Università degli Studi di Parma

DOTTORATO DI RICERCA IN SCIENZE MEDICO-VETERINARIE

- CICLO XXVIII -

NOVEL MICROBICIDAL AGENTS DERIVED FROM *Naja atra* CARDIOTOXIN 1 (CTX-1)

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“Ho controllato approfonditamente,” disse il computer, “e questa è sicuramente la risposta. Ad essere sinceri, penso che il problema sia che voi non abbiate saputo veramente qual è la domanda”

La vita, l’universo e tutto quanto - D.Adams

“I checked it very thoroughly,” said the computer… “I think the problem, to be quite honest with you, is that you’ve never actually known what the question is”

Life, the Universe and Everything - D.Adams

Summary:

Chapter 5:

Abstract:

During my PhD course, I focused my research on antimicrobial peptides (AMPs), in particular on the aspects of their computational design and development. This work led to the development of a new family of AMPs that I designed, starting from the amino acid sequence of a snake venom toxin, the cardiotoxin 1 (CTX-1) of *Naja atra*.

Naja atra atra cardiotoxin 1, produced by Chinese cobra snakes belonging to *Elapidae* family, is included in the three-finger toxin family and exerts high cytotoxicity and antimicrobial activity too. This toxin family is characterized by specific folding of three beta-sheet loops ("fingers") extending from the central core and by four conserved disulfide bridges. Using as template the first loop of this toxin, different sequences of 20 amino acids linear cationic peptides have been designed in order to avoid toxic effects but to maintain and strengthen the antimicrobial activity. As a result, the sequence NCP-0 (*Naja* Cardiotoxin Peptide-0) was designed as ancestor and subsequently other 4 variant sequences of NCP0 were developed. These variant sequences have shown microbicidal activity towards a panel of reference strains of Gram-positive and Gram-negative bacteria, fungi and an enveloped virus. In particular, the sequence designed as NCP-3 (*Naja* Cardiotoxin Peptide-3) and its variants NCP-3a and NCP-3b have shown the best antimicrobial activity together with low cytotoxicity against eukaryotic cells and low hemolytic activity. Bactericidal activity has been demonstrated by minimum bactericidal concentration (MBC) assay at values below 10 μg/ml for *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* (clinical isolates), *Moraxella catharralis* ATCC 25238, MRSA ATCC 43400, while towards *Staphylococcus aureus* ATCC 25923, *Enterococcus hirae* ATCC 10541 and *Streptococcus agalactiae* ATCC 13813 the bactericidal activity was demonstrated even below 1.6 μg/ml concentration. This potent antimicrobial activity was confirmed even for unicellular fungi

Candida albicans, *Candida glabrata* and *Malassezia pachydermatis* (MBC 32.26-6.4 μg/ml), and also against the fast-growing mycobacteria *Mycobacterium smegmatis* DSMZ 43756 and *Mycobacterium fortuitum* DSMZ 46621 (MBC 100 μg/ml). Moreover, NCP-3 has shown a virucidal activity on the enveloped virus Bovine Herpesvirus 1 (BoHV1) belonging to *herpesviridae* family.

The bactericidal activity is maintained in a high salt concentration (125 and 250 mM NaCl) medium and PB +20% Mueller Hinton Medium for *E. coli*, MRSA and *Pseudomonas aeruginosa* reference strains.

Considering these *in vitro* obtained data, we propose NCP-3 and its variants NCP-3a and NCP-3b as promising antimicrobial candidates. For this reason, the whole novel AMPs family has been protected by a national patent (n°102015000015951).

Introduction:

Worldwide there is a growing concern regarding the increase of microbial antibiotic resistance. Antibiotic resistance is associated with marked morbidity and mortality of a growing number of microorganisms. Therefore there is an urgent need to develop novel antimicrobial therapies to use against these pathogens (Taubes 2008).

In order to counteract this growing threat, useful molecules are antimicrobial peptides (AMPs), that are very ancient host defence effector molecules present in organisms across all the evolutionary spectrum (Avrahami and Shai 2002; Hiemstra and Zaat 2013). AMPs constitute a major component of the innate immune systems of most living organisms, including microorganisms, plants, invertebrates, and chordates.

AMPs possess a broad spectrum of antimicrobial activities against Gram-negative and –positive bacteria, viruses, fungi and parasites, and are divided in various family mainly based on their amino acid sequences and their structural characteristics.

One of the most important features of the AMPs in the fight against environmental microorganisms, is that they exhibit multiple mechanisms of action and, consequently, a low potential to induce *de novo* resistance, which allows the limited use of other antibiotics. Antimicrobial cationic peptides have attracted attention as alternative or complementary antibiotics due to their prospective potency, rapid action, and broad spectrum of activities against Gram-negative and -positive bacteria, viruses, fungi, and parasites. AMPs also seem to be a good candidate for treating biofilms, either alone or in combination with antibiotics (Dosler e Karaaslan 2014). Furthermore AMPs are becoming increasingly recognized as multifunctional mediators, with both antimicrobial activity and diverse immunomodulatory properties (Hiemstra and Zaat 2013).

An initial electrostatic interaction has been observed between cationic antimicrobial peptides and negatively charged structures exposed on the surface of the membranes of bacteria. In Gram-negative bacteria the mechanism involves anionic phospholipids and phosphate groups LPS-associated exposed on the surface of the outer membrane; Gram-positive organisms lack an outer membrane or LPS, however their cell envelopes are enriched in negatively charged teichoic and teichouronic acids. Subsequently, the mechanism of action of many AMPs is based on the direct interactions with phospholipid membranes (Brogden 2005). The more recent literature has shown that several AMP families do not directly alter membrane integrity but rather target conserved essential components of the bacterial membranes in a process that provides them with potent and specific antimicrobial activities (Alanis 2005).

Following the initial membrane binding, peptides begins to enter and traverse the lipid bilayer via different mechanism: in particular, they can create a toroid pore into the membrane or use a carpet mechanism leading to the membrane disruption. Definitely peptides kill microorganism by causing multiple and insurmountable defects in target microbial cell membranes (Sato and Feix 2006).

The amino acid sequence represents one of the most important feature in relationship with the antimicrobial activity of the AMPs; even minimal changes of the amino acid sequence can influence many physico-chemical parameters of the peptide, finally varying the antimicrobial activity and the cytotoxicity of this molecules towards eukaryotic cells (Huang, Huang et al. 2010).

AMPs are found in many tissue and secretions of living organisms and some peptides, like defensins or α-helical AMPs, have been also identified in animal venoms. Proteins found in venoms are the result of toxin recruitment events in which an ordinary secreted-protein gene, typically one involved in a key regulatory process, is duplicated, and the new gene is selectively expressed in the venom gland (Fry, Roelants et al. 2009). Genes that encode for

AMPs represent one of the superfamilies recruited. In some arthropod venoms, cationic peptides work sinergically with neurotoxins to paralyze prey or deter aggressors. When isolated from the venom these peptides exhibit, despite a certain degree of maintained cytotoxicity, common properties with AMPs including antimicrobial activity (Dubovskii, Vassilevski et al. 2015).

Snake venoms are complex mixtures of pharmacologically active peptides and proteins. Three-finger toxins (3-FTxs) belong to a superfamily of non-enzymatic proteins almost found in snakes belonging to *Elapidae* family. Among the 3-FTxs, cardiotoxins (CTXs) and α-neurotoxins are the main toxic proteins from elapid snake venoms. Although they all adopt the family-typical three-fingered loop-folding topology dominated by β-sheet, they can exhibit different pharmacological activities (Mackessy 2009). Cardiotoxins show many function including haemolysis, cytotoxicity and depolarization of muscles. Similarly to AMPs, these toxins can interact with anionic lipids or negatively charged oligosaccharides on the cell membrane and subsequently form an oligomeric toxin complex. Positively charged residues of CTXs critically contribute to their binding with phospholipids and membrane-damaging effect. Basing on these data, some of these venom proteins have been studied in order to assess any antimicrobial activities.

CTXs show similar mechanism of action to AMPs and damage phospholipid bilayers via the formation of a membrane pore structure. Specifically, CTX3 induce permeabilization both of the outer and the inner membrane, having a bactericidal effect directly related to its membrane-permeabilizing activity (Chen, Kao et al. 2011).

Because of their broad spectrum antimicrobial activity and the difficulties for the microbes to create *de novo* resistance, the use of AMPs to overcome antimicrobial resistance is recommended. In this view, is also useful to study venoms as possible sources of new antimicrobial peptides because of the demonstrated activity of several toxins on the cell

membranes. Moreover, the manipulation of the amino acid sequences of the different peptides could improve their efficacy against several microorganism and may heavily reduce the cytopathic effect on eukaryotic cells.

The specific aim of this study is focused on the development and the characterization of novel antimicrobials peptides, designed starting from the cardiotoxin I (CTX-1) of the Chinese cobra (*Naja atra atra*), in order to enhance the bactericidal activity and, at the same time, decrease the cytotoxicity than the ancestor against eukaryotic cells.

This study is framed within over three years of my PhD course research activities, carried on the synthesis of novel antimicrobials peptides. Together with the research team, I collaborated to the development of a family amino acidic sequences, other than those discussed in this context, subject of an article entitled "*Antimicrobial activity of 4 novel cyclic peptides against a panel of reference and multi-drug resistant clinical strains of animal origin*" (Cabassi, Taddei et al. 2015).

My PhD project on NCPs, presented in this study, has led to the filing of the patent n°102015000015951 on 19th May 2015.

Chapter 1:
Antimicrobial peptides (AMPs)

1.1 – Peptide Characteristics

Antimicrobials peptides (AMPs) are central components of the innate network of gene-encoded proteins and peptides that protect animals from microbial, viral or cellular intruders. Because they are gene-encoded, some AMPs are pre-deployed at barrier sites, including the skin or at places that are vulnerable to invasion in the respiratory, gastrointestinal and genitourinary tracts. Other AMPs provide reinforcement that are delivered rapidly by mobile convoys of neutrophils or produced locally in response to various molecular alarm signals with little or no delay (Hiemstra and Zaat 2013). This rapid response is crucial because activation and deployment of pathogen specific cell-mediated immune responses occur slowly relative to potential kinetics of microbial proliferation. Their broad-spectrum antibacterial activity, allied to their strategic locations, makes them central effectors molecules of innate immunity (Papagianni 2003).

The identification of cecropins, magainins and defensins in insects, amphibians and humans in the 1980s stimulated research on AMPs and led to the prediction or isolation and characterization of hundreds of new peptides (Wang 2010). AMPs are widely distributed in nature, being produced by vertebrates and invertebrates animals, plants and microorganisms (Papagianni 2003).

An understanding of mechanism of action of AMPs has evolved over time. As amphipathic, cationic peptides, it was apparent from the start that AMPs targeted the membranes of the microbes they killed, a conclusion consistent with studies utilizing model membranes. Their positive charge presumably facilitates interaction with the negatively charged bacterial phospholipid-containing membranes and or acidic bacterial cell walls, whereas their

amphiphilic character enables membrane permeabilization. In more recent years, there has been speculation that transmembrane pore formation is not the only mechanism of microbial killing (Wang 2010). In fact several observation suggest that translocated peptides can alter cytoplasmic membrane septum formation and may inhibit cell-wall synthesis, nucleic-acid synthesis, protein synthesis or enzymatic activity (Brogden 2005). Thanks to further investigations, scientists come to learn that many of these AMPs exhibit other biological properties in the context of injury and infection that promote recovery and healing (Wang 2010; Hiemstra and Zaat 2013).

Most of these peptides share several common properties; peptides often contain the basic amino acid residues lysine or arginine, the hydrophobic residues alanine, leucine, phenylalanine or tryptophan, and other residues such as isoleucine, tyrosine and valine. Some peptides contain amino acids repeats and ratios of hydrophobic to charged residues can vary from 1:1 to 2:1.

Their net charge is generally positive and size varies from 6 amino acids chains to greater than 59 amino acids residues molecules (Brogden 2005).

Almost all peptides are ribosomally synthesized as pre-propeptides, consisting of an N-terminal signal peptide and a variable C-terminal peptide. The leader region is eventually cleaved and the propeptide undergoes post-translational modification to become the final active antimicrobial (Papagianni 2003; Wang 2010).

1.2 – Classification of AMPs

AMPs are a unique and diverse group of molecules. The nomenclature and classification of naturally occurring AMPs are complex and have not been fully standardized.

AMPs can be roughly categorized into sub-groups on the basis of their amino acid composition and structure (Papagianni 2003):

- AMPs with high content of a certain amino acid
- AMPs containing intramolecular disulfide bridges
- AMPs that assumes an α-helical structure

In the first group are included proline- and arginine-containing peptides such as bactenecins from cattle (Bac7), sheep, goats (Bac5) and PR-39 from pigs.

To the second group belongs peptides that contain cysteine residues and form disulphide bonds and stable β-sheets. This sub-group includes protegrin from pigs and diverse families of defensins.

