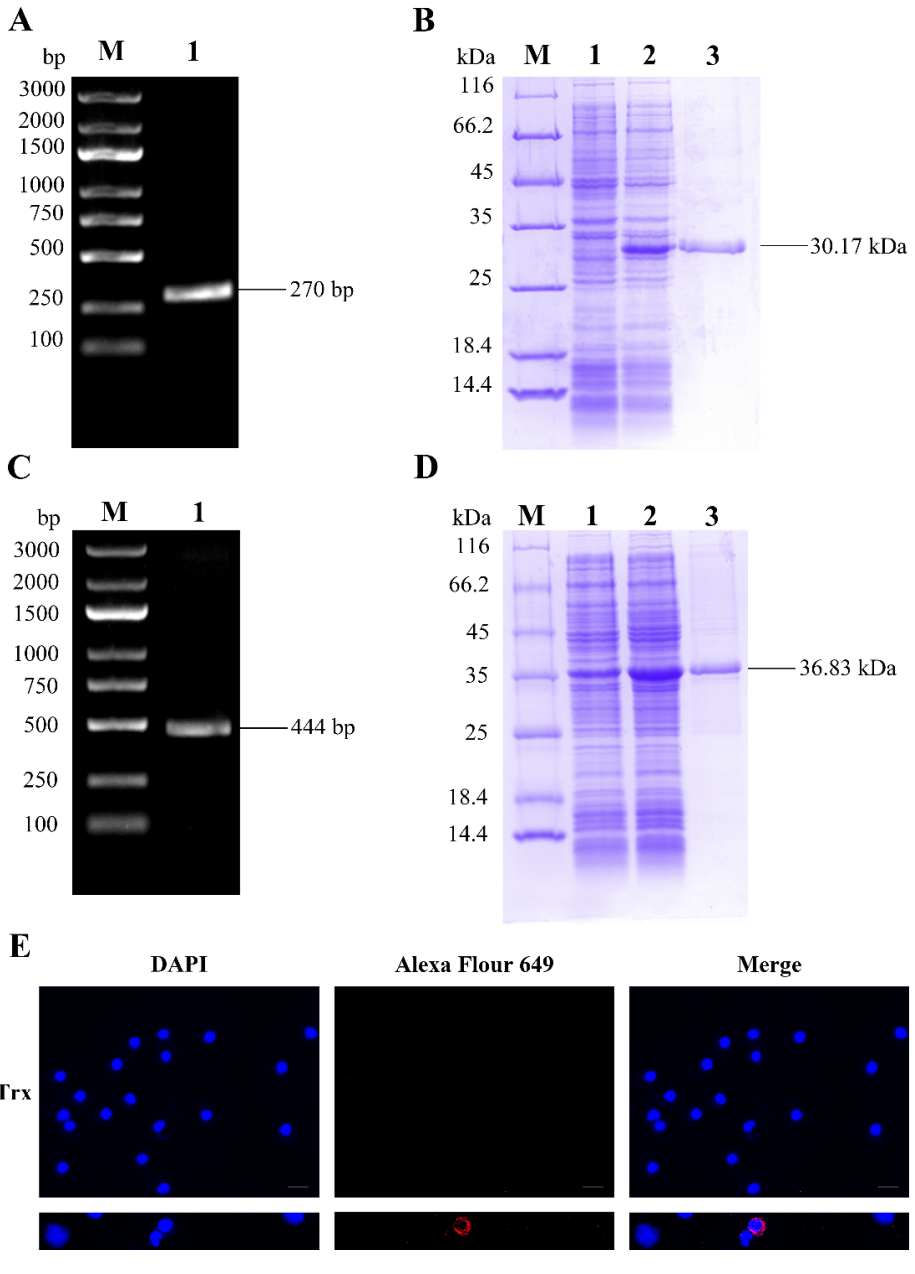

Relative express
120
80
40
0
Muscle
Intestine
Gill
Trunk Kidney
Head Kidney
Spleen
Blood
Liver
B
Relative expression of NK-lysin
40
30
20
10
0
Muscle
Liver
Intestine
Head Kidney
Trunk Kidney
Spleen
Gill
Blood

