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Fish-derived antimicrobial peptides: activity of a chionodracine mutant against bacterial models and human bacterial pathogens

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Abstract

The increasing resistance to conventional antibiotics is an urgent problem that can be addressed by the discovery of new antimicrobial drugs such as antimicrobial peptides (AMPs). AMPs are components of innate immune system of eukaryotes and are not prone to the conventional mechanisms that are responsible of drug resistance. Fish are an important source of AMPs and, recently, we have isolated and characterized a new 22 amino acid residues peptide, the chionodracine (Cnd), from the Antarctic icefish *Chionodraco hamatus*. In this paper we focused on a new Cnd-derived mutant peptide, namely Cnd-m3a, designed to improve the selectivity against prokaryotic cells and the antimicrobial activity against human pathogens of the initial Cnd template. Cnd-m3a was used for immunization of rabbits, which gave rise to a polyclonal antibody able to detect the peptide. The interaction kinetic of Cnd-m3a with the Antarctic bacterium *Psychrobacter* sp. (TAD1) was imaged using a transmission electron microscopy (TEM) immunogold method. Initially the peptide was associated with the plasma membrane, but after 180 min of incubation, it was found in the cytoplasm interacting with a DNA target inside the bacterial cells. Using fluorescent probes we showed that the newly designed mutant can create pores in the outer membrane of the bacteria *E. coli* and *Psychrobacter* sp. (TAD1), confirming the results of TEM analysis. Moreover, *in vitro* assays demonstrated that Cnd-m3a is able to bind lipid vesicles of different compositions with a preference toward negatively charged ones, which mimics the prokaryotic cell. The Cnd-m3a peptide showed quite low hemolytic activity and weak cytotoxic effect against human primary and tumor cell lines, but high antimicrobial activity against selected Gram – human pathogens. These results highlighted the high potential of the Cnd-m3a peptide as a starting point for developing a new human therapeutic agent.

Key words: antimicrobial peptides; chionodracine mutant; antibody production; interaction with bacterial membranes; antibacterial activity.

INTRODUCTION

The antimicrobial peptides (AMPs), fundamental components of the innate immune system of prokaryotes and eukaryotes, have a broad specificity towards different pathogens, like bacteria, fungi, parasites and viruses (Zasloff M., 2002; Bulet et al., 2004). AMPs show different size, structure and physico-chemical characteristics and, therefore, can be classified based on their biosynthesis, their biological activities or their structural properties (α-helices, β-sheets, extended or loop structures) (Campagna et al., 2007). The AMPs usually, show amphipathic features with a positively charged, and a non-polar face, that are fundamental for their interaction with the anionic group present on the bacterial cell membrane (White and Wimley, 1998). They have been investigated from some time in clinical research as new antibiotics for infectious conditions caused by multidrug-resistant microbes (Hancock and Lehrer, 1998), but only few AMPs, until now, have been approved for clinical use, like polymyxins in the treatment of Gram – bacterial infections (Zavascki et al., 2007; Mahlapuu et al. 2016).

Fish live in an aquatic environment rich in microbial load and, therefore, their innate immune system rely highly on AMPs. Many different peptides have been isolated from fish in the last thirty years showing various biological activities (see the reviews of Ravichandran et al., 2010; Rajanbabu and Chen, 2011; Shabir et al., 2018). They comprise molecules belonging to the defensin, cathelicidin and hepcidin families, and also a specific family found only in fish and called piscidin (homologous to cecropin) (Noga and Silphaduang, 2003). Fish AMPs are active against both bacterial and virus fish specific pathogens (Pan et al., 2007; Chia et al., 2010) and some are also involved in iron regulation (Shi and Camus, 2006). Piscidins have been firstly isolated from mast cells of hybrid striped bass as different isoforms (piscidins 1, 2 and 3) (Noga and Silphaduang, 2003) and, successively, piscidin-like peptides have been found in sea bream stored in granules of professional phagocytic granulocytes (Mulero et al., 2008). Piscidin 2 is active against fish ectoparasites (Colorni et al., 2008) and, more recently, a new piscidin isoform (piscidin 4), involved in the killing of different fish bacterial pathogens, has been identified in the gills of hybrid striped bass (Corrales et al., 2009). Piscidins are produced as pre-pro-peptides and then activated, after cleavage, into a biologically active cationic molecule (about 22 amino acids in length); their pore-forming activity on bacterial cell wall is more likely achieved by a toroidal mechanism (Campagna et al., 2007), but recent results suggest that transient distortions of the membrane bilayer rather stable pores are responsible for membrane disruption (Perrin et al., 2016). Piscidin-like antimicrobial peptides have shown also interesting potential applications as chemotherapeutic drugs

due to their cytotoxic activity against breast cancer cells (Hilchie et al, 2011) and human fibrosarcoma, histiocytic lymphoma and epithelial carcinoma (Hsu et al., 2011).

We have identified a piscidin-like AMP from the icefish (*Chionodraco hamatus*) (Buonocore et al., 2012), an Antarctic teleost of the Channichthyidae family, and we have determined its localization in gill mast cells and its antimicrobial activity against two psychrotolerant and psychrophilic bacterial strains (*Psychrobacter* sp. TAD1 and TAD144). This 22 amino acid residues long peptide, named chionodracine (Cnd), did not show both significant lytic activity on human erythrocytes and antimicrobial activity against human bacterial pathogens. Successively, we have demonstrated that chionodracine is able to make discrete pores on the membranes of both *Psychrobacter* sp. TAD1 and *Escherichia coli*, and that it folds into an amphipathic α-helix in the presence of lipid vesicles (Olivieri et al., 2015). In this work, we designed a new mutant starting from the sequence of the Cnd, namely Cnd-m3, with the aim to improve its antimicrobial activity against human pathogens. We produced an antibody against this new generated peptide that was used to investigate its internalization within bacterial membranes by transmission electron microscopy (TEM). Moreover, we studied its ability to form pores on *Psychrobacter* sp. TAD1 and *Escherichia coli* membranes and its binding capacity on lipid vesicles mimicking bacterial membranes. Finally, we determined the Cnd-m3 hemolytic activity and cytotoxic effect against human primary and cancer cell lines, and its antimicrobial activity against both Gram – and Gram + human pathogens to assess its potential use as a new therapeutic drug.

MATERIALS AND METHODS

2.1 Peptide and lipids

The peptide (98% purity) was purchased from United Biosystem Inc. USA. Peptide concentration was determined for each sample preparation by UV light absorption at 280 nm. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The alignment between Cnd and Cnd-m3a was performed using Clustal Omega (https//www.ebi.ac.uk/Tools/msa/clustalo/). The net charge and the hydrophobic moment of the peptide have been calculated using the prediction tools of the APD3 Antimicrobial Peptide Database (http://aps.unmc.edu).

2.2 Antibody production and ELISA assay testing

The two synthetic mutant peptides (KS-Cnd NH_2-WFGHLYRGITKVVKHVHGLLKG-COOH, see Olivieri et al., 2018, and Cnd-m3a NH_2-WFGKLYRGKTKVVKKVKGLLKG-COOH) were employed as antigens to immunize two New Zealand rabbits for obtaining anti-peptides antisera by a Company (Primm Srl, Milano, Italy). The immunization was performed subcutaneously with the two synthetic peptides conjugated to KLH, resuspended in 0.1M phosphate buffered saline (PBS) and with the use of complete Freund's adjuvant (Serva, Heidelberg, Germany) in the first two inoculi.