The last sub-group represents one of the most widely distributed in nature. These AMPs assume an amphipathic, α-helical conformation that permits insertion of a well-defined hydrophobic sector into the lipid bilayer. Cecropins and melittin from insects, and molecules such as magainins, bombinin and dermaseptin from anurans, are only few examples of peptides included inside of this large group (Brogden 2005).

1.3 – α-helical AMPs

An exhaustive analysis of all the categories of AMPs listed above is beyond the purpose of this work, however, it may be helpful to further examine some aspects concerning α-helical AMPs.

Of all AMPs discovered, the linear α-helical peptides are among the most abundant and widespread in nature, and appear to represent a particularly successful structural arrangement in innate defense (Tossi, Sandri et al. 2000).

The antimicrobial and hemolytic activity in amphipathic AMPs are related to multiple physico-chemical parameters, including length, sequence, charge, helicity, hydrophobicity, amphipaticity, the hydrophobic/hydrophilic angle and self-association. All these parameters

are intimately interconnected, thus altering one will cause changes to the others, which makes it complicated to assess the influence of a single factor to the activity (Huang, Huang et al. 2010).

1.3.1 – Length

Natural α-helical AMPs are generally small with <50 amino acid residues. Considering the minimal requirements of 3-4 amino acid residues per turn in an alpha helix, 7-8 amino acid residues (two turns) are required for the formation of amphipathic faces (Huang, Huang et al. 2010). Length information could be useful to better understand the type of mechanism of action of the peptide considered: for example, mechanisms of action which provide the formation of pores, require sequences composed by at least 22 residues to transverse the lipid bilayer (Sato and Feix 2006; Huang, Huang et al. 2010).

1.3.2 – Sequence

Natural AMPs have different primary sequences, with approximately 50% hydrophobic residues (Huang, Huang et al. 2010). In an investigation Giangaspero *et al.* compared the N-terminal α-helical domain of over 150 AMPs found in nature and did not find a conserved pattern (Giangaspero, Sandri et al. 2001) and the positional residue conservation is also very poor, with the exception of position 1 (70% Gly) and 8 (50% Lys). However, the positional conservation of residue types (for example, charged, neutral or hydrophobic) was presented in a relatively defined way.

1.3.3 – Charge

It is generally accepted that the interaction of AMPs with membrane is largely dependent on the electrostatic force. However, the correlation between peptide charge and biological activity is complicated. Alteration of the peptide charge will inevitably cause changes to other parameters (Huang, Huang et al. 2010). In natural AMPs while the charge varies widely, from

0 to +16, most active peptides fall into an intermediate range. Various studies have confirmed a clear correlation between charge and potency, particularly below the optimum charge range. These data suggest that there is a critical threshold for the net positive charge or positive charge density on a given α-helical AMP that governs antimicrobial and hemolytic activities and the optimal charge, for maximal antimicrobial activity, is around +4 (Tossi, Sandri et al. 2000; Huang, Huang et al. 2010).

1.3.4 – Helicity

Multiple studies demonstrated that peptide helicity might be more important for toxicity (neutral eukaryotic membranes) than antimicrobial activity (negatively charged prokaryotic membranes) of AMPs (Huang, Huang et al. 2010). The most convincing evidences are from D-amino acid substitution on model peptides on model peptides. Chen *et al*. reported a systematic study to determine impacts of D-amino acid substitution in the nonpolar face of an amphipathic α-helical peptide on secondary structure. The results showed the feasibility of controlled disruption of helical structure by D-amino acid substitution in aqueous medium, whilst still allowing peptides to be fully folded as α-helical structure in a hydrophobic environment (Chen, Mant et al. 2002). D-diastereomers often shows negligible cytotoxic effects on mammalian cells a retained antibacterial activity. Moreover D-amino acid substitution seems to prevent the diastereomers from enzymatic degradation and maintain their activity in serum, whereas the L-amino acid peptides are fully cleaved by trypsin or proteinase K with low activity in serum (Huang, Huang et al. 2010).

1.3.5 – Hydrophobicity

It is believed that the cytoplasmic membrane is the main target of AMPs and hydrophobicity is a crucial parameter for the biological activities. In general, increased hydrophobicity of the non-polar face of an amphipathic α-helical peptide improves antimicrobial activity (Avrahami

and Shai 2002). Chen *et al*. in a study found that there was an optimal window in which high antimicrobial activity could be obtained. Decreased or increased hydrophobicity beyond this window dramatically inhibit antimicrobial activity, and higher hydrophobicity is also correlated with stronger hemolytic activity. It can be explained by the strong peptide self-association that prevents the peptide from passing through the cell wall in prokaryotic cells, whereas such peptide self-association does not affect the access to eukaryotic membranes (Chen, Guarnieri et al. 2007).

1.3.6 – Amphipathicity and hydrophobic moment

Amphipathicity is usually quantitated by the hydrophobic moment defined as the vector sum of the hydrophobicity of individual amino acids (Eisenberg 1984). Several studies have shown that on the angle of the helix content the mean hydrophobic moment is a more important factor affecting antimicrobial activity than hydrophobicity (Pathak, Salas-Auvert et al. 1995; Fernández-Vidal, Jayasinghe et al. 2007).

1.3.7 – Angle subtended by the hydrophilic/hydrophobic faces on the wheel projection

In an ideal helical peptide, the angle sustained by polar and nonpolar facets can be clearly exhibited using a helix wheel projection. It is proposed that the angle influence the location of the peptide within the membrane and the structure of the transmembrane pores (Dathe and Wieprecht 1999).

1.3.8 – Self-association

Peptide self-association (i.e., the ability to dimerize) in aqueous solutions is an important parameter for antimicrobial activity (Chen, Mant et al. 2005). If the self-association ability of a peptide is too strong in aqueous medium, it could decrease the ability of the peptide to

dissociate, to pass through the capsule and cell wall of microorganisms, and to reach and penetrate into the cytoplasmic membrane to kill target cells.

1.4 – Mechanisms of action

Many studies have been conducted aiming at elucidating the mechanism by which positively charged antimicrobial peptides kill their target microorganisms. In general, the peptides cause damage to the bacterial membrane in the form of pores, through induction of non-lamellar lipid phases or membrane disintegration, which results in the collapse of the transmembrane electrochemical gradients (Shai 2002). There is good experimental evidence for each of these processes, and it may be that different peptides utilize different mechanisms to exert their effects on the microbial membrane. These mechanisms also need not be mutually exclusive, one process may represent an initial or intermediate step and another may be its consequence. Additional factors such as lipid-to-peptide ratio and target membrane composition may also be involved (Sato and Feix 2006).

Nevertheless, to reach their target membrane, antimicrobial peptides need first to transverse the bacterial wall, which is different in Gram-negative and –positive bacteria. Gram-negative bacteria have a smaller cell wall peptidoglycan layer than Gram-positive bacteria. Furthermore, Gram-negative bacteria contain an outer membrane in addition to the common cytoplasmic membrane. Therefore, antimicrobial peptides need to pass through two lipidic membranes in Gram-negative bacteria compared with one membrane in Gram-positive bacteria. It has been proposed that antimicrobial cationic peptides utilize a specific uptake pathway across the outer membrane of Gram-negative bacteria, termed self-promoted uptake, and, therefore, they usually work as well or better against Gram-negative than Gram-positive bacteria (Shai 2002). Antimicrobial peptides must first be attracted to bacterial surfaces, and one obvious mechanism is the electrostatic bonding between cationic peptides and the net

negative charges that exist on the outer envelope of Gram-negative bacteria (for example, anionic phospholipids and phosphate groups on lipopolysaccharides (LPS)), or the lipoteichoic acids in the surface of Gram-positive bacteria.

Once close to the microbial surface, peptides must traverse capsular polysaccharides before they can interact with the membrane, which contains LPS in Gram-negative bacteria, and transverse capsular polysaccharides, teichoic acids and lipoteichoic acids before they can interact with the cytoplasmic membrane in Gram-positive bacteria (Brogden 2005).

Upon the addition of AMPs to these membranes, AMP monomers bind to the outer leaflet of the phospholipid bilayer as a first step in the processes of membrane lysis; the initial binding state of most α-helical AMPs is parallel to the lipid bilayer surface. It has been suggested that such binding induces positive membrane curvature, increasing the surface area of outer leaflet, leading to a decrease in the thickness of hydrophobic core and thinning of the lipid bilayer (Heller, He et al. 1997; Mecke, Lee et al. 2005). Although the initial association of amphipathic α-helical AMPs with the membrane surface increases the volume and surface area of the outer leaflet, the inner leaflet is affected by this mismatch (Sato and Feix 2006). These changes in the physical properties of the lipid bilayer appear to facilitate further penetration of AMPs.

When the peptide concentration reaches a critical threshold, membrane-bound AMPs reorient and penetrate or insert into the hydrophobic core of lipidic bilayer (Huang 2000).

Subsequent disruption of the membrane permeability barrier may occur by a variety of mechanisms, described below, leading ultimately to loss of cytoplasmic membrane integrity and cell death.

1.4.1 – "Barrel stave" model

In early studies it was suggested that, following binding, linear amphipathic α-helical AMPs form transmembrane pores, presumably via a "barrel stave" mechanism (Westerhoff, Juretić

et al. 1989). This has been demonstrated by the ability of the peptides to induce single channels in planar lipid membranes (Fig. 1). The pore acts as a conductance channel that disrupts transmembrane potential and ion gradients, leading to a leakage of cell components and cell death. Dissipating the transmembrane electrochemical gradient causes a loss of bacterial cell's ability to synthesize ATP, and the increase in water and ion flow that accompanies loss of the permeability barrier leads to cell swelling and osmolysis (Sato and Feix 2006).

Peptides that act via "barrel stave" mechanism must fulfill several criteria:

1) They need to be hydrophobic to penetrate into the lipidic core of the membrane and, therefore, their interaction with the target membrane is governed predominantly by hydrophobic interactions.
2) They should self-associate in the membrane-bound state to form bundles of transmembrane pores.
3) The pore would increase due to the recurrent of more monomers.
4) A minimal length of ≈22 amino acids is required to transverse the lipid bilayers with α-helical peptides.
5) Only a few of transmembrane pores are required to dissipate the transmembrane potential in cells. Therefore, the minimum bactericidal concentration of the peptides should be far below micromolar concentrations, the actual concentration required to kill bacteria, and under which the peptides cover the outer surface of the bacterial membrane.

Studies on membrane-permeating peptides demonstrate that only a few of them could be considered as channel-forming peptides (Shai 2002). A prototype for the formation of "barrel stave" channels is the 20-residues hydrophobic peptide alamethicin, produced by the fungus *Trichoderma veidae* (Cafiso 1994).

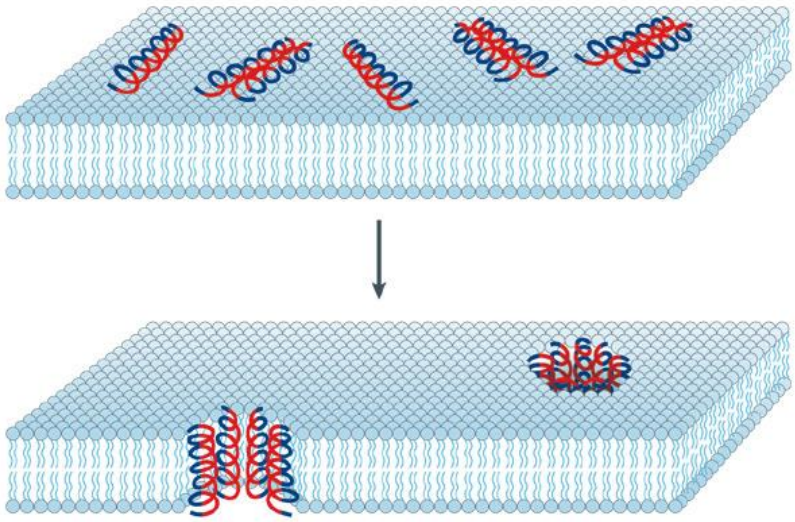

Figure 1: The barrel-stave model mechanism. The peptides aggregate and insert into the membrane bilayer so that the hydrophobic peptide regions (colored in blue) align with the lipid core region while the hydrophilic regions (colored in red) face the interior region of the pore (Brogden 2005).

1.4.2 – "Toroidal-pore" model

In the "toroidal-pore" model, AMPs induced expansion of the lipid head group region results in a bending of the bilayer back on itself followed by connection of the outer and inner leaflets, and the pore is composed of both peptide and phospholipid. In forming a toroidal pore, the polar faces of the peptides associate with the polar head groups of the lipids. Positive curvature of the membrane surface, resulting from accumulation of AMP at the bilayer interface, facilitates the bending of phospholipids and toroidal pore formation (Fig. 2). The structure of this model addresses the fact that many AMPs are simply too small to span an unperturbed phospholipid bilayer in an α-helical conformation (Sato and Feix 2006).