Fig. 3


A
bp
M
1
3000
2000
1500
1000
750
500
250
100
270 bp
B
kDa
M
1
2
3
116
66.2
45
35
25
18.4
14.4
30.17 kDa
C
bp
M
1
3000
2000
1500
1000
750
500
250
100
444 bp
D
kDa
M
1
2
3
116
66.2
45
35
25
18.4
14.4
36.83 kDa
E
DAPI
Alexa Flour 649
Merge
Anti-rTrx

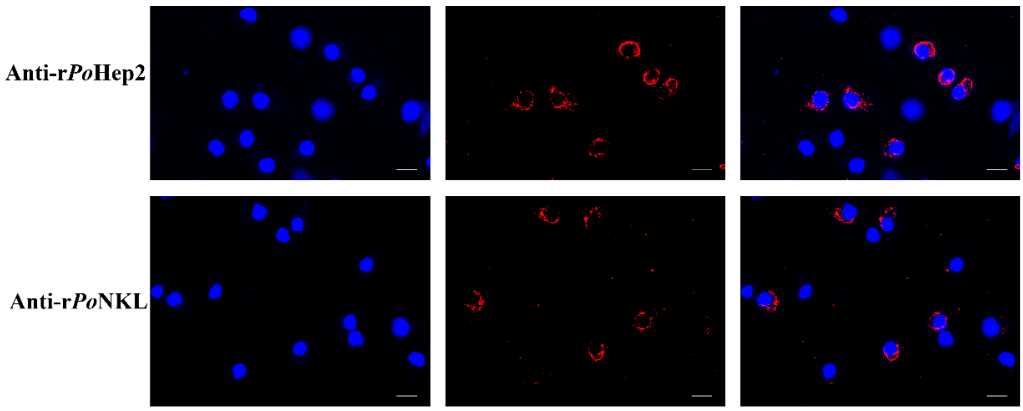

Anti-r*Po*Hep2
Anti-r*Po*NKL

Fig. 4

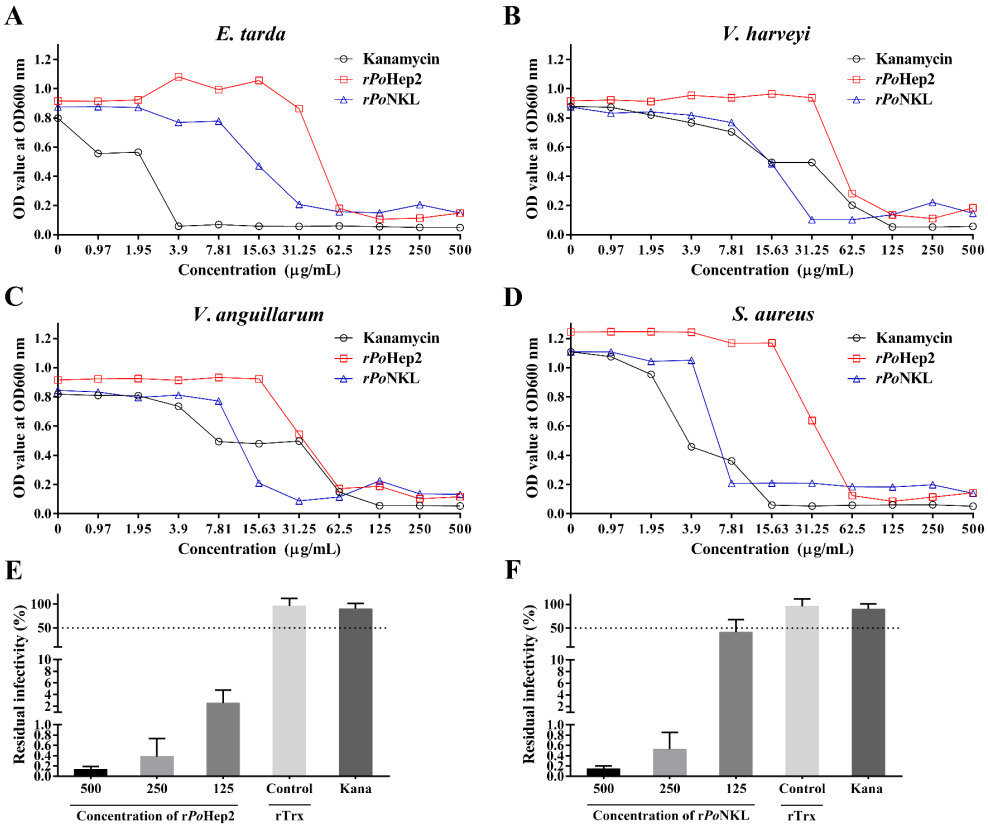

A
E. tarda
B
V. harveyi
C
V. anguillarum
D
S. aureus