The obtained rabbit sera were tested in indirect ELISA against adsorbed immunization peptide Cnd-m3a. Briefly, lyophilized peptide was resuspended in distilled water at 30 µg/ml, and polystyrene wells were coated with 100 µl/well of peptide at a final concentration of 300 ng/well in 0.05 M carbonate-bicarbonate buffer pH 9.4. The wells were then washed three times with Tris-HCl 50 mM pH 7.4 containing 0.05% Tween-20 and 0.15 M NaCl (TTN). After blocking remaining sites with 3% BSA in TTN (TBT), 100 µl/well of the two antisera Peps1 and Peps2, separately diluted at 1:10, 1:100, 1:1000, 1:5000 in TBT, were added, incubating the plates for 3 hours at 4°C. Each experimental point was analysed in triplicate and control wells without adsorbed antigen were used as a control. The wells were carefully washed with TTN and then incubated for 2 hours with a HRP-conjugated anti-rabbit Ig secondary antibody solution (Cappel) in TBT. The wells were then washed with 50 mM phosphate-citrate buffer (pH 5.0) and the reaction was visualised using *o*-phenylenediamine (Sigma) as substrate. The absorbance values were read at 450 nm with an automatic plate reader (Labsystems). Optical density values (OD at 450 nm) of control wells were automatically subtracted from positive wells and the ELISA assays data have been calculated as the mean absorbance ± SD of the triplicate wells.

2.3 Immunoelectron microscopy

Psychrobacter sp. TAD1 bacteria cells were grown to the mid-log phase in Luria Bertani (LB broth from Sigma) medium. Cnd-m3a peptide at a concentration of 15.0 µM was added to the cultures and they were incubated at different time points (0, 10 and 180 min at 15 °C) with shaking. At the end of the incubation time, samples were centrifuged (5,000 rpm for 5 min) and the supernatant was discarded. Samples at time 0, 10 and 180 minutes after incubation and untreated bacteria were collected and fixed with 100 µl of a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 6.9, for 20 min at room temperature. After rinsing in the same buffer for 10 min, samples were dehydrated in a graded ethanol series and embedded in medium grade LR White resin. The resin was polymerised in tightly capped gelatine capsules for 24 h at 50°C. Ultrathin sections were obtained using a Reichert Ultracut ultramicrotome with a diamond knife, and collected on nickel grids.

For immunogold staining (IGS) non-specific antigens were blocked with 0.5% BSA in 0.05 M TRIS-HCl buffer, pH 7.6 for 15 min. Sections were incubated overnight in a moist chamber with the polyclonal antibody Peps2 diluted 1:100 in TRIS-HCl buffer, pH 7.6. The grids were washed in 0.05 M TRIS-HCl, pH 7.6, for 20 min and then in 0.05 M TRIS-HCl, pH 7.6, containing 0.1% BSA for 10 min. Sections were incubated with a secondary goat anti-rabbit antibody conjugated to 10 nm gold particles (British BioCell International, UK), diluted 1:10 in 0.02 M TRIS-HCl buffer, pH 8.2. After rinsing in 0.05 M TRIS-HCl buffer containing 0.1 % BSA for 10 min and in 0.05 M TRIS-HCl buffer for 20 min, the grids were washed three times with distilled water (for 5 min). Sections were subsequently stained with uranyl acetate and lead citrate and observed with a Jeol JEM EX II transmission electron microscope at 100 kV. Pre-immune serum substituted the primary antibody in control sections.

2.4 LUV Preparation

LUVs (large unilamellar vesicles) composed, respectively, of 100% POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) and 70%/30% (w/w) POPC/POPG (POPG 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol) were prepared according to general procedures previously reported (Olivieri et al., 2015). Briefly, the lipids dissolved in chloroform were dried under nitrogen flow and then overnight under high vacuum. The lipid film was then hydrated in 1 mL of buffer (20 mM phosphate buffer at pH 7.4 with 150 mM NaCl and 0.8 mM EDTA) and subjected to 5 freeze-thaw cycles. The suspension was extruded through a polycarbonate membrane with an Avanti Polar mini-

extruder (20 times through two-stacked polycarbonate membranes with pore sizes of 100 nm) and the obtained LUVs were used within 48 hours of preparation.

2.5 Steady state fluorescence experiments

All the steady state fluorescence experiments were performed using a Perkin Elmer LS55 operating at 25 °C in a thermostatic cell holder. The spectra were corrected by subtracting the corresponding blanks.

2.5.1 Outer membrane permeabilization assay

The permeabilization assay was carried-out using the fluorescent probe ANS (1-aminonaphtalene-8-sulfonic acid) as previously described (Domadia et al., 2010; Olivieri et al., 2015; Olivieri et al., 2018). *E. coli* BL21 (DE3) and *Psychrobacter sp*. TAD1 cells were grown, at 37 °C and 15 °C respectively, to mid-log phase in Luria Bertani broth (LB broth from Sigma) were centrifuged, washed and suspended in 10 mM Tris-HCl, 150 mM NaCl, and 0.8 mM EDTA (pH 7.4) buffer to give an OD_{600} of ~1.2. Subsequently, increasing amounts of the Cnd-m3a peptide (from 1.0 to 15.0 μM) were added to a quartz cuvette containing 1.0 mL of cell suspension and 5.0 μM ANS. Fluorescence spectra were recorded at wavelengths between 400 and 600 nm with an excitation wavelength of 360 nm. The excitation and emission slit widths were 5 nm. The ANS was incorporated into the membrane and, consequently, the fluorescence intensity increased and blue-shifted.

2.5.2 Partition studies

The ability of peptides to associate with and partition into lipid vesicles was studied by measuring the enhancement of tryptophan fluorescence upon addition of LUVs. Trp-1 fluorescence spectra were recorded at wavelengths between 305 and 500 nm considering an excitation wavelength of 295 nm. Measurements were performed with a cross-oriented configuration of polarizers ($Pol_{em}=0°$ and $Pol_{exc}=90°$) to reduce contributions from vesicles (Ladokhin et al., 2000). A 1.0 μM peptide solution in 20 mM phosphate buffer at pH 7.4 containing 0.8 mM EDTA and 150 mM NaCl was added to a cuvette and then titrated with LUVs of different compositions (100% POPC and 70%/30% POPC/POPG) with a lipid/peptide ratio ranging from 50 to 500 as described previously (Olivieri et al., 2015; Olivieri et al., 2018). The background effects of both buffer and vesicles were subtracted from each spectrum. Mole fraction partition coefficients, K_x, were obtained calculating the fraction of peptide, f_p, which partitioned into the LUVs (Wimley and White, 1993; Rathinakumar and Wimley, 2008; Ladokhin, 2009; Fernández-Vidal et al., 2011). The values of K_x were obtained as described before (Olivieri et al., 2015; Olivieri et al., 2018).

2.5.3 Iodide quenching experiments

Peptide solutions (5.0 µM) in both absence and presence of LUVs (in a peptide:lipid ratio 1:100), were excited at 295 nm and fluorescence spectra were recorded from 305 to 500 nm. The samples were titrated by adding increasing amount of potassium iodide in the range 0.01 - 0.28 M and spectra were recorded with excitation and emission band widths of 2.5 nm. All the fluorescence spectra were corrected for dilution. Fluorescence intensities were extracted and the data were fitted according to the Stern-Volmer equation as described previously (Park et al. 2011; Olivieri et al., 2015; Olivieri et al., 2018).