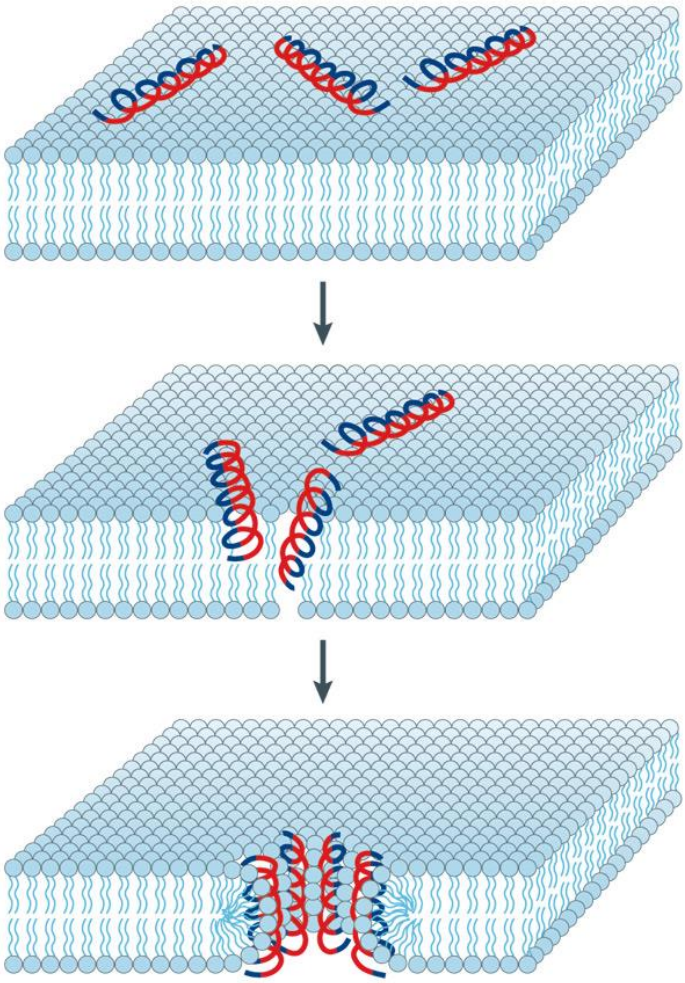

Figure 2: The toroidal model mechanism. The attached peptides aggregate and induce the lipid monolayers to bend continuously trough the pore. Hydrophobic regions of the peptide are shown in blue while hydrophilic regions in red (Brogden 2005).

This type of transmembrane pore is induced by magainins, protegrins and melittin (Brogden 2005).

1.4.3 – Detergent-like "carpet" model

A conceptually different model for the way in which AMPs disrupt membranes is the carpet mechanism. In this model peptides, electrostatically attracted to the anionic phospholipid head groups, cover the bilayer surface in a carpet-like manner. Above a critical threshold concentration of monomers, the peptides form toroidal transient holes, allowing additional peptides to access the membrane. Finally, the membrane is permeated and disintegrated in a detergent-like manner and forms micelles after disruption of the bilayer curvature (Fig. 3) (Brogden 2005).

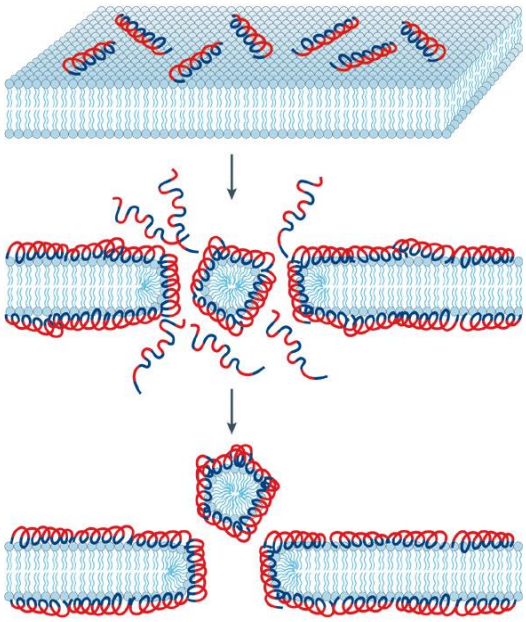

Figure 3: The carpet model mechanism. The peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer and forming an extensive carpet. Hydrophobic regions of the peptide are shown in blue while hydrophilic regions in red (Brogden 2005).

1.5 – Animal α-helical peptides

The amphipathic α-helical family of AMPs is varied and versatile. Peptides of this type are produced by organisms that are evolutionarily quite distant, ranging from insects and tunicates to humans. They have evolved to function in many different contexts, such as the hemolymph of insects, amphibian skin secretions, gastric mucosa and intestinal epithelia, mammalian phagocytic vacuoles, wound and blister fluids and epithelia. These molecules are often potent and broad spectrum antibiotics, but their activity spectra can differ considerably (Giangaspero, Sandri et al. 2001).

1.5.1 – α-helical AMPs from invertebrates

Insects are one of the major sources of antimicrobial peptides. Most insect AMPs are small and cationic, and they show activities against bacteria and/or fungi, as well as some parasites and viruses. Insect AMPs can be classified into four families based on their structures or unique sequences:

- the α-helical peptides (e.g. cecropin and moricin)
- cysteine-rich peptides (e.g. insect defensing and drosomycin)
- proline-rich peptides (e.g. apidaecin and drosocin)
- glycine-rich peptides (e.g. gloverin)

Among the α-helical peptides group, cecropins were the first animal inducible antibacterial peptides to be characterized, purified from hemolymph of the pupae of *Hyalophora cecropia* in 1980 (Yi, Chowdhury et al. 2014). Cecropins are a family of cationic AMPs of 31-39 residues with common structural features: a basic N-terminal part, which may form an amphiphilic α-helix, and relatively hydrophobic C-terminal helical part, which is joined by a hinge sequence containing proline and/or glycine. The C-terminal carboxyl group is amidated, and amidation is important for interaction with membranes and liposomes (Papagianni 2003). Cecropins adopt a random coil structure in aqueous solution but convert to α-helical structure in hydrophobic environments. Cecropins have a broad spectrum of activity against Gram-negative and Gram-positive bacteria, as well as fungi. In addition to antimicrobial activity, cecropins and cecropins derivatives are also active against parasites, including *Plasmodium* and *Trypanosome*, and can inhibit replication of HIV-1 virus and proliferation of cancer cells (Yi, Chowdhury et al. 2014).

Moricins, together with cecropins, are included inside the α-helical peptides group. These AMPs have been found only in haemolymph of lepidopteran insects so far (Yi, Chowdhury et al. 2014).

The structure of these AMPs is similar to that of cecropins, except lacking a hinge region in moricins. The tertiary solution structure of moricins show a long α-helix with eight turns along nearly the full-length peptide, except for a few residues at the N- and C-terminal regions. The N-terminal segment of the α-helix is amphipathic and is responsible for increase in membrane permeability for killing of bacteria, while the C-terminal segment is hydrophobic and is critical for antimicrobial activity (Hemmi, Ishibashi et al. 2002). Similarly to cecropins, moricins have activity against Gram-negative and Gram-positive bacteria, and against filamentous fungi and yeasts.

Although attacins and gloverins are included in glycine-rich peptides group, circular dichroism (CD) spectrum showed that many of them adopt mainly random coil structure in aqueous solution but, similarly to cecropins, undergoes conformational transition to α-helical structure in the hydrophobic environment (Gunne and Steiner 1993; Axén, Carlsson et al. 1997). These AMPs are active mainly against *Escherichia coli*, but activity against Gram-positive bacteria (*Flavobacterium* sp. and *Bacillus cereus*) and fungi (*Saccharomyces cerevisiae* and *Cryptococcus neoformans*) is also reported for some of them (Yi, Chowdhury et al. 2014).

1.5.2 - α-helical AMPs from fish

Several α-helical peptides have been identified in many species of fish. Many have been isolated from mucosal and epithelial surfaces, and are likely to be especially important defense factors for aquatic animals because these surfaces are in constant contact with an environment crowded with a variety of opportunists or potential pathogens (Papagianni 2003).

Among this large group of peptides, pleurocidin is a potent and wide spectrum antibacterial peptide from the skin of winter flounder (*Pseudopleuronectes americanus*). This peptide, quite basic and rich of histidine residues, forms an amphipathic α-helix and is active against both Gram-positive and Gram-negative bacteria in a salt-independent manner. Another example is given by parasin, isolated from the epithelial mucosa of the catfish *Parasilurus asotus*, active against a wide spectrum of bacteria and fungi (Giangaspero, Sandri et al. 2001). Both peptides sequences share large stretches of homology with AMPs isolated from the granular glands of the skin of many anuran species.

1.5.3 - α-helical AMPs from amphibians

Peptides with antimicrobial activity have been reported from many frog species across eleven families (mostly of them were found in *Ranidae* and *Hylidae* families) (König, Bininda-Emonds et al. 2015).

An impressive number of α-helical AMPs, which evidently arose via gene duplications events, have been isolated from the specialized skin structures called granular glands or poison glands, in which they are stored. It is generally assumed that these molecules serve to protect the organism against a wide range of pathogens. Recently, many authors voiced skepticism about the contribution of these peptides to ranid host defense (Conlon, Kolodziejek et al. 2004; König, Bininda-Emonds et al. 2015).

Typically these peptides share some main characteristics, as for the net cationic charge at physiological pH, hydrophobicity, amphipathicity and α-helical structure in membrane-mimetic solvents (Papagianni 2003; Hiemstra and Zaat 2013). On other hand, the large differences in features such as the size and sequence among them, the amino acid sequences of the signal peptide and acidic pro-region have been well conserved (Conlon, Kolodziejek et al. 2004).

Amphibian AMPs are structurally distinguished in two classes:

- linear α-helical peptides without cysteines
- peptides with one disulfide bridge forming a C-terminal loop

The first AMP was found in the skin of the European frog (*Bombina orientalis*), but the decisive spur to further research is represented by the subsequent discovery of magainins in 1987.

The complete description of the activity of all several AMPs discovered to date, that compound this group, it's beyond the needs of the present work, however amphibian AMPs display a remarkable range of microbicidal activity versus both Gram-positive and Gram-negative bacteria, yeasts, protozoa and viruses (Papagianni 2003).

1.5.4 - α-helical AMPs from amniotes

All amniotes (reptiles, birds and mammals) produce a number of α-helical AMPs belonging to the cathelicidin family of antimicrobial peptide precursors (Giangaspero, Sandri et al. 2001). The cathelicidins peptides comprise one of several families of AMPs that are found in heterophils/neutrophils and in a wide range of tissues (such as the respiratory and gastrointestinal tracts and multiple lymphoid organs) as components of the early host defenses against infections (Zanetti 2005). These peptides are typically short, usually less than 40 amino acids, which are characterized by a highly conserved cathelin-like domain. They are produced as pre-propeptides containing an N-terminal signal peptide, the cathelin-like domain and the C-terminal mature peptide. The signal peptide is cleaved off before secretion of the peptide, but the pro-cathelin-like domain remains attached and is cleaved off by serine proteases once the peptide is secreted (Cuperus, Coorens et al. 2013).

Most of them are α-helical cationic peptides, with an amphipathic structure. Other types of cathelicidins have been described, like short cyclic peptides that have β-sheets and form intramolecular disulfide bridges, or peptides containing a high amount of specific amino acids (Brogden 2005). However the presence of at least one α-helical cathelicidin peptide in every

amniote animal investigated is significant and suggests that a cathelicidin of this type was the prototype from which the family differentially expanded, generating a variety of peptides in selected species by several rounds of gene duplication and rapid divergence of the peptide coding sequence (Zanetti 2005).

A wide variety of bacteria, both Gram-positive and Gram-negative, fungi and viruses has been shown to be susceptible to killing by cathelicidins at micromolar or sub-micromolar concentrations, with a generally broad spectrum but significant differences in specificity and potency (Zanetti 2005; Cuperus, Coorens et al. 2013; van Hoek 2014).

In addition to direct killing of bacteria, most cathelicidins also have important immune-modulatory effects. These effects have been extensively studied for the human cathelicidin LL-37 (Hiemstra and Zaat 2013).

Cathelicidins have been identified in mammals (e.g. primates, rabbit, mouse rat, Guinea pig, dog, cow, sheep, goat, horse and pig) and birds (e.g. chicken, pheasant and quail) (Zanetti 2005; Cuperus, Coorens et al. 2013). In reptiles, cathelicidins have been identified and characterized in the snake family: highly related cathelicidins were identified in elapid snakes such as *Bungarus fasciatus*, *Ophiophagus hannah* and *Naja atra*. Cathelicidin-like peptides have been also reported, by BLAST searching, in the genome of other snake species (e.g. *Crotalus durissus* and *Pseudonaja textilis*), lizards (e.g. *Anolis carolinensis*), turtles (e.g. *Chrysemys picta belli*, *Pelodiscus sinensis* and *Chelonia mydas*) and crocodilians (e.g. *Alligator mississipiensis* and *Alligator sinensis*) (van Hoek 2014).