Kanamycin
rPoHep2
rPoNKL
OD value at OD600 nm
Concentration (μg/mL)
0 0.97 1.95 3.9 7.81 15.63 31.25 62.5 125 250 500
E
F
Residual infectivity (%)
500 250 125 Control Kana
Concentration of rPoHep2
Concentration of rPoNKL
rTrx

Fig. 5

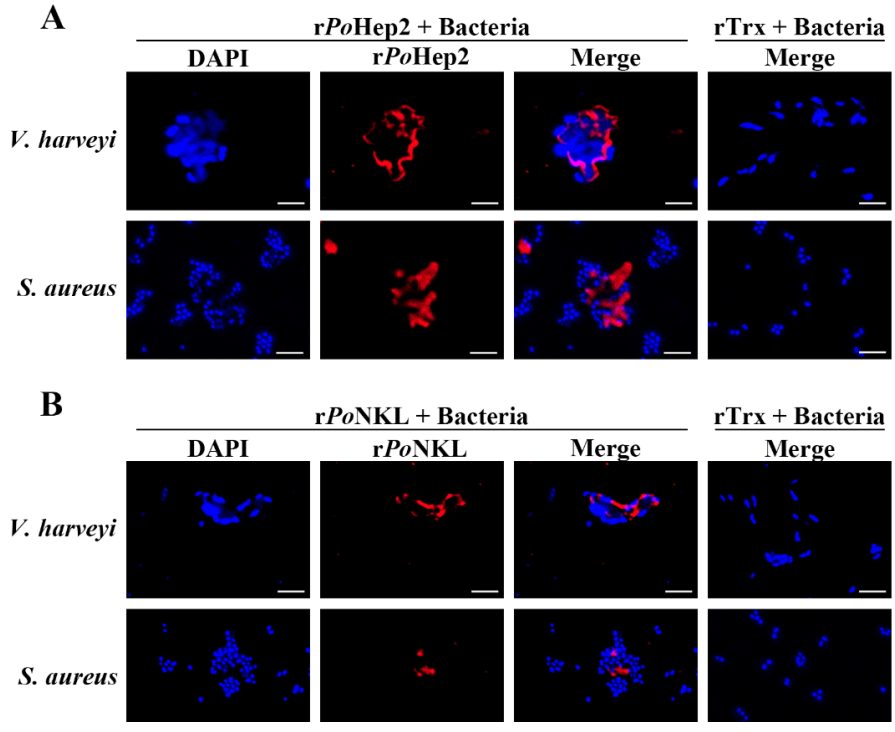

A
rPoHep2 + Bacteria
rTrx + Bacteria
DAPI
rPoHep2
Merge
Merge
V. harveyi
S. aureus
B
rPoNKL + Bacteria
rTrx + Bacteria
DAPI
rPoNKL
Merge
Merge
V. harveyi
S. aureus