2.6 Negative staining

Samples at the peptide/lipid ratio of 1:100 were prepared with a final lipid concentration of 70%/30% (w/w) POPC/POPG vescicles. They were incubated for 10 minutes with 15.0 µM of the Cnd-m3a peptide. Untreated vesicles and lipids at time 0 and 10 min after incubation with the peptide were fixed using 4% PFA in phosphate buffered saline (PBS, Gibco). Droplets of sample suspensions (10 µl) were placed on formvar-carbon coated grids and allowed to adsorb for 60 sec. Excess liquid was removed gently touching the filter paper. The adsorbed specimen was then processed for negative-staining by first washing the specimen grid on a drop of negative stain (2% uranyl acetate in distilled water) and, successively, blotting and repeating this step once more leaving, in this case, the specimen grid for 60 seconds on a new drop of negative stain solution. Samples were observed at a JEOL 1200 EX II electron microscope. Micrographs were acquired with an Olympus SIS VELETA CCD camera equipped the iTEM software.

2.7 Hemolytic assay

The hemolytic assay was performed as indicated by Belokoneva et al. (2003). In brief, a 2.5 % (v/v) suspension of human red blood cells from healthy donors in PBS (Gibco) was incubated with serial dilutions of the mutant peptide. Red blood cells were counted by a haemocytometer and adjusted to about 8.0 X 10^6 cell/ml. Erythrocytes were incubated at 37 °C for 2 h with 10% Triton X-100 solution (positive control), PBS (negative control) and with different concentrations of the Cnd-m3a peptide (five dilutions from 50 µM to 0.5 µM, in triplicates). The supernatant was separated from the pellet by centrifugation at 1500x *g* for 5 min and the absorbance measured at 570 nm. The relative OD compared to that of the positive control defined the percentage of hemolysis. The

experiments were performed in triplicate and date shown as mean ± SD, the statistical analysis was made using the one-way analysis of variance and Bonferroni's post test.

2.8 Cell proliferation assay

The peptide Cnd-m3a was dissolved in 100% DMSO (Sigma-Aldrich, USA) to a concentration of 10 mM and then diluted and transferred to the bottom of a tissue culture 384 well plate (Greiner Bio One, Austria); the plate was subjected to acoustic droplet ejection (ATS-100, EDC Biosystem, USA) in order to reach the desired final peptide concentration in the culture volume of 40 µL (the final percentage of DMSO was 0.5%).

The cell types reported in Table SI (see Supplementary files) were then plated on compound containing microplates to a density of 2000 cells/well in the final volume of each used medium (40 µL) (see Table SI).

After 48 h (72 h for HUVEC only) of incubation at 37 °C and 5% CO_2 in humidified atmosphere, cell proliferation was assessed by CellTiter-Glo following manufacturer's instruction (Promega, USA). Ten different 1:2 dilutions of the peptide Cnd-m3a were tested starting from a concentration of 50 µM and three independent experiments were performed for each selected concentration. Data analysis was performed by considering four parameter-logistic regression, using GraphPad software (Prism, USA).

2.9 Evaluation of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

The effects of the Cnd-m3a peptide on microorganism's vitality have been analyzed for Gram – and Gram + positive bacteria. Clinical isolates with assessed resistance profiles were used in this study: *E. coli* ESBL, *Klebsiella pneumoniae* KPC, *Acinetobacter baumanni* XDR, *Psuedomonas aeruginosa* MDR, *Staphylococcus aureus* MRSA, *Staphylococcus epidermidis* MRSE and *Enterococcus* spp. VRE. Frozen glycerol stocks were streaked on fresh Trypticase soy agar with 5% sheep blood plate (bioMerieux), incubated at 37° C for 18 h and sub-cultured to produce fresh colonies. A single bacterial colony was used to grow in Mueller-Hinton liquid medium.

The CLSI (CLSI, 2015) susceptibility testing protocol was used for the determination of the MICs of the antibacterial agent. The peptide was dissolved in an appropriate buffer and diluted in Mueller-Hinton broth to reach a final concentration of 200 µg/ mL. Logarithmic phase bacterial cultures were suspended in saline solution to achieve a turbidity equivalent to that of a 0.5 McFarland standard and then diluted to a final concentration of $1.2X10^5$ CFU/mL. Bacterial

suspensions were added to serial dilutions of the peptide (from 0.097 to 100 µg/mL) in a 96-well flat-bottom Microtiter® plate. Both positive (no peptide) and negative (no bacteria) controls were included. The MIC was defined as the lowest compound concentration which prevented visible growth after 24 h of incubation at 37° C. To determine the MBC, aliquots of 100 µL were removed from the wells with no visible microbial growth and plated on Trypticase soy agar plates with 5% sheep blood, than incubated overnight at 37 °C. The MBC was defined as the lowest peptide concentration at which more than 99.9% of the cells were killed compared with an untreated control. All tests were performed in triplicate in two different experimental sessions. A panel of conventional antibiotics have been used as a positive control (see Supplementary files, Table SII).

RESULTS

3.1 Peptides design

AMPs are short peptides (10-50 amino acids) with an overall positive net charge (usually from +1 to +9) and an amount of hydrophobic residues in general close to the 30% of the total molecule. Upon interaction with biological membranes, AMPs fold into amphipathic conformations with the polar residues facing towards the negatively charged head groups of the membrane phospholipids. Starting from the sequence of Cnd (Buonocore et al, 2012), bearing a net charge of +2 at pH 7, we designed a new mutant called Cnd-m3a. Serines 11 and 22, histidines 4, 15, and 17, and, finally, isoleucine 9 have been replaced by lysines (see Figure 1). After the mutation the overall charge is now +8 and the hydrophobic moment 0.564.

3.2 Antibody selection

To detect the interaction of the Cnd-m3a peptide with the bacterial membranes we produced two polyclonal antibodies from rabbits and we selected for the performed experiments the antiserum (named Peps2) showing the best peptide detection in ELISA (see Table I).

3.3 Penetration of Cnd-m3a into target bacterial cells

To evaluate the mechanism of action of Cnd-m3a against bacterial cells, the polyclonal antibody Peps2 has been used and the peptide localization performed by immunoelectron microscopy (Fig. 2). The peptide, visualized by gold particles, interact with the target bacterial cells, *Psychrobacter sp*., by electrostatic forces between its positive amino acid residues and the negative exposed charges present on bacterial cell surface. Upon initial investigation at time 0, it was immediately noted that few peptides were closely associated with the bacterial membranes. Cnd-m3a is able to spontaneously traverse *Psychrobacter sp*. outer and inner membranes and, after 10 minutes of incubation, it was found inside the cells, targeting intracellular molecules such as nucleic acids. Once inside the cells, AMPs accumulated in the cytoplasm in a time dependent manner and evident morphological cell damages were induced at 180 minutes, leading to the consequent microbial cell death.