Chapter 2:
Animal venoms

2.1 – Animal venoms

Venom systems are key evolutionary innovations in broad phylogenetic range of animal lineages and are used for defense, competitor deterrence or predation. Venomous animals have been identified in a variety of metazoan phyla, including Cnidaria, Plathelmintha, Anellida, Arthropoda, Mollusca, Echinodermata and Chordata. Venomous vertebrates include representatives from fishes (Chondrichtyes and Osteichtyes), Amphibia and Reptilia (snakes and venomous lizards) (Kordiš and Gubenšek 2000; Fry, Roelants et al. 2009).

Venoms are complex mixtures that include variable combinations of proteins (ranging from multi-unit globular enzimes to small peptides), salts, and organic molecules such as polyamines, amino acids and neurotrasmitters (Mackessy 2009).

Throughout evolution, numerous proteins have been convergently recruited into the venoms of various animals. These proteins are the result of toxin recruitment events in which an ordinary protein gene is duplicated, and the new gene is selectively expressed in the venom gland. Such toxin genes were often amplified to obtain multigene families with extensive neofunctionalization (but with a fundamentally conserved basal activity), followed by deletion of some copies and degradation of other nonfunctional copies or pseudogenes (Fry, Roelants et al. 2009). The new multigene family preserve much more the three-dimensional structure of the ancestral protein than its primary structure (the key functional residues outside the core scaffold are modified to acquire a plethora of newly derived activities). In fact on average only 3-4% of amino acid residues are crucial for maintaining a functional protein structure (Kordiš and Gubenšek 2000).

Fry *et.al* observed that even if protein families that have been recruited include a broad spectrum of different structures and biochemical activities, many of them share a number of features:

- All known toxins represent secreted proteins;
- Protein families from which toxins have been convergently recruited are functionally versatile, but proteins within each family seem to share an underlying biochemistry that has remained uniform throughout the Kingdom Animalia (and often throughout eukaryotes);
- Most toxins are recruited from body proteins involved in one or several short-term physiological processes;
- Toxins seem to be often recruited from body proteins with stable tertiary structures maintained, for example, by a high degree of disulfide cross-linking (Fry, Roelants et al. 2009).

2.2 – Toxins as AMPs

Considering the characteristics mentioned above, it is not surprising that genes involved in the synthesis of secreted AMPs were recruited for the production of toxins. These molecules possess well-established structures and are highly conserved in many living organisms. Several reports have noted structural homology among AMPs and toxin classes elaborated in different animal species. Despite the toxic activities exerted by these AMPs-derived toxins, it is interesting to observe how shared structural features, such as charge or hydrophobic topologies, are consistent with conserved antimicrobial effects (often lower than their ancestors) and cytotoxic functions (frequently even enhanced) (Torres-Larios, Gurrola et al. 2000; Fry, Roelants et al. 2009; Yount, Kupferwasser et al. 2009).

2.2.1 – Defensin-derived toxins

Defensins are among the most widely distributed innate immunity-related AMPs. Two structural classes are recognized: the phylogenetically related α-, β- and θ-defensins that are exclusive to vertebrates and the α/β (CSαβ) defensins that are found in plants, fungi, nematodes and mussels as well as in insects and arachnids (Papagianni 2003; Yi, Chowdhury et al. 2014). The defensins contain a highly conserved cysteine-array that affords structural rigidity and promotes hypervariability for accelerated evolutionary adaptation. The β-defensin-scaffold toxins characterized from *Crotalus* snake venom (e.g. crotamine) are neurotoxins that modify voltage-gated Na^+ channel, resulting in a potent analgesic effect and significant myotoxicity (Mackessy 2009). Also the defensing-like peptides isolated from platypus venom are related to the β-defensin class found only in vertebrates, but their bioactivities remain to be elucidated (Whittington, Papenfuss et al. 2008).

For what concern invertebrates, most scorpions venom neurotoxins adopt the CSαβ motif (Bontems, Roumestand et al. 1991); gene-structure conservation and phylogenetic analyses support a paralogous relationship between scorpion defensins and three families of voltage-gated K^+ channel blockers from scorpion venom, likely derived from independent recruitment events (Fry, Roelants et al. 2009). All these defensin-scaffold toxins showed partially conserved antimicrobial activity.

2.2.2 - α-helical toxins

A number of cationic α-helical toxins, that show antimicrobial activity, have been isolated from the venoms of many species.

Mellitins are the major component found in bee (*Apis mellifera*) venom, and have highly hemolytic activity, but have also broad-spectrum antibacterial activity. These 26-residue peptides, like the ceropins, appear to structure into two α-helical regions separated by a hinge, and are amidated, but the N-terminal tract is more hydrophobic in this case. Among the AMPs

found in arthropods venoms, it is possible to count also crabrolin, a 13-residue peptide from the venom of the European hornet (*Vespa crabro*). In addition, a group of molecules (ponericins) were isolated from venom glands of the predatory ant *Pachycondylas goeldii* (Giangaspero, Sandri et al. 2001; Bulet, Stöcklin et al. 2004).

In arachnids, almost all AMPs identified so far have been isolated from the venom, in particularly of spiders and scorpions. Two antimicrobial α-helical peptides named hadrurin and opistoporin were isolated from the venom of the scorpion *Hadrurus aztecus* and *Opistophtalmus carinatus* respectively. Cupiennins, lycotoxins and oxyopinins have been isolated from the venom of the hunting spider *Cupiennus salei*, the spider *Lycosa carolinensis* and the wolf spider *Oxyopes kitabensis* respectively (Giangaspero, Sandri et al. 2001; Bulet, Stöcklin et al. 2004).

A number of α-helical AMPs are even employed as toxins in venomous secretions of many chordates; for example, besides the well-known cytolytic peptides elaborated by the cutaneous granular glands of anuran amphibians (frogs and toads), a group of AMPs, named pardaxins, have been isolated from the shark repelling defense secretions of different species of sole (Giangaspero, Sandri et al. 2001; König, Bininda-Emonds et al. 2015).

2.2.3 – Other toxins with antimicrobial activity

Previous reports point towards the association of some snake venom with antibacterial activity (Theakston, Phillips et al. 1990; Talan, Citron et al. 1991; Perumal Samy, Gopalakrishnakone et al. 2007). Snake venom is an extremely complex secretion that contains many proteinauceous components (including neurotoxins, cardiotoxins, myotoxins, cytotoxins, proteases and nucleases). Among some of the common antimicrobial components that have been isolated from snake venom are L-amino acid oxidase (LAAO) and phospholipase A_2 (PLA_2). While the action of the former appears to result from hydrogen peroxide generated by his oxidative action, the bactericidal effect of PLA_2 enzymes is thought

to depend on direct membrane-permeabilizing and damage mechanism (Perumal Samy, Gopalakrishnakone et al. 2007). Apart from enzymatic proteins, several antimicrobials peptides including cathelicidins, vgf-1 and omwaprin have been isolated from the venom of *Bungarus fasciatus*, *Naja atra*, and *Oxyuranus microlepodotus*, respectively (Chen, Kao et al. 2011).

Another large family of toxins, called three-finger toxins (3-FTxs), have shown bactericidal activity through direct membrane-damaging activity. All proteins in this family have a similar three-dimensional structure that consists of three loops extending from a central core. Similarly to what happens for AMPs, positively charged residues of 3-FTxs confer the ability to interact with anionic lipids or negatively charged oligosaccharides on the cell membrane and critically contribute to their membrane-damaging effect (Chen, Kao et al. 2011).

Phylogenetic studies have showed how PLA_2 and 3-FTx have arisen from recruitment events of genes that encode for proteins other than AMPs employed by the innate immunity (Fry 2005).

2.3 – Three-finger toxins

The three-finger toxins (3-FTxs) are nonenzymatic proteins containing 60 to 74 amino acid residues and usually 4 -5 disulfide bonds. This family of toxins is found abundantly in the venoms of elapids (including cobras, kraits, mambas and sea snakes). The characteristic feature of all 3-FTxs is their distinct protein fold: three β-stranded loops extending from a small, globular, hydrophobic core that is cross linked by four conserved disulfide bridges (Mackessy 2009).

In all 3-FTxs there are structurally conserved regions that contribute to the proper folding and structural integrity of the protein. Their amino acid sequences can be readily aligned, using eight conserved cysteine residues found in the core region; Tyr25 or a homologous aromatic

residue Phe27 is also conserved in most toxins and is required for proper folding and stability of the antiparallel β-sheet structure (Ohno, Menez et al. 1997; Mackessy 2009). Despite their overall similarity in structure, these polypeptides differ from each other in their amino acid sequences, enabling a wide variety of biological effects and binding to different receptors/acceptors (Fry, Wüster et al. 2003). Members of this family include: short- and long- chain α-neurotoxins, which antagonize muscle and neuronal nicotinic acetylcholine receptors; κ-bungarotoxin, which recognize neuronal nicotinic receptors; muscarinic toxins, which are selective agonists/antagonists of distinct subtypes of muscarinic acetylcholine receptors; calciseptine and related toxins, which block the L-type Ca^{2+} channels; dendroaspins, which are antagonists of various adhesion processes; and cardiotoxins/cytotoxins, which exert their toxicity by forming pores in cell membranes (Mackessy 2009).

The endogenous three-finger peptides of vertebrates that play a significant role in cell-cell adhesion may be the ancestors of the three-finger toxins. Related peptides are used in the complement system (CD59) and lymphocytes/keratinocytes (Ly6 and SLURP1) and are also secreted in the brain (Lynx1) (Miwa, Ibaňez-Tallon et al. 1999; Chimienti, Hogg et al. 2003; Fry 2005).

2.3.1 – Cardiotoxins

Snake venom cardiotoxins (CTXs) are a group of 3-FTxs that are found abundantly in the venoms of most elapid snakes, particularly cobras. Cobra venoms may be constituted by CTXs up to 50% of their dry weight; since CTXs are highly lethal, this fraction is suspected to be the leading cause of death and morbidity (Mackessy 2009).

They are composed of 60-63 amino acids in a single polypeptide chain cross-linked by four disulfide bond, folded in a three-loop structure typical of 3-FTxs (Fig. 4) (Chang, Huang et al. 2000).

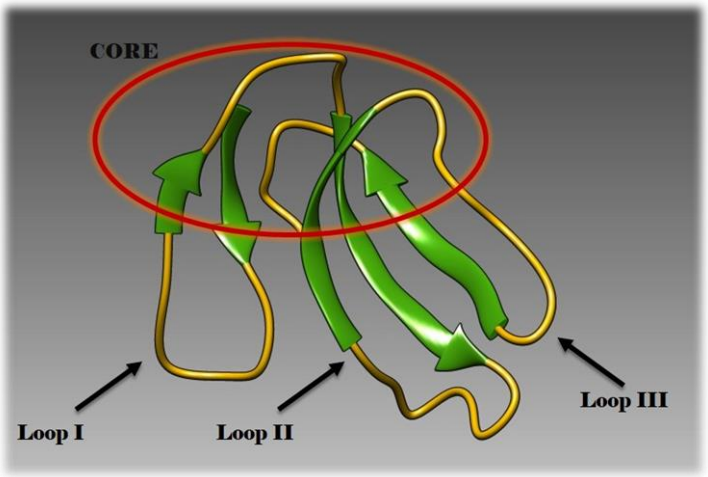

Figure 4: Three-dimensional structure of CTX-1 (P60304) from *Naja atra* (Chinese cobra), in ribbon representation. Beta sheets are colored in green.
Visualized using University of California, San Francisco (UCSF) Chimera.

These toxins can induce various pharmacological effects on prey that precipitate the death of the animal:

- Deactivation of Na^{+}- K^{+}-activated ATPase of axonal membranes
- Contracture induction in the skeletal muscle
- Increase of Ca^{2+} influx via nonspecific calcium channels, Na^{+}- Ca^{2+} exchange, and mobilization of intracellular Ca^{2+} in electrically induced in a single ventricular myocytes
- Contraction of smooth muscle
- Activation of tissue phospholipase C and arachidonic acid-associated phospholipase A_2
- Anticoagulation effects, induction and inhibition of platelet aggregation
- Hemolytic activity
- Decrease the force of contraction and increase in heart rate due to ventricular tachycardia
- Lysis of various cell types, such as epithelial cells, fetal lung cells, and certain types of tumor cells

Although much information about the various pharmacological activities of CTXs is known, the specific molecular targets or mostly mechanisms of action are yet to be determined (Mackessy 2009). There are currently about two hundred genes in the sequence databases and more than 100 determined sequences of cardiotoxins from various of elapid snakes. It is found that CTX isoforms could be simultaneously isolated from the venom of a single snake species (Chang, Huang et al. 2000; Mackessy 2009).