Fig. 6

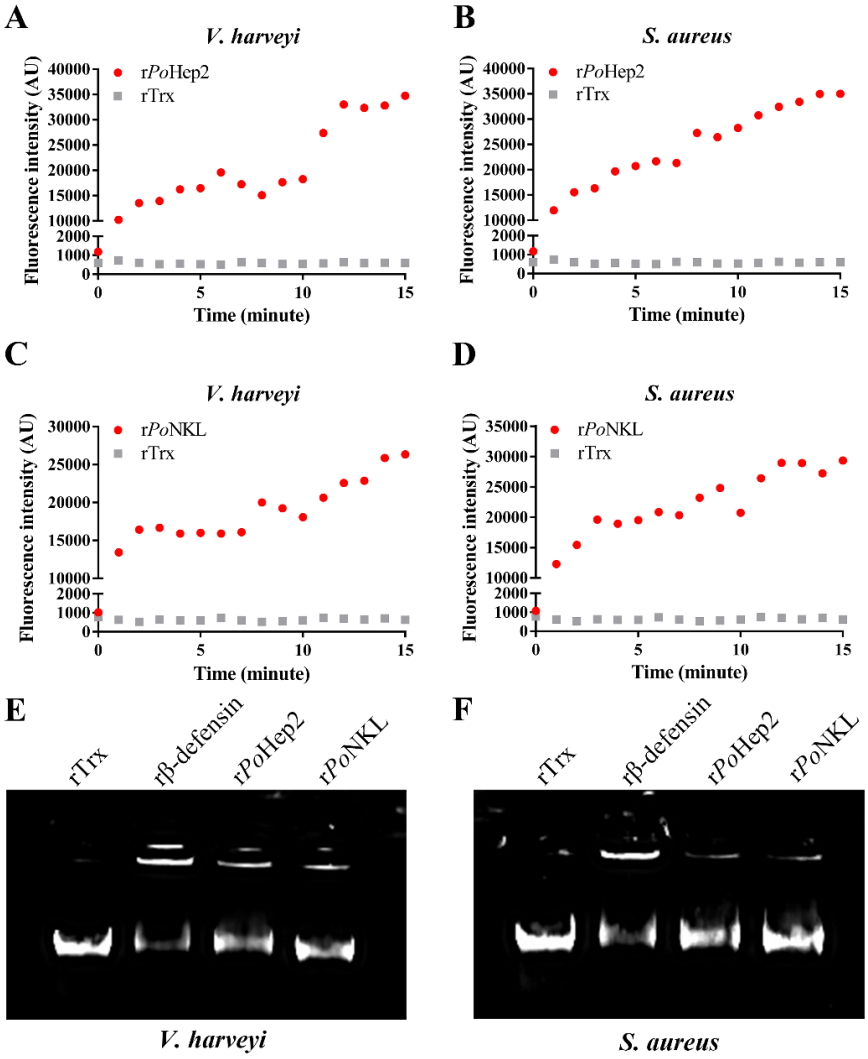


Fig. 7

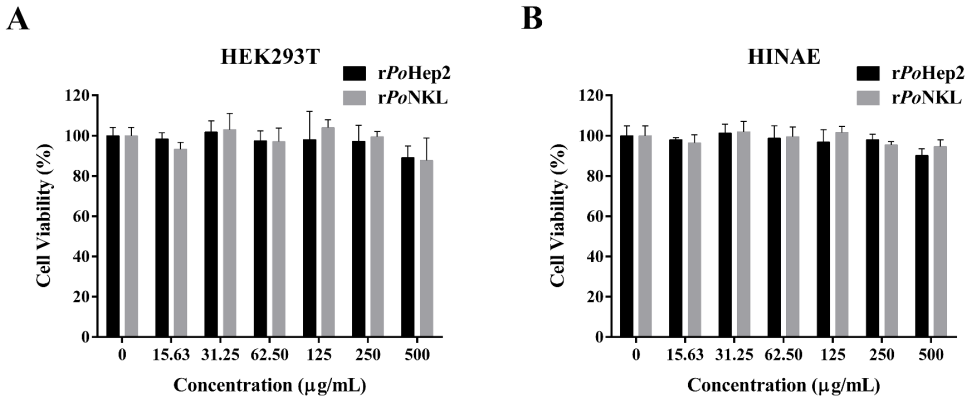
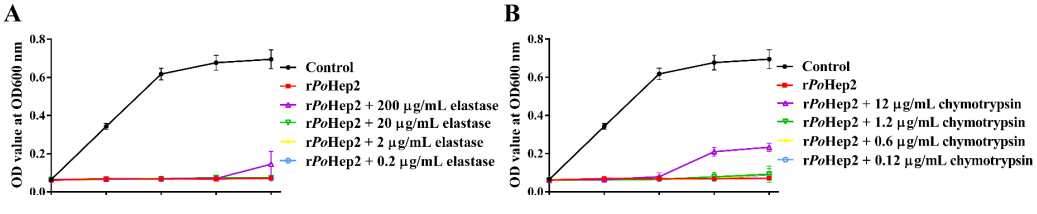