3.4 Outer membrane permeabilization assay

Permeabilization of *E. coli* BL21 (DE3) and *Psychrobacter sp*. TAD1 outer membrane has been studied using the ANS fluorescence. ANS displays a weak fluorescence in aqueous solutions but a very high fluorescence in a hydrophobic environment. Because of its structure, ANS is not able to

cross the intact outer membrane and thus ANS cannot normally enter in the cell. Upon perturbation or disruption of lipid bilayer, operated by an antimicrobial agent, the ANS can penetrate the cell and its fluorescence emission increases drastically shifting towards lower wavelength (λ). In Figure 3 (A and B) the percentage of ANS uptake for the permeabilization of *E.coli* and *Psychrobacter sp*. upon addition of Cnd and Cnd-m3a is reported. Each experiment has been carried out in triplicate and the standard deviation was ~5%. It is evident that the mutant peptide is able to induce a higher perturbation of the bacterial outer membranes compared to the wild type Cnd (see Olivieri et al., 2015). In presence of Cnd-m3a or of Cnd, at a concentration of 1.0 μM, the percentage of ANS uptake is ~38% and ~21% for *Psychrobacter sp.* and ~56% and ~40% for *E. coli*, respectively. Moreover, at a concentration of 5.0 μM, Cnd-m3a induces an ANS uptake of ~70% for both *E. coli* and *Psychrobacter sp.*, whereas to obtain the same effect it should occur a 10.0 μM and 15.0 μM concentration of Cnd, respectively. The fluorescence spectra for the permeabilization assay using Cnd-m3a are also reported (C and D).

3.5 Partition studies

To study the interaction of peptides with the eukaryotic and prokaryotic membrane we used as membrane models LUVs of different composition. Specifically, a mixture of POPC/POPG (70/30 w/w) was used as mimicking system for bacterial membranes and the zwitterionic POPC was used as mimicking system for eukaryotic membranes (Herbig et al., 20005; Reid et al., 2018). The ability of Cnd-m3a to have interaction with and partition into lipid vesicle of different compositions was determined by fluorescence spectroscopy of Trp-1 for each peptide. Tryptophan fluorescence is commonly used to determine the polarity of the local environment. Specifically, as the polarity of the environment decreases the Trp fluorescence shifts to a lower wavelength (blue shift) and increases its intensity. The shift and the enhancement in the fluorescence emission spectrum (Figure 4) suggest that the Cnd-m3a peptide is able to interact and bind to the lipid vesicles of different composition, 100% POPC and 70%/30% POPC/POPG. LUVs entirely composed of POPC, a zwitterionic lipid with no net charge, were used to mimic mammalian cell membranes, whereas LUVs containing also POPG, a lipid head group bearing a net negative charge, where chosen to mimic the net anionic surface charge of bacterial membrane. To evaluate the mole fraction partition coefficients K_x, we plotted the binding isotherms derived from the titration of peptides (Figure 4). The values of K_x have been determined and reported in Table II with the selectivity ratio defined as the ratio between the partition coefficient determined for 70%/30% POPC/POPG and the one for 100% POPC. Partitioning data suggest that Cnd-m3a has weaker interactions with LUVs composed

412 exclusively of POPC (K_x = (5.1 ± 0.2) x 10^4), thus showing a higher affinity and selectivity towards
413 POPC/POPG LUVs (K_x = (25.0 ± 0.9) x 10^4), which mimics the prokaryotic cell membranes.
414 Noteworthy, is the high value of the selectivity ratio, ~ 5, indicating its higher preference for
415 vesicles mimicking the bacterial cell membrane if compared to the wild type Cnd (see Olivieri et
416 al., 2015).
417
418 ***3.6 Iodide quenching***
419 Successively, the accessibility of tryptophan to the iodide dynamic quencher was investigated and
420 in Figure 5 the Stern-Volmer plots in absence and presence of lipid vesicles are reported for both
421 Cnd (Olivieri et al., 2015) and Cnd-m3a. Stern-Volmer plots for peptides fit linearly, indicating a
422 collision mechanism of quenching, and the corresponding K_{SV} (Stern-Volmer constants) determined
423 values are reported in Table III. The Trp-1 showed high solvent accessibility for both peptides in
424 buffer and high values of K_{SV} have been determined (10.4 and 8.3 M^{-1}, for Cnd and Cnd-m3a,
425 respectively). The smaller values of K_{SV} obtained in presence of LUVs, clearly indicate a great
426 solvent protection of Trp-1 due to a strong interaction with the bilayer lipid environment. The
427 similar trend observed (Figure 5) for the wild type and the mutant peptides in presence of lipid
428 vesicles of different composition suggest that both interact with the LUVs and that the Trp-1 is not
429 solvent-exposed.
430 The net accessibility factors (NAF) were defined by the formula:

$$NAF = \frac{(K_{SV})_{LUV}}{(K_{SV})_{buffer}}$$

431

432 as the ratio between the Stern-Volmer constant in presence and in absence of LUV (Saikia et al.,
433 2018). The small values of NAF suggest that the Trp residue is largely inaccessible to the quencher,
434 due to a strong interaction with the lipid bilayer. In presence of 100% POPC vesicles, the membrane
435 mimicking system for eukaryotic cells, the values of NAF are nearly the same for both peptides.
436 However, in presence of 70%/30% POPC/POPG vesicles, the Cnd-m3a peptide shows a smaller
437 value of NAF, 0.18, with respect to 0.24 for Cnd, thus indicating an improved preference for
438 anionic lipid vesicles.
439
440 ***3.7 Effect of Cnd-m3a on POPC/POPG LUVs***
441 TEM analysis (Figure 6) showed that the Cnd-m3a peptide alters the 70%/30% POPC/POPG
442 LUVs membranes, leading to the formation of roughening and surface ruptures if compared to the
443 smooth membranes found in untreated vesicles. Moreover, Cnd-m3a peptide induced, after 10

minutes of incubation, membrane curvature and change in the POPC/POPG LUVs shape, which is evident by the largely found non spherical LUVs compared with the nearly spherical shape with round edges present in the untreated ones.

3.8 Hemolytic and citotoxicity assasys on Cnd-m3a

The hemolytic effect of the Cnd-m3a peptide has been tested on human erythrocytes to investigate its capacity to induce membrane lysis. Five concentrations have been used (starting from a concentration of 50 µM with successive dilutions) that correspond for the first point to about 120 µg of peptide (Figure 7). The percentage of hemolysis is quite low at 3 µM (about 5 %) and it reaches the maximum (about 30 %) at the highest tested concentration value (50 µM). All differences were statistically significant compared to the lowest tested peptide concentration (3 µM).

The cytotoxity of the Cnd-m3a peptide was investigated, in a dose response manner starting from 50 µM and performing ten 1:2 successive dilutions, on one primary human endothelial cell line (HUVEC) and on a panel of cancer cell lines for the determination of its anti-proliferative potential. No inhibition of cell proliferation was appreciable at all tested concentrations both in the case of normal as in tumoral cell lines (Table IV).

3.9 Antibacterial activity of Cnd-m3a

In vitro experiments were conducted to investigate the antibacterial activity of Cnd-m3a. A total of 7 clinical isolates belonging to clinically-relevant multidrug-resistant bacteria were tested. Strains were previously identified by standard morphological, cultural and biochemical tests. The MICs and MBCs values are summarized in Table V**.** The obtained data showed that the peptide exerted the strongest bactericidal activity on Gram – bacteria, as expected by the high affinity of this molecule towards their cell membrane and evidenced by the results of the previously reported partition and iodide quenching studies. Conventional antibiotics have been used as positive controls of the tested bacterial strains resistance (see supplementary files Table II).

DISCUSSION

During last years the antibiotic resistance, the ability of bacteria to withstand to the effects of usual medications, is highly increasing and it is now a serious global public health threat. According to the Centers for Disease Control and Prevention (CDC), 2 million people in the U.S. develop antibiotic-resistant infections each year, and at least 23.000 people die from those infections (https://www.cdc.gov/drugresistance/). Intensive research is therefore directed towards the identification of new and non-conventional anti-infective molecules, and AMPs have been considered as possible candidates. They have been identified in all living organisms showing also immunomodulatory properties, which make them the compounds most intensively studied for the development of novel therapeutics in this field (Mahlapuu et al., 2016).