Many lines of evidences suggest that these toxins can bind strongly to zwitterionic or acidic phospholipids, resulting in the aggregation/fusion of phospholipid vescicles. CTXs possess also the capability to cause lysis of many different cells presumably by perturbing the structure of membrane lipid bilayers, which grants to these proteins a potent hemolytic/cytolytic activity. Correlations of the primary and tertiary structures reveals two lipid binding sites at the positions 6-13 and 24-37 localized near the tip of the loop 1 and loop 2, respectively (Chien, Chiang et al. 1994). Interestingly, alignments of the amino acid sequences of cardiotoxins from the venoms of *Naja* species show that amino acid substitution of cardiotoxin isoforms frequently occur just within these regions (Chang, Huang et al. 2000). These regions are characterized by the coexistence of an exposed hydrophobic surface (covering nearly 40% of the molecular surface) flanked by conserved basic residues, that form a border around this hydrophobic patch and help in the recognition of natively charged head groups of phospholipids in membranes (Chien, Chiang et al. 1994; Mackessy 2009). Subsequently, the hydrophobic tip of loop I (a continuous hydrophobic patch that extends from amino acid position 6 to 13) interacts with hydrophobic parts of the phospholipidic membranes and leads to membrane insertion of the molecule (Mackessy 2009).

On the basis of the amino acid residues types of the two aforementioned lipid binding sites, two distinct types of cardiotoxins were described: P- and S-type CTXs. In general, the amino acid residues near Pro-31 (at the tip of loop II) are more hydrophobic in P-type CTXs than S-

type CTXs. In addition, there is a continuous hydrophobic patch from 24 to 37 amino acid positions for P-type CTXs, which has no equivalent in S-type CTXs because the amino acid residues at 29-33 positions are more hydrophilic and some possess strong hydrogen bounding capabilities. Due to these features, two possible mechanism of CTX-phospholipids interaction have been hypothesized. While P-type CTXs bind to phospholipid membranes by anchoring their two lipid binding sites on the tip of loops I and II, bringing the CTX molecule closer to the membrane surface and possibly to penetrate deeper into the phospholipid bilayers, the S-type CTX binds to phospholipid membranes only by their binding site on loop I (Fig. 5). That binding mode would permit the Ser and Thr amino acid residues near the tip of loop II to form hydrogen bonds with other proteins at the membrane surfaces (Chien, Chiang et al. 1994).

Few CTXs, on the basis of these information, were investigated as potential antibacterial agents; in a study, Chen *et.al* has noted how CTX-3 (belonging to the P-type CTXs) exerts direct microbicidal effects on *Escherichia coli* and *Staphylococcus aureus* through its membrane-permeabilizing activity, with a mechanism of action similar to that of AMPs (Chen, Kao et al. 2011).

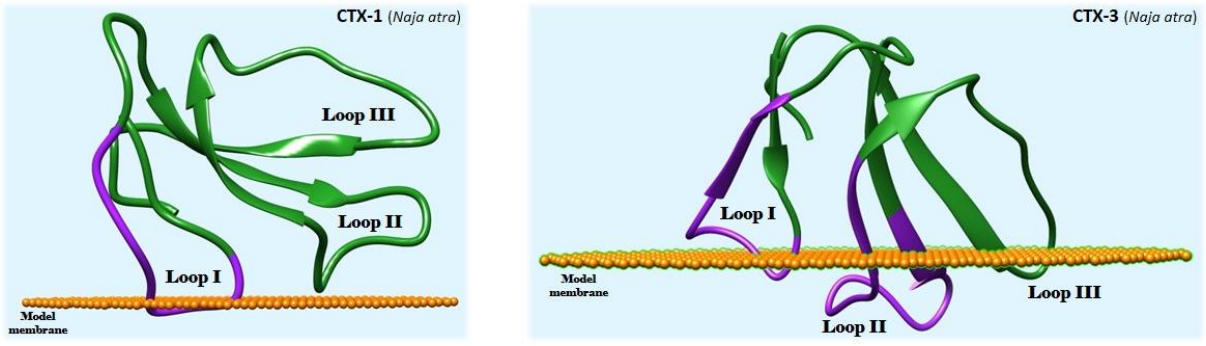

Figure 5: Interactions between CTXs and membranes. On the left, is represented the three dimensional structure of the S-type CTX-1 (P60304) from *Naja atra* (Chinese cobra), which penetrate the membrane model (colored in orange) with loop I only. On the right, is represented the three dimensional structure of the P-type CTX-3 (P60301) from *Naja atra* (Chinese cobra), which penetrate the membrane model with both loop I and loop II. CTXs lipid binding sites involved in this interaction are colored in purple.
Visualized using University of California, San Francisco (UCSF) Chimera.

Chapter 3:
Peptides design

3.1 – Sequences generation

Starting from the amino acid sequence of S-type cardiotoxin 1 (CTX-1) produced by the Chinese cobra (*Naja atra atra*), and on the basis of what exposed in the previous chapters, I began to design the sequence of novel experimental AMPs composed of 20 residues. From the complete pro-protein sequence, the signal peptide has been previously detected using "*SegnalP 4.1 Server*" web server (Gautam, Chaudhary et al. 2015) and subsequently removed (Fig. 6).

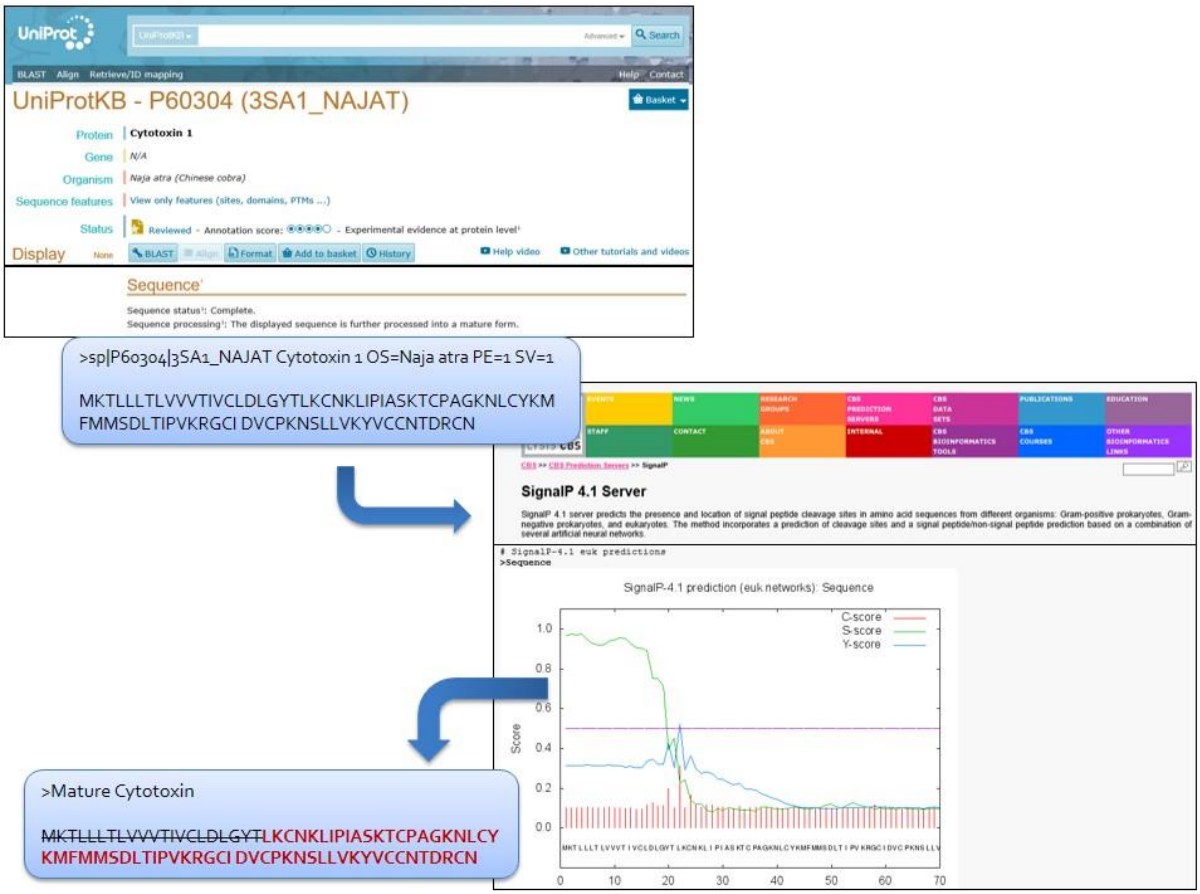

Figure 6: Signal peptide removal. The complete sequence of CTX-1 (P60304), avaliable on Uniprot database (http://www.uniprot.org/), through *"SignalP 4.1 Server"* (http://www.cbs.dtu.dk/services/SignalP) was deprived of the signal peptide. In the lower right is shown the results page and on the right the mature protein sequence.

The CTX-1 sequence was then scanned for the search of stretches suitable as cell-penetrating peptide (CPP). CPPs are short peptides (<50 amino acid) that have the inherent ability of interacting and penetrating phospholipid membranes. Since α-helical AMPs are able to interact with eukaryotic and prokaryotic membranes, they share many structural and chemical features with this kind of peptides (Nathan and Cars 2014). To identify CTX-1 sections of interest, the protein sequence have been scanned throughout its length using "*CellPPD – Protein Scanning*" web server in order to predict CPP fragments. SVM (Support Vector Machine) + motif based were the prediction mode selected, with an *E-value* cut off of 0,0001 and a SVM threshold of 0,0, selecting a window of 20 amino acid. The window length determine the length of fragments in which the protein has to be fragmented in "sliding window" fashion. Only sequences predicted as CPP were considered among the 42 fragments contained inside the tabular output. Then, I sorted the 23 CPP fragments so obtained upon SVM score to get the most potent CPP, obtaining by this way the final candidate sequence (Fig. 7) (Petersen, Brunak et al. 2011).

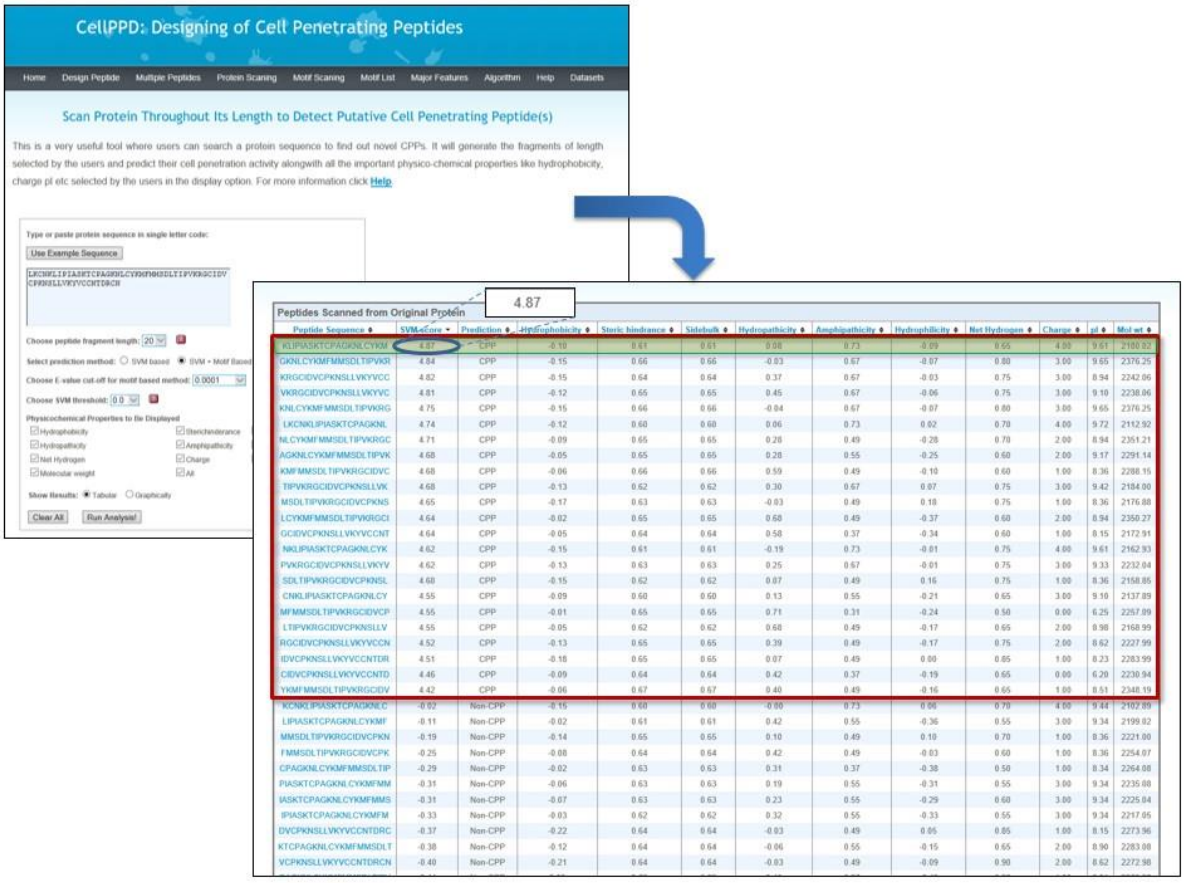

Figure 7: At the top the protein scan module of CellPPD web server is shown (http://crdd.osdd.net/raghava/cellppd/submit_prot.php) filled with the mature CTX-1 sequence and related search parameters. Below is reported the results screenshot, with the fragments obtained along with their prediction score and prediction status and the physico-chemical properties (hydrophobicity, hydrophilicity, molecular weight, etc.). CPP stretches are included inside the red rectangle, while the final candidate with the highest prediction score is highlighted in green.