Fig. 8



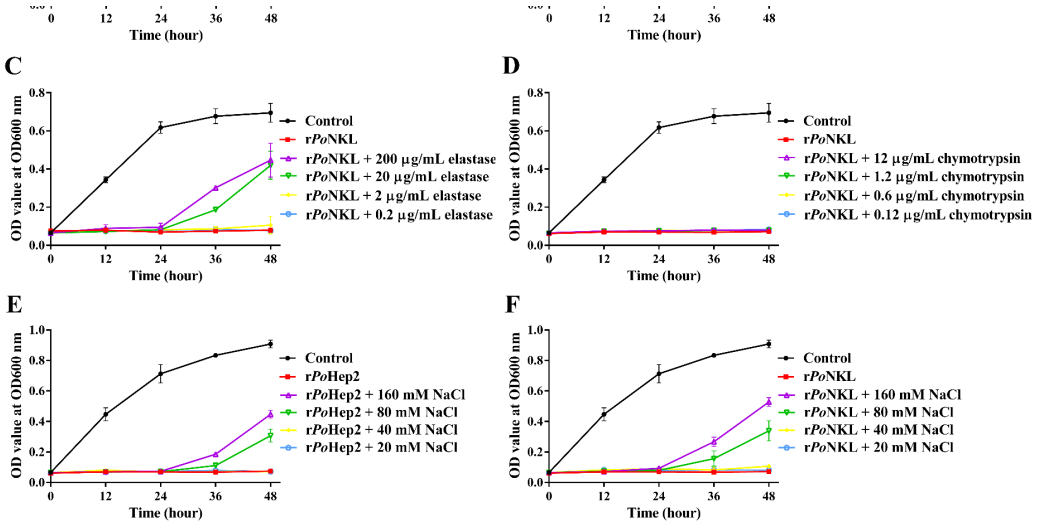


Fig. 9

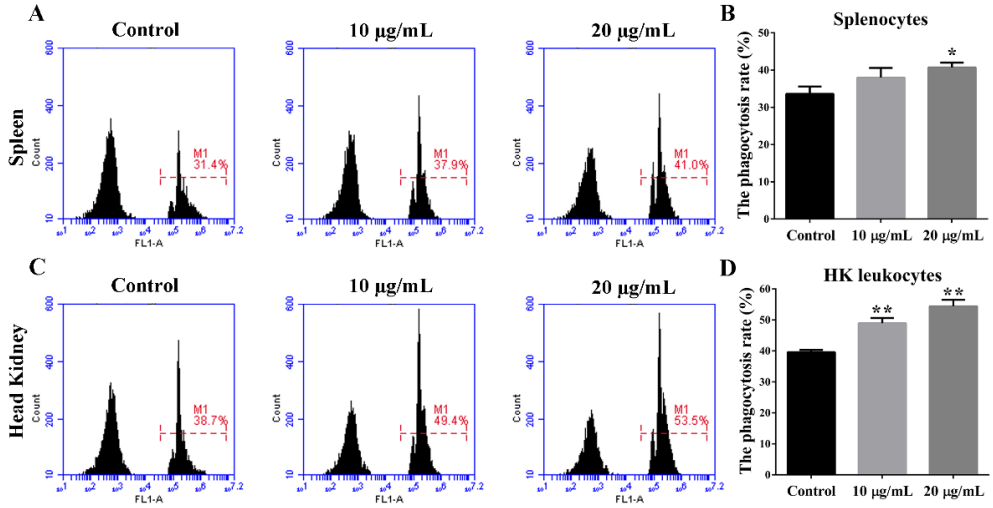
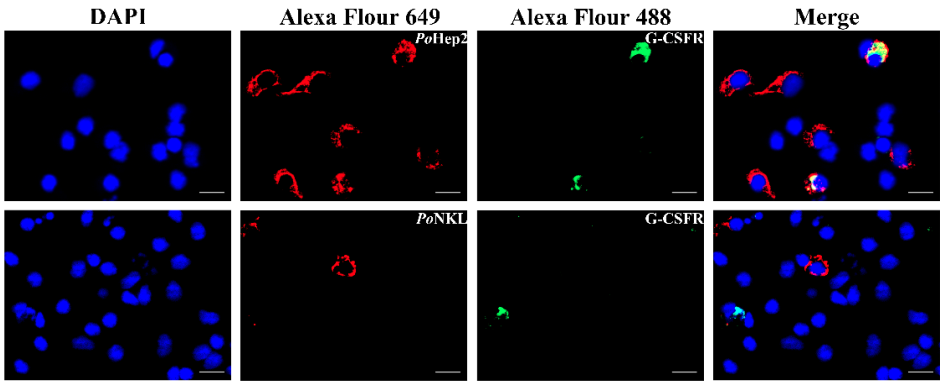
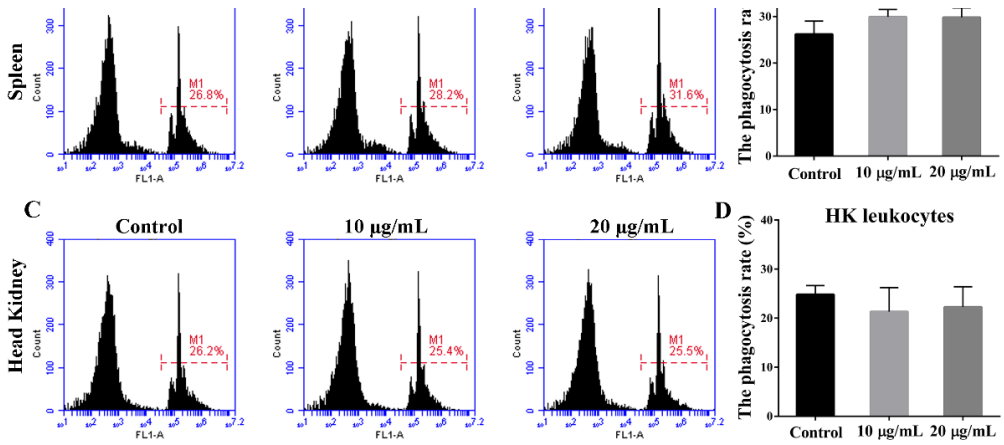


Fig. 10



Supplementary data:

Fig. S1. The phagocytosis of leukocytes from spleen (A and B) or head kidney (C and D) with the treatment of r*Po*NKL at doses of 10 and 20 μg/mL. Fluorescence histograms were used to represent the phagocytosis rate of leukocytes from the spleen (A) or head kidney (C). Three biological replicates were performed and the data were analyzed (B and D).


Spleen
Count
FL1-A
M1
26.8%
M1
28.2%
M1
31.6%
The phagocytosis ra
Control
10 μg/mL
20 μg/mL

C
Control
10 μg/mL
20 μg/mL
Head Kidney
Count
FL1-A
M1
26.2%
M1
25.4%
M1
25.5%
D
HK leukocytes
The phagocytosis rate (%)
Control
10 μg/mL
20 μg/mL