A high number of AMPs, starting from the first peptide isolated from the skin of the winter flounder in 1997 (Cole et al., 1997), have been isolated from fish that, living in an aquatic environment with a high microbial load, have evolved a potent innate immune system (Rakers et al., 2013; Shabir et al., 2018). Thus, we decided some years ago to investigate the presence of AMPs in a teleost, the *Chionodraco hamatus* (Perciformes: Channichthyidae), adapted to resist in an extreme environment like the Antarctica, as this species was considered a model for its adaptations to subzero temperatures that involved peculiar physiological features (Ruud, 1954; Bargelloni et al., 1994). The isolated peptide was named chionodracine (Cnd), and its molecular characterization and bactericidal activity was firstly investigated (Buonocore et al., 2012). The peptide showed all the expected features of a fish AMP and it was biologically active against endemic bacteria from Antarctica (*Psychrobacter* sp. TAD1 and *Psychrobacter* sp. TAD144). Unfortunately, although it showed low hemolytic effect, it was not active against human bacterial pathogens as another Antarctic AMP recently identified (Shin et al, 2017). Successively, we studied its structure, its interaction with different membranes and we determined that it adopts a canonical α-helical structure upon the interaction with lipid membranes, with a preference towards negatively charged lipids and *E. coli* extracts (Olivieri et al., 2015). Taking into account our previous results, starting from the Cnd template, we designed a new mutant peptide with the aim to obtain a molecule more active against human bacterial pathogens. Therefore, we introduced in this new sequence, named Cnd-m3a, more positive charged amino acids (the total net positive charge is now +8), as this aspect is crucial for the interaction with the negatively charged membranes of bacteria, and, moreover, we disrupted the amphipathicity of the nonpolar face to improve the antimicrobial activity by increasing the specificity and selectivity towards bacterial membranes and decreasing the cytotoxicity towards mammalian membranes (Chen et al., 2005). A similar strategy was used in the

designing of other three mutants, KS-Cnd, KH-Cnd and KHS-Cnd, but with a maximum total net charge of +7, in KSH-Cnd, and preserving the original α-helix conformation (Olivieri et al, 2018).

Using a polyclonal antibody rose against Cnd-m3a we demonstrated by immunoelectron microscopy that the peptide absorbs on the membrane surface of the bacteria *Psychrobacter* sp. TAD1 and, after entering inside the cell it targets the DNA causing cell death. Very recently, He and colleagues identified an antimicrobial peptide from red drum (*Sciaenops ocellatus*) (He et al., 2018) and demonstrated *in vitro* that it was able to induce degradation of bacterial nucleic acids (both DNA and RNA). However, this is the first time, on our knowledge, that this kind of mechanisms has been demonstrated in *in vivo* studies, by immunoelectron microscopy on target bacteria, for a fish AMP. Successively, we investigated the interaction of the Cnd-m3a peptide with two different synthetic unilamellar vesicles, one formed only of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), which mimics mammalian membranes that are composed of zwitterionic lipids with no net charge (Zasloff, 2002), and the other composed of 70% POPC and 30% POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol), which is more similar to bacterial membranes as POPG is negatively charged (Yeaman and Yount, 2003). Both outer membrane permeabilitation assay and partition studies evidenced that the mutant peptide acts preferentially against POPC/POPG vesicles with a higher activity compared to the wild type Cnd and also with a higher selectivity ratio compared to the other three mutants previously designed (Olivieri et al., 2018). The action on synthetic membranes of piscidin 1 has been simulated in a recent paper and the obtained results evidenced that the membrane disruption was not due to the formation of stable pores but, rather than, to the formation of transitory distortions of the bilayer/water interface (Perrin et al., 2016): this results is in agreement with the mechanism of bacteria degradation we demonstrated for Cnd-m3a and with its effects on POPC/POPG LUVs we determined by TEM-analysis.

To evaluate the possible use of the Cnd-m3a peptide as a new therapeutic drug, we studied its hemolytic activity against human erythrocytes and it resulted of about 5 % at the lowest tested concentration (3 µM), a slightly higher value if compared to the same data obtained for both Cnd and the three mutants (Olivieri et al., 2015; Olivieri et al., 2018). We investigated also the cytotoxic effect of the Cnd-m3a mutant against a primary human endothelial cell line (HUVEC) with no evident effect at all tested concentrations (the maximum was 50 µM). Therefore the peptide should have no or very low effects on normal mammalian cells, as it was expected.

We further investigated the potentiality of the peptide studying its antibacterial activity against multidrug resistant human pathogens. The results were very interesting as the Cnd-m3a was

active, with MIC values around 5-10 µM, especially against Gram – bacteria. This bacteria, in contrast to Gram + positive bacteria where cytoplasmic membrane is surrounded by a thick peptidoglycan layer, present a cytoplasmic membrane surrounded by a thin peptidoglycan layer as well as an outer membrane (Lin and Weibel, 2016). The MBC and MIC values are very similar to the ones obtained for the other three Cnd mutants on the same tested bacteria (Olivieri et al., 2018) and lower than the MIC values presented in a recent paper (Jiang et al., 2014) for piscidin-1 against *Pseudomonas aeruginosa*. It has also to be considered that, at a peptide concentration of 5-10 µM we detected a low effect of Cnd-m3a on mammalian cells, as described before. Finally, we determined the Therapeutic Index (TI) for the peptide (data not shown), a widely accepted parameter to represent the specificity of antimicrobial peptides for prokaryotic *versus* eukaryotic cells (Jiang et al., 2014) that is calculated by the ratio of HC_{50} (50% of hemolytic activity) and MIC, in relation to Gram – bacteria, and we obtained values in accordance to the results obtained for the other studied three Cnd mutants (Olivieri et al., 2018)

In conclusion, our data makes the Cnd-m3a peptide an excellent candidate for the development of a possible new antimicrobial agent. However, the further addition of a positive net charge have not dramatically increased the antimicrobial activity of this new mutant compared to the other three already designed. Future studies will be addressed to investigate, *in vivo* on animal models, the toxicity, the pharmacokinetics and the pharmacodynamics of the Cnd-m3a peptide to finally address its use on clinical trials.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Alignment of the wild type peptide (Cnd) and the mutant (Cnd-m3a). The positively changed amino acids have been evidenced in bold for Cnd and in bold and underlined for Cnd-m3a. The conserved amino acids are indicated with an "*" below the sequences, while "." and ":" show amino acids with conserved physical and/or chemical properties.

Figure 2. Representative TEM immunogold images of *Psychrobacter* sp. cells incubated with Cnd-m3a. ***(A, B, C)*** Bacteria incubated with Cnd-m3a peptide for 0, 10 and 180 minutes, respectively. ***(D)*** Untreated bacteria. The peptide first interacts with the membranes and once inside the cells preferentially targets the DNA. The black arrows in panel ***(G)*** indicate cytoplasm disruptions. Bars: from A to H = 500 nm.

Figure 3. Percentage of permeabilization for *E. coli* (A) and *Psychrobacter* sp. (B) outer membranes obtained using Cnd or Cnd-m3a. The experiments were performed in triplicate and an illustrative result is showed. Fluorescence spectra for the permeabilization assay using Cnd-m3a are also reported (C and D).