The final sequence has been therefore examined using the prediction tool of "*CAMP*$_{R3}$" web server, in order to predict the antimicrobial activity of the submitted sequence through four prediction models:

- Support Vector Machine (SVM)
- Random Forests (RF)
- Artificial Neural Network (ANN)
- Discriminant Analysis (DA)

The submitted sequence was confirmed as potential AMP by all prediction models employed (Fig. 8).

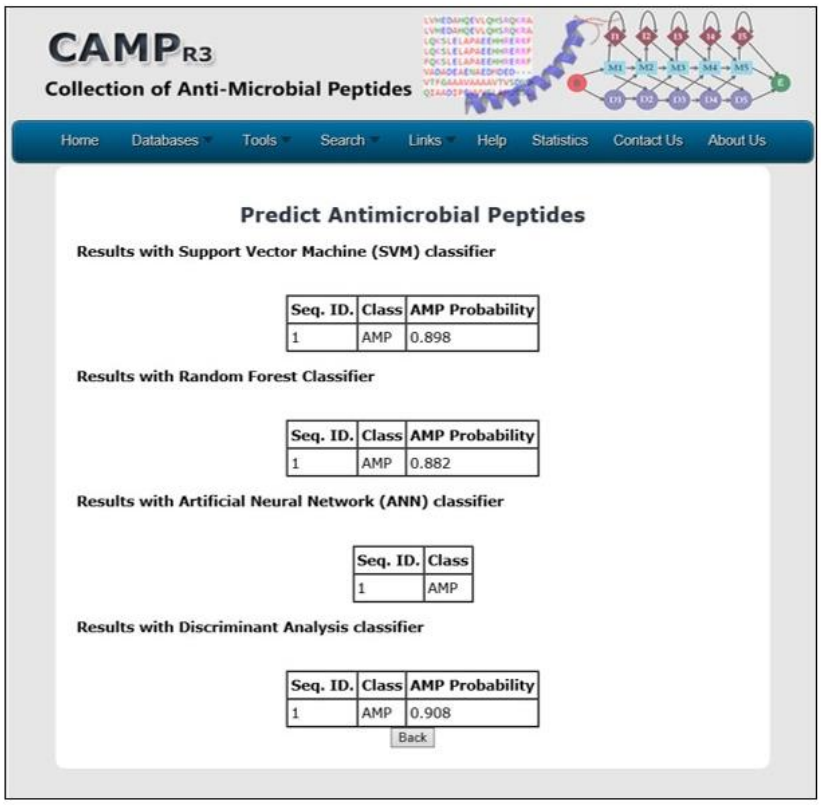

Figure 8: Results page of $CAMP_{R3}$ web server prediction tool (http://www.camp.bicnirrh.res.in/predict/)

Subsequently, through single-residue substitutions, I produced 2 mutant sequences (NCP-0 and NCP-1) with the purpose of improving mainly the amphipathicity of the resulting peptides. These AMPs were named with the acronym NCP (*Naja* Cardiotoxin Peptide) followed by the progressive number of the sequence. In addition, 8 NCP-1 variants (identified by letters: *e.g.* NCP-1a) were designed by using a proprietary screening software developed *ad hoc* to mine putative AMPs. The script consists of four main blocks: the generation unit (*random amino acid sequence generation*), the filtering unit (*first—pass sequence filtering*), the screening unit (*prediction of potential antimicrobial activity*), and the scoring unit (*second-pass sequence filtering*). The screening unit that uses the response of five neural

networks to pick out potential AMP sequences represents the core of this script. The proportion of potential AMPs mined by the software was around 0,01 and 0,2% (Schmitt, Rosa et al. 2015). From the NCP-1a, through the substitution in sequence of Ala6 with one residue of leucine, and subsequently of Pro4 with a residue of tryptophan, I obtained NCP-2 and NCP-3, respectively. NCPs 3a and 3b are additional variants of NCP-3 that I created by replacing Trp4 with Phe4 and Leu4 respectively (Fig. 9). The patterns of physico-chemical/structural characteristics of all of these sequences (positive charge, hydrophobicity, amphipathicity, polar angle, etc.) are consistent with those observed for the AMPs.

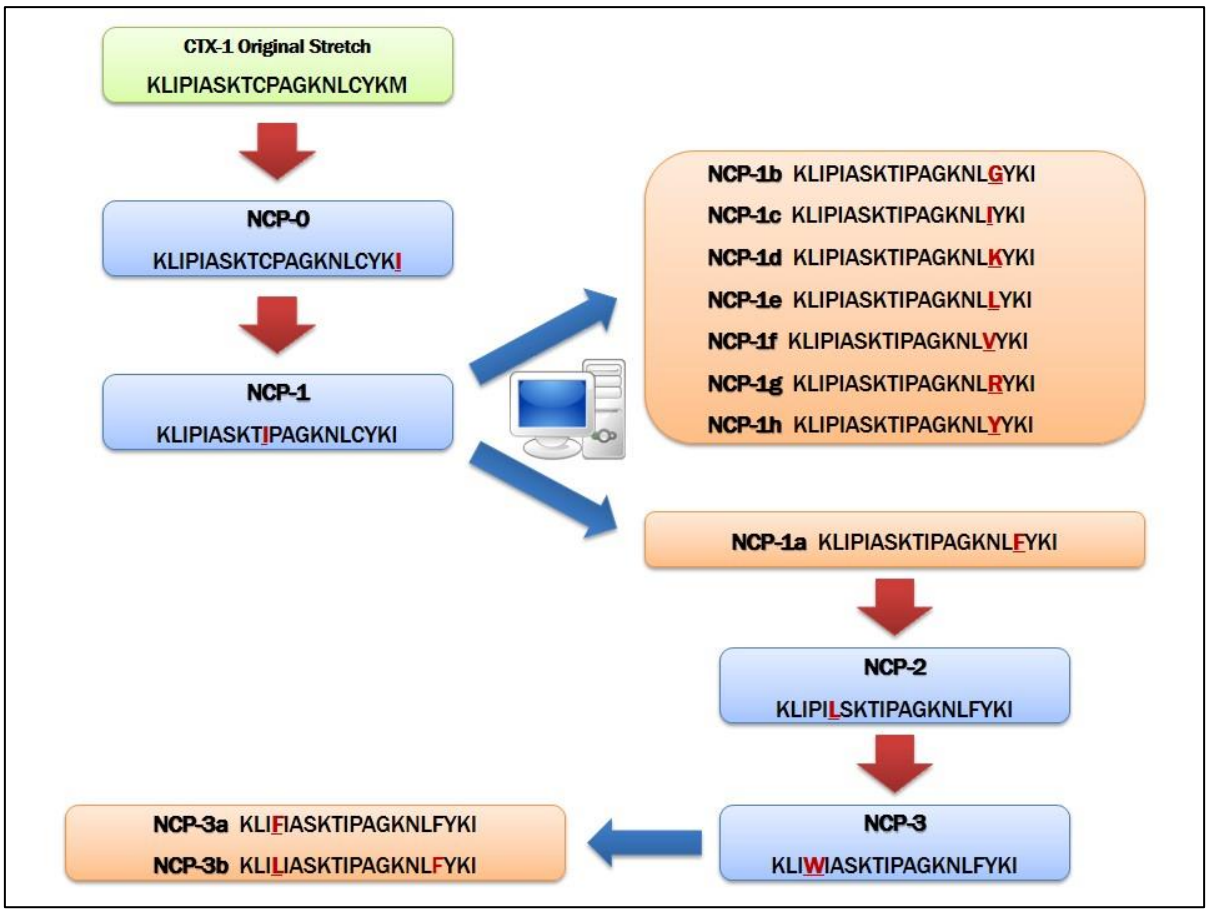

Figure 9: NCPs creation diagram. In the green box the original CTX-1 fragment selected is reported. In those blue the main NCPs while in those orange their variants are reported.

NCP-0, NCP-2, NCP-3 and, at a later stage, NCP-3a and NCP-3b were artificially synthesized a commercial company.

The aim of my PhD course, is focused on the evaluation of antimicrobial activity of these novel AMPs towards a panel of reference and field-isolated bacterial strains. In addition, also antifungal, antiviral and cytotoxic activities were assessed.

The structural characteristics of these peptides were inferred from a computational model. They assume α-helical conformation, stabilized 15 interstrand hydrogen bonds. The charge of peptides range from +4 to +5 (NCP-1d and NCP-1f). The electrostatic potential of the peptides NCP-2 and NCP-3, revealed a positive-charge presenting surface with 4 Lys, with a large hydrophobic area on the opposite side of the molecule (Figg. 10 and 11).

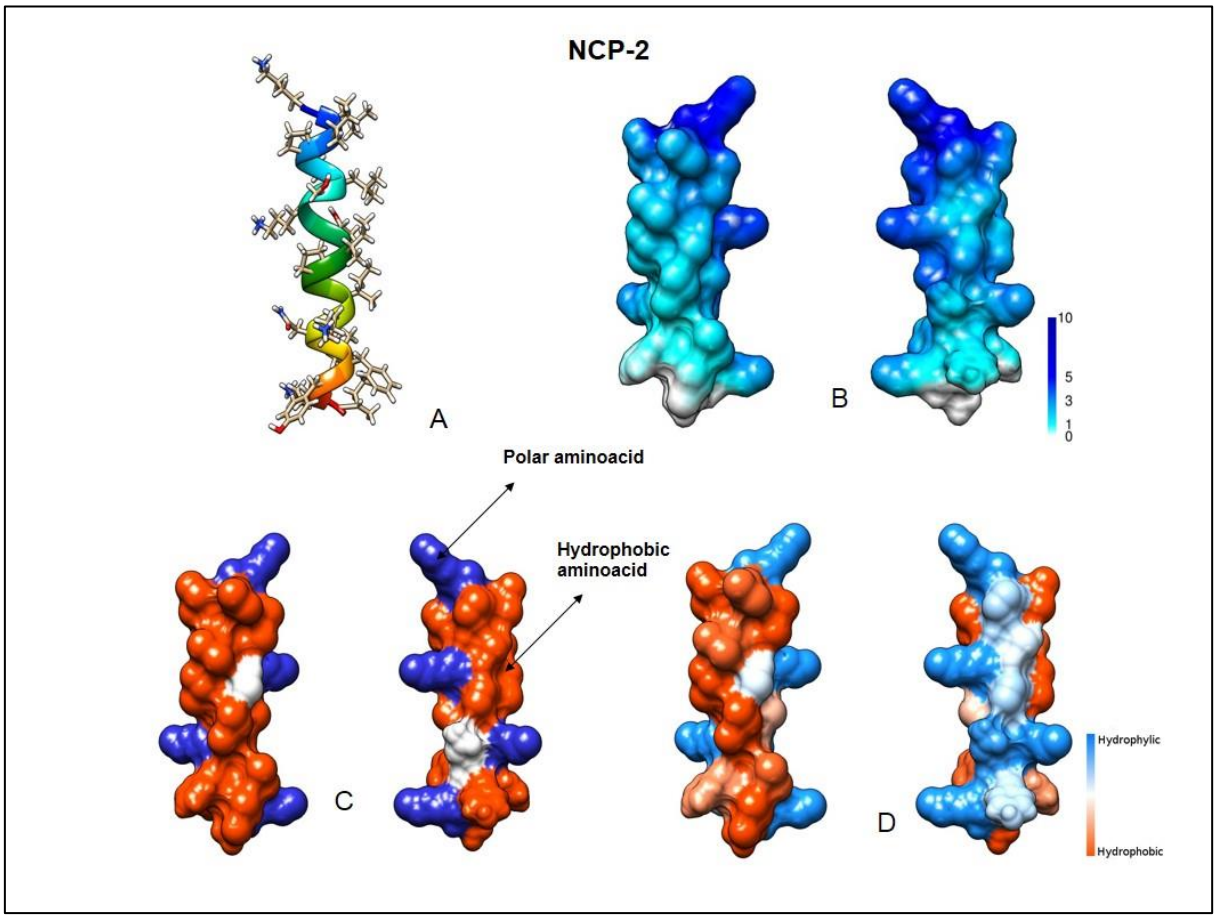

Figure 10: NCP-2 structural features: (A) Three dimensional structure. (B) Surface charge distribution. (C) Distribution of basic (blue) and hydrophobic amino acids (red). (D) Distribution of hydrophobic (red) and hydrophilic (light blue) surface portions.
Visualized using University of California, San Francisco (UCSF) Chimera.