Figure 4. Binding isotherm (25 °C) for Cnd-m3a interacting with LUVs with different lipid compositions. The fluorescence of tryptophan was measured; lipid vesicles were added to samples containing 1.0 μM peptide.

Figure 5. Stern-Volmer plot showing the Trp-1quenching by KI for Cnd and Cnd-m3a. The experiments were performed in quadruplicate and representative results are shown.

Figure 6. ***Negative staining of 70%/30/% POPC/POPG LUVs. (A)*** Untreated LUVs and ***(B)*** higher magnification showing the smooth membranes. ***(C)*** Vesicles incubated with Cnd-m3a peptide at time 0 and ***(D)*** higher magnification membrane roughening (arrow heads). ***(E)*** Cnd-m3a-treated LUVs after 10 minutes of incubation and ***(F)*** higher magnification showing membrane curvature and change in the vesicles shape (arrows). Bars: A, C and E 500 nm; B, D and F 200 nm.

Figure 7. Hemolytic activity of the Cnd-m3a peptide against human erythrocytes. Five different concentrations have been tested, starting from 50 μM with successive dilutions. The values represent the mean ± SD (n=3). A positive control was determined using 10% Triton X-100 solution and it was considered as 100% of hemolysis. **= $p < 0.01$ with respect to the 3 μM; ***= $p < 0.001$ with respect to 3 μM; N=3.

TABLE LEGENDS

Table I. ELISA assay to define the best antiserum against Cnd-m3a. Optical density values (OD at 450 nm) are the mean absorbance ± SD of triplicate wells at different dilutions of the antiserums.

Table II. Partition parameters for the mutant and the wild type peptide in the presence of 100 % POPC or 70%/30% POPC/POPG.

Table III. Stern–Volmer quenching constants (K_{SV}) and NAF values obtained for the two peptides in the presence of different lipid vesicles. The peptide/lipid molar ratio was 1:100 in all performed experiments.

Table IV. Cytotoxicity assays results for all tested cell lines. The data are presented as the mean ± SD of three independent experiments.

Table V. *In vitro* susceptibility of 70 clinical isolates of *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *S. aureus*, *S. epidermidis* and *Enterococcus* spp with known resistance profiles to Cnd-m3a. The reported MICs and MBCs are the average values ± SD from 10 isolate experiments for each species. The range of the obtained values is also presented.

Table I.

Peps1		Cnd-m3a
	Dil. 1:10	0.202±0.020
	Dil. 1:100	0.060±0.008
	Dil. 1:1000	0.043±0.005
	Dil. 1:5000	0.030±0.003

Peps2		Cnd-m3a
	Dil. 1:10	1.328±0.045
	Dil. 1:100	0.899±0.040
	Dil. 1:1000	0.656±0.020
	Dil. 1:5000	0.131±0.012

Table II.

Peptide	*Lipid Mixture*	*K_x ($x10^4$)*	*Selectivity ratio*
Cnd	100% POPC	3.43 ± 0.26	1.43
	70%/30% POPC/POPG	4.91 ± 0.29	
Cnd-m3a	100% POPC	5.1 ± 0.2	4.90
	70%/30% POPC/POPG	25.0 ± 0.9	

Table III.

	100% POPC		70%/30% POPC/POPG		Buffer	
	K_{SV} (M^{-1})	NAF	K_{SV} (M^{-1})	NAF	K_{SV} (M^{-1})	NAF
Cnd	2.9 ± 0.1	0.28	2.5 ± 0.1	0.24	10.4 ± 0.3	1
Cnd-m3a	2.5 ± 0.1	0.30	1.5 ± 0.1	0.18	8.3 ± 0.3	1

Table IV.

Conc (nM)	HUVEC prol. (72h inc)		OVCAR5 prol. (48h inc)		A549 prol. (48h inc)		K562 prol. (48h inc)		HUH7 prol. (48h inc)		HeLa prol. (48h inc)		PC3 prol. (48h inc)		SH-SY5Y prol. (48h inc)	
	% viable cells	SD	% viable cells	SD	% viable cells	SD	% viable cells	SD	% viable cells	SD	% viable cells	SD	% viable cells	SD	% viable cells	SD
64000	96,15537	3,8446	100,3706	0,3706	105,3556	5,3556	90,90531	9,0947	92,05963	7,9404	101,8847	1,8847	95,60587	4,3941	106,2453	6,2453
32000	88,75033	11,25	103,1796	3,1796	88,62649	11,374	102,6698	2,6698	95,29737	4,7026	100,6868	0,6868	105,5163	5,5163	100,1379	0,1379
16000	104,533	4,533	93,1307	6,8693	92,07398	7,926	105,933	5,933	106,4775	6,4775	88,80672	11,193	90,63387	9,3661	103,5688	3,5688
8000	95,71216	4,2878	102,1754	2,1754	92,77121	7,2288	88,61385	11,386	109,426	9,426	106,1609	6,1609	94,51285	5,4871	106,8167	6,8167
4000	112,0595	12,06	104,69	4,69	88,34805	11,652	93,59661	6,4034	89,82394	10,176	112,2077	12,208	91,29074	8,7093	105,1012	5,1012
2000	101,9691	1,9691	92,78011	7,2199	99,81451	0,1855	93,0051	6,9949	91,33071	8,6693	103,7148	3,7148	92,56275	7,4372	88,80294	11,197
1000	104,956	4,956	107,3523	7,3523	110,9368	10,937	109,4318	9,4318	105,2652	5,2652	101,9829	1,9829	102,8112	2,8112	106,3035	6,3035
500	92,94393	7,0561	111,4265	11,427	97,39573	2,6043	91,89226	8,1077	88,38365	11,616	104,5596	4,5596	96,5681	3,4319	90,73578	9,2642
250	94,46924	5,5308	99,74733	0,2527	110,712	10,712	99,74444	0,2556	94,40342	5,5966	104,2904	4,2904	100,8805	0,8805	101,877	1,877
125	94,47279	5,5272	96,40509	3,5949	102,6199	2,6199	97,08855	2,9114	100,1267	0,1267	110,7138	10,714	101,9513	1,9513	111,3053	11,305

Table V.

Bacterial Strains	MIC (μg/mL)		MBC (μg/mL)	
	Median±SD	Range	Median±SD	Range
Gram – bacteria				
E. coli ESBL	6.25±0.0	6.25-6.25	25±0.0	25-25
K. pneumoniae KPC	12.5±5.1	12.5-25	25±0.0	25-25
A. baumanni XDR	6.25±3.1	3.12-6.25	12.5±6.5	12.5-25
P. aeruginosa MDR	12.5±0.0	12.5-12.5	25±0.0	25-25
Gram + bacteria				
S. aureus MRSA	25±10.2	25-50	50±0.0	50-50
S. epidermidis MRSE	25±0.0	25-25	50±10.2	25-50
Enterococcus spp. VRE	25±10.2	25-50	50±0.0	50-50

Figure 1

A

```
Cnd-m3a      WFGKLYRGKTKVVKKVKGLLKG
Cnd          FFGHLYRGITSVVKHVHGLLSG
             :**:**** *.***:*:***.*
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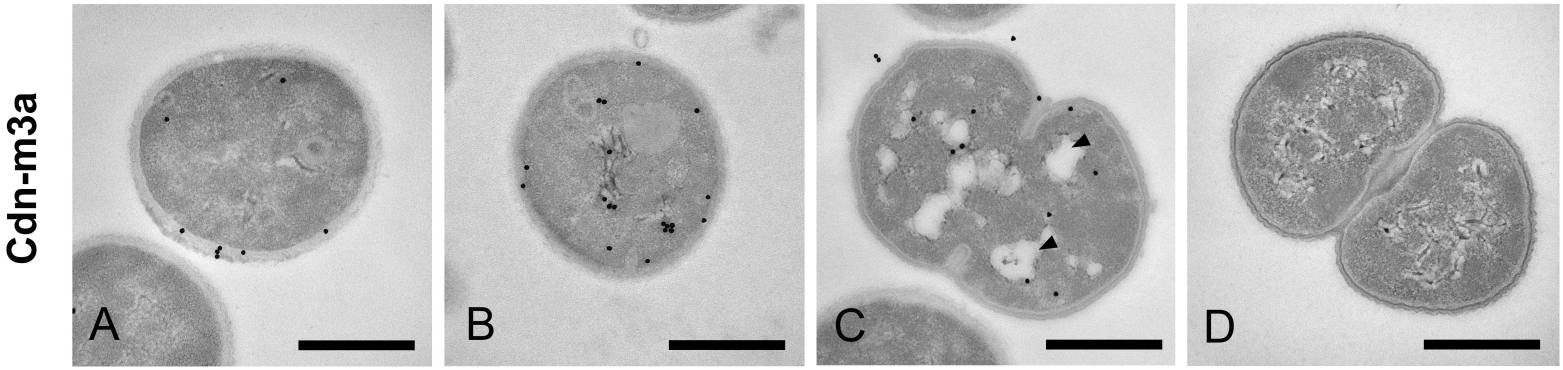


Figure 2

Figure 3.

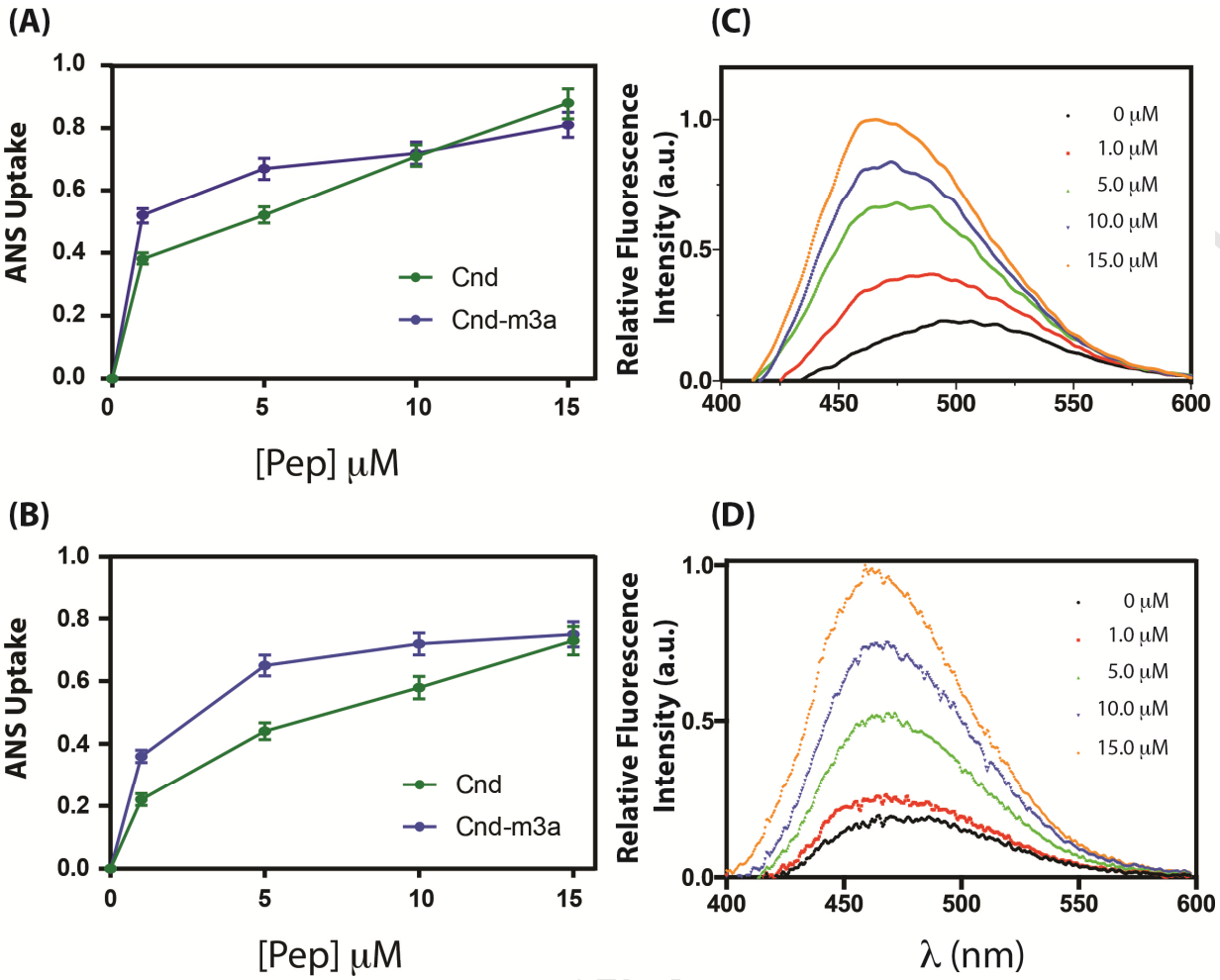


Figure 4.

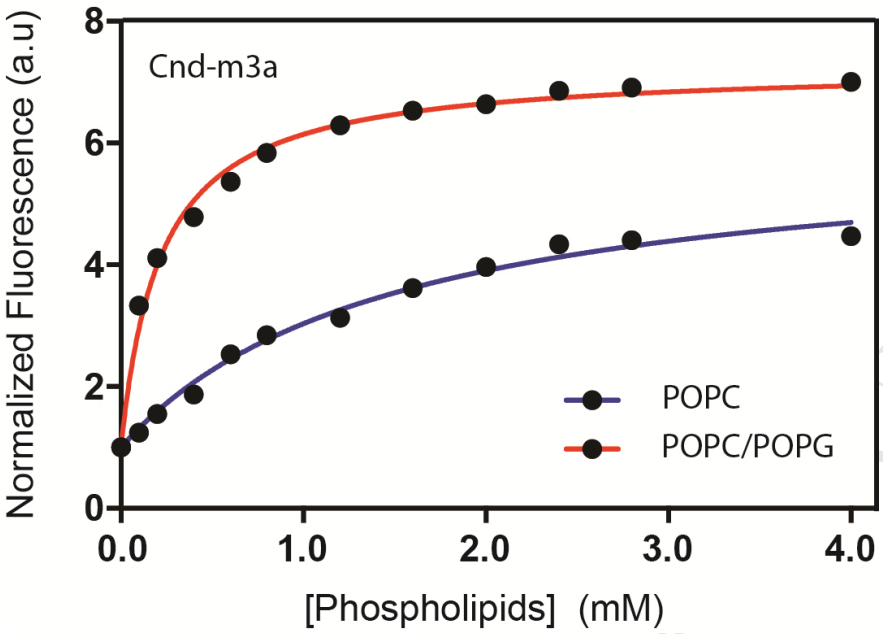
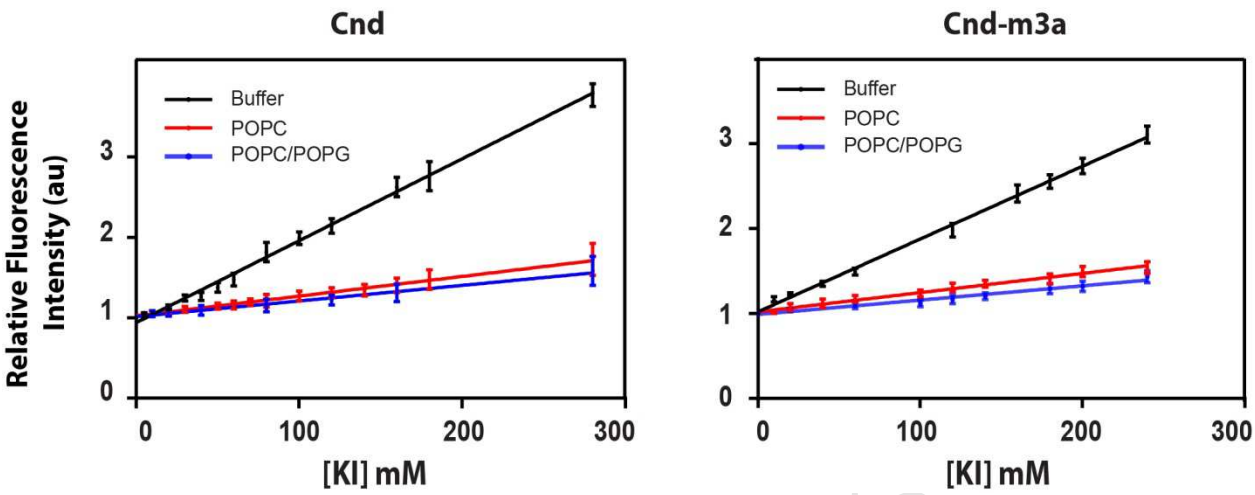


Figure 5.