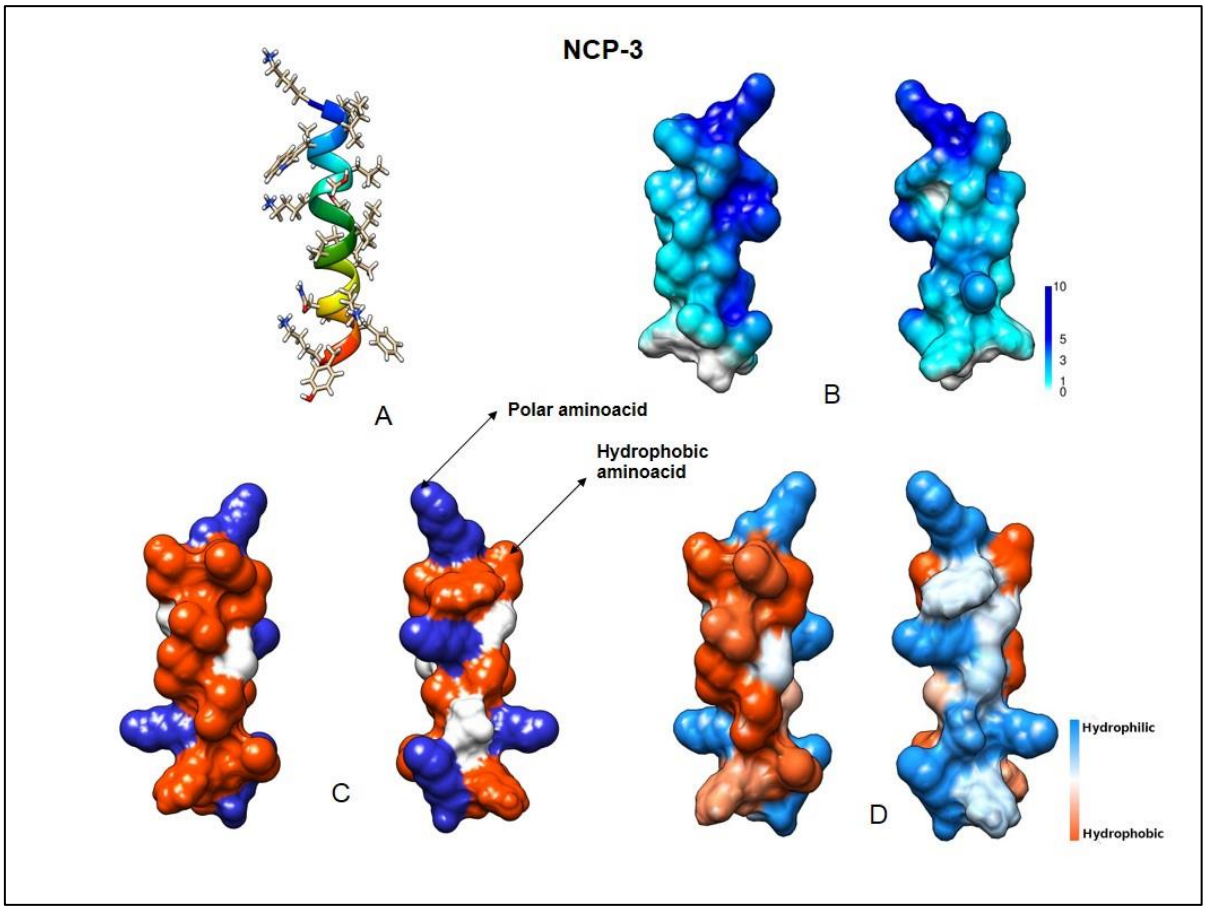

Figure 11: NCP-3 structural features: **(**A) Three dimensional structure. (B) Surface charge distribution. (C) Distribution of basic (blue) and hydrophobic amino acids (red). (D) Distribution of hydrophobic (red) and hydrophilic (light blue) surface portions.
Visualized using University of California, San Francisco (UCSF) Chimera.

Chapter 4:
Materials and Methods

4.1 – Antimicrobial activity

This section describes the different assays carried out to evaluate the antimicrobial activity of NCP-0, NCP-2, NCP-3, NCP-3a and NCP-3b. These tests were conducted initially in optimal medium for the activity of the peptides (low concentrations of salts, Phosphate Buffer, PB pH 7.0) and, subsequently, in more complex media, with different salts and peptones concentrations. It was also evaluated the antimicrobial activity on fixed concentrations of the peptides at regular intervals of time, useful for understanding the kinetic of the different NCPs.

4.1.1 – Peptides

The peptide sequences NCP-0, NCP-2, NCP-3, NCP-3a and NCP-3b have been synthesized from SelleckChem (Houston, TX, USA). The purity of the peptides (>90%), the sequences and concentrations have been assessed and provided by SelleckChem using the techniques of HPLC (High Pressure Liquid Chromatography) and mass spectroscopy. The lyophilized peptides were resuspended in a 10mM phosphate buffer solution (PB, 0.8709 g/L K_2HPO_4, 0.6804 g/L KH_2PO_4) at a stock concentration of 1 mg/ml.

4.1.2 – Bacterial strains

The following bacterial reference strains, purchased from Mast Diagnostic (Germany) were used for this study: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 14153, *Burkholderia cepacia* ATCC 17759, *Moraxella catharralis* ATCC 25238, *Staphylococcus aureus* ATCC 25923, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *Streptococcus agalactiae* ATCC 13813 and *Enterococcus*

hirae ATCC 10541. In addition, also the following field strains of animal origin were tested: *Acinetobacter baumannii, Enterobacter cloacae* and *Klebsiella pneumonia* subsp. *pneumoniae*.

4.1.3 – MBC evaluation

MBC was considered as the ability of the peptides to kill 99.9% of bacteria. MCB assay was conducted in liquid media, by incubating 50 µl of each peptides solutions between 0,1 µM (0,2 µg/ml) and 45 µM (100 µg/ml) with 50 µl of bacterial suspension 10^6 CFU/ml added in 96 wells microtiter plates.

In particular, for each bacterial strain 3-5, morphologically similar colonies from fresh cultures were inoculated in Brain Heart Infusion broth (BHI), then incubated at 37°C in a shaker at 225 r.p.m. for about 3-4 hours. After being pelletted at 1000 *g* for 20 minutes, the bacterial suspension was adjusted spectrophotometrically at 600 nm to an optical density value in the range 0,08-0,13 containing approximately 10^8 CFU/ml in PB pH 7, 10mM. The obtained bacterial suspension was further diluted 1:100 in PB to obtain a bacterial concentration of about 10^6 CFU/ml. Within 30 minutes 50 µl of the suspension 10^6 CFU/ml were inoculated in each well of the microtiter plate, already containing the peptide solution, to obtain a bacterial concentration of approximately $5x10^5$ CFU/ml. Bacterial cultures were cultivated without peptide as growth control.

The bacterial suspension and peptides were in contact for 2 hours at 37°C. After this incubation time, aliquots of 20 µl were withdrawn from every well and spread onto adequate solid culture medium, generally Columbia agar with 5% of bovine erythrocytes for almost bacterial strains (agar Mc Conkey was used for *E. coli, P. mirabilis, E. cloacae* and *K. pneumoniae*). The *inoculum* was evenly distributed on the agar medium using a single use

sterile handle. After an incubation time of 24 hours at 37°C, the bacterial colonies (CFU) were counted.

4.1.4 – Time kill assay

The antibacterial activity in function of time (time-kill assay) was evaluated by broth microdilution assay, as described above (4.1.3 – MBC evaluation). In particular, the bacterial suspension 10^6 CFU/ml was incubated with the different peptides 5,4 µM (12,5 µg/ml). Twenty µl aliquots were withdrawn at fixed intervals (15', 30', 60' and 120') from every well and spread onto adequate solid culture medium, Columbia agar with 5% of bovine erythrocytes or agar Mc Conkey. After an incubation time of 24 hours at 37°C, the bacterial colonies (CFU) were counted.

4.1.5 – Anti-mycobacterial activity

Mycobacteria were incubated in Middlebrook 7H9 broth at 37°C for 24 hours. The bacterial suspension was passed through a 25G needle 5-10 times to break up cells. Using a spectrophotometer with absorbance of 600 nm the turbidity of the bacterial suspension was immediately measured; the range 0,08-0,13 equates to about 10^8 CFU/ml. The obtained bacterial suspension was further diluted 1:100 in PB 10mM to obtain a bacterial concentration of about 10^6 CFU/ml. Within 15 minutes 100 µl of the suspension 10^6 CFU/ml were inoculated in every well of the microtiter plate, to obtain a bacterial concentration of about $5x10^5$ CFU/ml.

The stock peptide NCP-3 was diluted in PB at concentration of 166,4 µM (400 µg/ml), to obtain a final peptide concentration of 41,6 µM (100 µg/ml) inside the well. In order to investigate the activity of NCP-3, 50 µl of the diluted peptide were put into wells of a microtiter plate. In the wells (final volume: 200 µl) there was 25% Middlebrook 7H9 and one

of the following bacteria: *Mycobacterium fortuitum* DSMZ 46621 and *Mycobacterium smegmatis* DSMZ 43756 at the concentration indicated above.

In addition, 50 µl of the same peptide were put into wells (final volume: 200 µl) containing EDTA 0,25 mM, 50% Middlebrook 7H9. Since EDTA presents an intrinsic antimicrobial activity, it has been used a concentration low enough not to interfere with the bacterial growth, but that allow to highlight a synergy with NCPs, if there existed. Given the possible disturbing effect operated by a culture medium on the antimicrobial activity of many cationic AMPs, the test was performed with the lowest concentration of media achievable without compromising the growth of the employed strains. Bacterial cultures were cultivated without peptide as growth control. The microtiter plates were incubated at 37°C for about 72 hours.

After incubation of 72 hours at 37°C, 20 µl of resazurin 0,01% were added in each well. Resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) is a blue dye, until it is irreversibly reduced to the pink colored resorufin by living cells. For that reason, it is used as oxidation-reduction indicator in cell viability assays for bacteria and mammalian cells, and for measuring aerobic. Results were evaluated after other 24 hours of incubation at 37°C.

4.1.6 – Antifungal activity

The antifungal activity was also evaluated on unicellular mycetes *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 2001 and *Malassetia pachydermatis* ATCC 14522. These fungi were cultivated in Czapek-Dox broth (DIFCO) medium for 48-72 hours. The day of the test the fungal culture was pelletted at 1700 *g* for 15 minutes and resuspended in PB, then vortexed to disrupt fungal cell aggregations. The suspension was diluted to get a final optical density of 0,5 of the McFarland scale (about 1-5x10^6 cell/ml).

Fifty microliters (50 µl) of each peptides solutions between 0,1 µM (0,2 µg/ml) and 45 µM (100 µg/ml) were added in 96 wells microtiter plates with one of these fungi:

Candida albicans ATCC 10231, *Candida glabrata* ATCC 2001 and *Malassetia pachydermatis* ATCC 14522. Fungal cultures were cultivated without peptides as growth control.

Incubation time was 2 hours at 37°C. After then, aliquots of 20 µl were withdrawn from every well, and spread on solid culture medium, Sabouraud agar. The *inoculum* was then distributed on the agar medium using a single use sterile handle. After an incubation time of 24 hours at 37°C, the bacterial colonies (CFU) were counted.

4.1.7 – Antiviral activity

The peptides antiviral activity was valuated through cytopathic effect on Madin-Darby Bovine Kidney cells (MDBK). In particular NCP-3 was tested against Bovine Herpesvirus-1 (BoHV-1), an enveloped virus belonging to the *Herpesviridae* family, subfamily *Alphaherpesvirinae*, *Varicellovirus* genus. The envelope is typically derived from portions of the host cell membranes, rich in phospholipids and proteins, thus representing a potential target for NCPs action.

MDBK cells were infected with BoHV-1 in DMEM medium with 2% of fetal bovine serum (FBS). To obtain a final concentration of 100 TCID50 in 25 µl of DMEM medium, virus was diluted in DMEM medium without FBS.

NCP-3 was serially diluted in 25 µl of DMEM medium in 96 wells microtiter plate, to obtain a peptide final concentration between 41,6 µM (100 µg/ml) and 1,3 µM (3,125 µg/ml) after the viral inoculum addition.

After 2 hours of incubation of the microtiter plates with NCP-3 and BoHV-1, 50 µl of DMEM medium with 10% of fetal bovine serum and MDBK cells ($2x10^5$ cell/ml) in every wells were added. Subsequently, incubation of about 72 hours at 37°C with 5% of CO_2 follows. After this

step, the NCP-3 virus-neutralizing activity was evaluate through the virus cytopathic effect on cells using a phase contrast microscope.