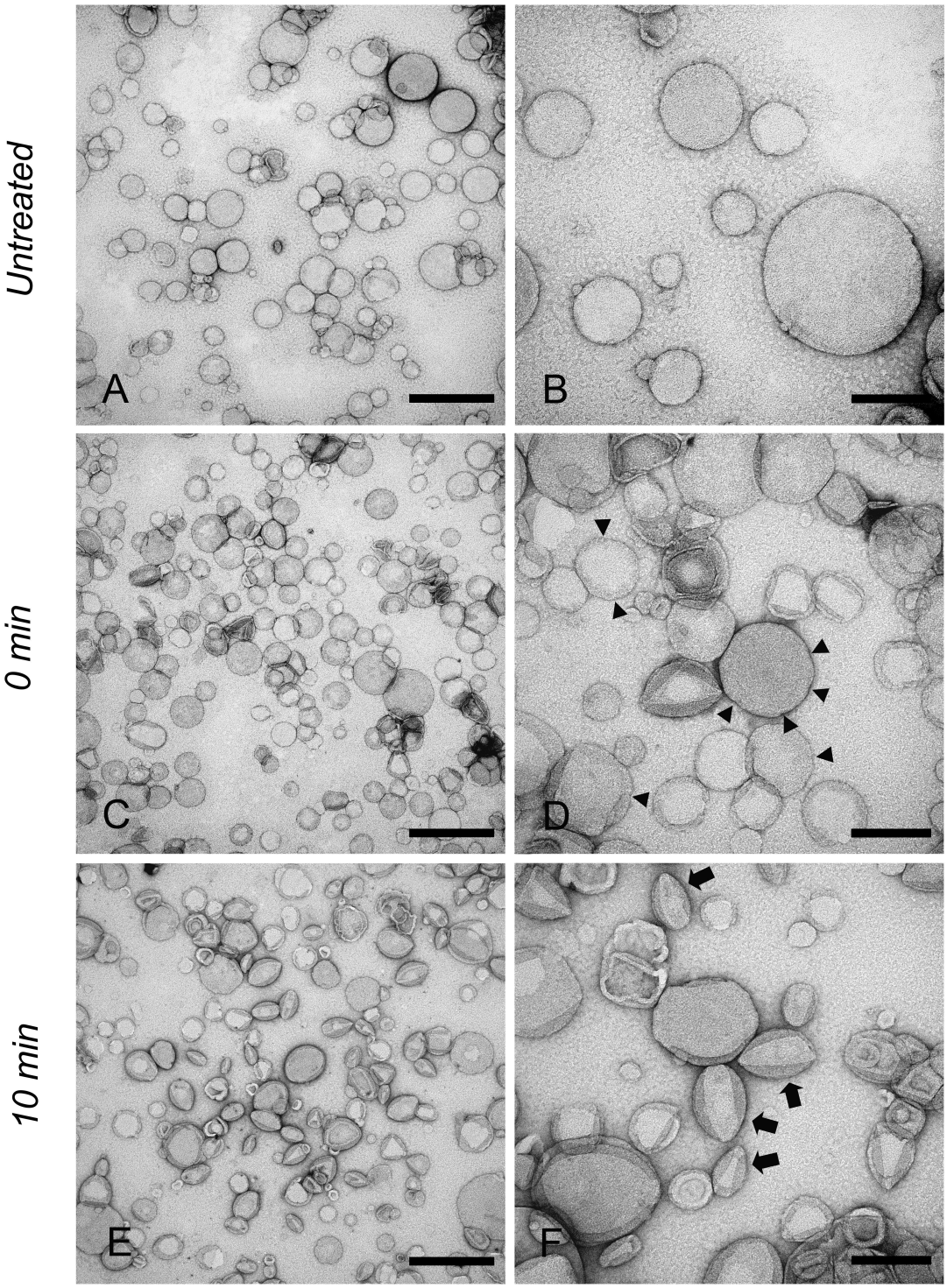
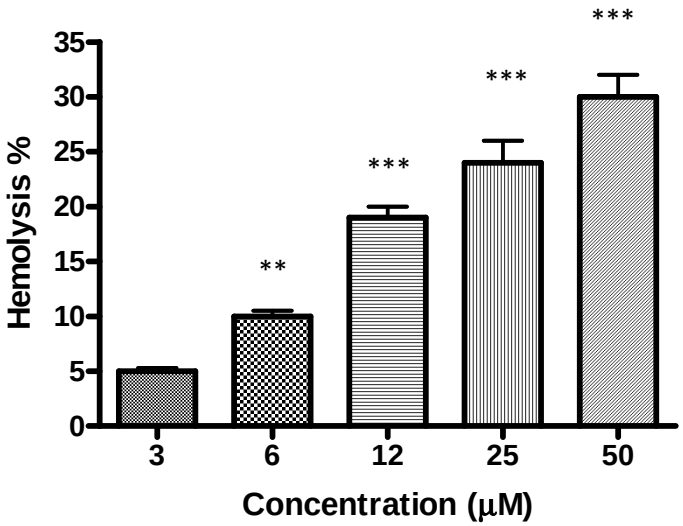


Figure 6

Figure 7.



Highlights

1) Fish antimicrobial peptides are relevant as new possible antibiotics
2) A new fish-derived antimicrobial peptide has been designed
3) Its interactions with bacterial membranes have been investigated in detail
4) The peptide shows high antimicrobial activity against known pathogenic Gram – bacteria