4.1.8 – Antimicrobial activity in presence of NaCl

Peptides were also tested for their ability to inhibit bacterial growth in liquid medium. Fifty microliters (50 µl) of peptides at a concentration between 0,1 µM (0,2 µg/ml) and 45 µM (100 µg/ml) were added in microtiter plate wells with PB 10 mM with 125-250 mM of NaCl and one of these bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and methicillin-resistant *Staphylococcus aureus* ATCC 43300.

The bacterial suspension and peptides were incubated for about 2 hours at 37°C. After then, aliquots of 20 µl from each well were spread on solid culture medium, Mc Conkey agar for *E. coli* and Columbia agar with 5% of bovine erythrocytes for others bacteria. The *inoculum* was then distributed on the agar medium using a single use sterile handle. After an incubation time of 24 hours at 37°C, the bacterial colonies (CFU) were counted.

4.1.9 – Antimicrobial activity in presence of 20% of Mueller-Hinton broth

This test verify the peptides antimicrobial activity in presence of a concentration of 20% of Muller-Hinton liquid medium, characterized by the presence of salts with high concentration. This concentration is the minimal concentration of the medium that allow to a bacterial growth comparable to the growth control.

Fifty microliters (50 µl) of peptides at a concentration between 1,3 µM (3,125 µg/ml) and 41,6 µM (100 µg/ml) were added in microtiter plate wells filled with PB, Muller-Hinton liquid medium al the final concentration of 20% and one of these bacteria: *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *Staphylococcus aureus* ATCC 43300. After 2 hours incubation at 37°C,

bacteria were plated on solid culture medium. Bacterial cultures were cultivated without peptides as growth control.

4.2 – Mechanism of action

This section describes the two tests carried out to evaluate the mechanism of action of these novel AMPs. These tests were conducted using NCP-2 and NCP-3.

4.2.1 – Permeation of the bacterial internal membrane by propidium iodide (PI) assay

To assess the ability of AMPs to alter the permeability of inner membrane (IM) of *P.aeruginosa*, propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI), as counterstaining, were used. PI is membrane impermeant and therefore does not enter viable cells with intact membranes; when membrane damage occurs, PI gain access to nucleic acids and intercalates with them, its fluorescence dramatically increases and it can be used to identify dead cells. DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA. Differently from PI, DAPI can pass through an intact cell membrane, so it can be used to stain both live and fixed cells.

For each bacterial strain 3-5 morphologically similar colonies from fresh cultures were inoculated in Brain Heart Infusion broth (BHI), then incubated at 37°C for 24 hours. After being pelletted at 1000 *g* for 20 minutes, the bacterial suspension was adjusted spectrophotometrically at 600 nm to an optical density value in the range 0,08-0,13 containing approximately 10^8 CFU/ml in PB pH 7, 10 mM.

Immediately after mixing together respectively 15 µl of obtained bacterial suspension, 15 µl of 5 µg/ml DAPI solution, 15 µl of 3 µg/ml PI solution and 15 µl of peptide 20,8 µM (50 µg/ml) suspended in PB, 4 µl of that suspension were put on the glass slide and bacteria were observed by fluorescence microscopy.

Negative and positive controls were obtained in the absence of peptides and in the presence of 1 mM EDTA and 0.5% Triton X-100, respectively.

4.2.2 – Permeation of bacterial membranes assay

To test the permeabilization of the bacterial membranes caused by NCP-2 and NCP-3, *E. coli* ML-35pYC strain was used. This bacterial strain express ampicillin resistence, a constitutive expression of cytoplasmic β-galactosidase and is engineered with a plasmid for a periplasmic β-lactamase synthesis.

The bacteria were inoculated in 15 ml Mueller Hinton broth with 50 μg/ml ampicillin and incubated overnight at 37 °C. The suspension was then centrifuged at 1000 *g* for 10 minutes and the pellet was resuspended in PB. Sixty microliters (60 μl) of the *E. coli* ML-35pYC strain suspension were added to 60 μl of ONPG (15 mM), or of CENTA (1,5 mM), and to 360 μl of PB. Subsequently were added 120 μl of peptide, to obtain a final concentration of 5,4 μM (12,5 μg/ml). The absorbance value was registered at 405 nm for ONPG and CENTA, every 10 minutes for 240 minutes at all, to assess the reaction kinetics.

4.3 – Cytotoxicity assessment

Antimicrobial peptides cytotoxicity was evaluated by haemolysis assay on sheep erythrocytes and staining with Trypan Blue vital dye of epithelial eukaryotic cells.

4.3.1 – Haemolysis test

Sheep heparinized erythrocytes, sampled immediately before the test, were centrifuged at 100 *g* for 15 min and washed three times with PBS to obtain clear supernatant. Then, the RBCs were centrifuged again at 1000 *g* for 10 min, and resuspended to concentration of 2% (v/v) in PB with 308 mM of sucrose to maintain cell osmolarity. Fifty microliters (50 µl) of the washed RBCs were incubated with 50 µl of different peptide concentrations for 1 hour at ambient temperature. After this, the solution was centrifuged at 1000 *g* for 5 min; the haemolysis in the supernatants was quantified with a spectrophotometer at 450 nm. Negative control (0% haemoglobin release) and positive control (100% haemoglobin release) were obtained by using PB + 308 mM sucrose and PB + 1% of Tween 20, repectively. The haemolysis percentage was calculated as follows:

$$\left(1-\frac{A_{pep}-A_{PB}}{A_{Tween}-A_{PB}}\right)x100$$

where A_{pep} represents the optical density of the sample at 450 nm, A_{tween} the optical density of the positive control and A_{PB} the optical density of the negative control. MHC10 was the minimal peptide concentration able to lyse 10% of RBC.

4.3.2 – Cytotoxicity test

Peptides cytotoxicity against eukaryotic cells: peptides cytotoxicity against epithelial MDBK cells (Madin Darby Bovine Kidney) was determined using cell cultures of about $5x10^5$ cells/ml with and without peptides solutions 45 µM (100 µg/ml), with overnight incubation at 37°C with CO_2. The negative control was in medium without peptide; positive control was in medium with 0,2% of Triton x100, to obtain complete cell lysis. Cells vitality was assessed adding every well with 20 µl of Trypan Blue, incubating other 5 minutes at 37°C. MCC10 was the minimal peptide concentration with 10% of cytotoxicity.

Chapter 5:
Results

5.1 – Antimicrobial activity

5.1.1 - MBC evaluation

Table 1 reports LD90 and LD99 values for the tested peptides against Gram-negative bacteria while Table 2 contains LD90 and LD99 values against Gram-positive bacteria.

None or only slight activity of NCP-0 (reference peptide) was observed against almost all the tested bacteria.

NCP-2 showed a good activity against Gram-negative bacteria, unlike what has been observed against Gram-positive bacteria, where it has shown poor activity. Among Gram-negative bacteria, NCP-2 was found particularly active against *P. aeruginosa* ATCC 27853 (LD90 = 1,6 µM), and also very effective against 3 out of 4 *A. baumannii* field strains (LD99=1,6 µM). Also worthy of note is the activity of NCP-2 against *P. mirabilis* ATCC 14153 (LD90 = 9,1 µM), the best observed among NCPs against this microorganism. Among Gram-positive bacteria, only *S. agalactiae* have shown a certain sensitivity to NCP-2.

NCP-3 is the sequence which has shown the best broad-spectrum antimicrobial activity against all Gram-negative and –positive tested bacteria. Similarly to NCP-2, NCP-3 has shown excellent activity against all *A. baumannii* field strains (LD99 = 0,7/2,7 µM), and even better activity against *P. aeruginosa* ATCC 27853 (LD99 = 1,3 µM). In addition, the activity of NCP-3 against Gram-positive bacteria has proven to be excellent, with LD99 ranging from 3,5 µM to 0,7 µM.

At last, worthy of note is the great sensitivity shown by *M. catharralis* ATCC 25238 against all NCPs tested.

Table 1: LD90 and LD99 values for NCP-0, NCP-2 and NCP-3 against Gram-negative bacterial strains.

	NCP-0		NCP-2		NCP-3	
	LD 90	LD 99	LD 90	LD 99	LD 90	LD 99
Escherichia coli ATCC 25922	152,02 µg/ml (70,4 µM)	ND	10,445 µg/ml (4,5 µM)	ND	7,01 µg/ml (2,9 µM)	14,01 µg/ml (5,8 µM)
Pseudomonas aeruginosa ATCC 27853	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	3,77 µg/ml (1,6 µM)	9,67 µg/ml (4,2 µM)	1,76 µg/ml (0,7 µM)	3,2 µg/ml (1,3 µM)
Acinetobacter baumnanii from cloaca (herpetological)	142,80 µg/ml (66,1 µM)	202,98 µg/ml (94 µM)	0,8 µg/ml (0,4 µM)	1,6 µg/ml (0,7 µM)	0,8 µg/ml (0,3 µM)	1,6 µg/ml (0,7 µM)
Acinetobacter baumnanii from skin (herpetological)	ND	117,96 µg/ml (54,6 µM)	0,8 µg/ml (0,4 µM)	1,6 µg/ml (0,7 µM)	0,8 µg/ml (0,3 µM)	1,6 µg/ml (0,7 µM)
Acinetobacter baumnanii from cloaca (ornithological)	126,205 µg/ml (58,4 µM)	127,26 µg/ml (59 µM)	24,46 µg/ml (10,6 µM)	55,98 µg/ml (24,2 µM)	5,98 µg/ml (2,5 µM)	6,4 µg/ml (2,7 µM)
Acinetobacter baumnanii (human)	123,77 µg/ml (57,3 µM)	131,55 µg/ml (61 µM)	0,8 µg/ml (0,4 µM)	1,6 µg/ml (0,7 µM)	0,8 µg/ml (0,3 µM)	1,6 µg/ml (0,7 µM)
Klebsiella pneumoniae subsp. *Pneumonia* (herpetological)	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	168,8 µg/ml (72,9 µM)	201,1 µg/ml (86,9 µM)	37,63 µg/ml (15,7 µM)	50 µg/ml (20,8 µM)
Enterobacter cloacae (herpetological)	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	155,9 µg/ml (67,3 µM)	221,5 µg/ml (95,7 µM)	19,53 µg/ml (8,1 µM)	25 µg/ml (10,4 µM)
Burkholderia cepacia ATCC 17759	38,35 µg/ml (17,8 µM)	39,43 µg/ml (18,3 µM)	152,0 µg/ml (65,7 µM)	>152,0 µg/ml (>65,7 µM)	40,25 µg/ml (16,7 µM)	50 µg/ml (20,8 µM)
Proteus mirabilis ATCC 14153	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	21,06 µg/ml (9,1 µM)	ND	73,37 µg/ml (30,5 µM)	100 µg/ml (41,6 µM)
Moraxella catharralis ATCC 25238	0,8 µg/ml (0,4 µM)	1,6 µg/ml (0,7 µM)	0,8 µg/ml (0,4 µM)	1,6 µg/ml (0,7 µM)	0,8 µg/ml (0,3 µM)	1,6 µg/ml (0,7 µM)

Table 2: LD90 and LD99 values for NCP-0, NCP-2 and NCP-3 against Gram-positive bacterial strains.

	NCP-0		NCP-2		NCP-3	
	LD 90	LD 99	LD 90	LD 99	LD 90	LD 99
Methicillin-resistant *Staphylococcus aureus* ATCC 43300	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	6,09 µg/ml (0,3 µM)	8,34 µg/ml (3,5 µM)
Staphylococcus aureus ATCC 22953	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	36,81 µg/ml (15,9 µM)	50 µg/ml (21,2 µM)	0,8 µg/ml (0,3 µM)	1,6 µg/ml (0,7 µM)
Enterococcus hirae ATCC 10541	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	155,09 µg/ml (65,6 µM)	240,12 µg/ml (103,7 µM)	0,8 µg/ml (0,3 µM)	1,6 µg/ml (0,7 µM)
Streptococcus agalactiae ATCC 13813	100 µg/ml (46,3 µM)	>100 µg/ml (>46,3 µM)	4,91 µg/ml (2,1 µM)	8,73 µg/ml (3,7 µM)	0,8 µg/ml (0,3 µM)	1,6 µg/ml (0,7 µM)

Of particular interest are the activities shown by the NCP-3 variants, NCP-3a and NCP-3b. Results are reported on Figure 12. While NCP-3 shows the best activity against *P. aeruginosa* ATCC 27853, the substitution on amino acid position 4 of Trp with Phe have improved the antimicrobial activity of NCP-3a against a Gram-positive bacteria such as MRSA ATCC 43300, with respect to its ancestor (NCP-3). Conversely, the substitution on the same amino acid position of Trp with Leu (NCP-3b) have improved antimicrobial activity against *E. coli* ATCC 25922, with respect to NCP-3 and NCP-3a, that present an aromatic residue in this position. Moreover, differently from NCP-3, both NCP-3a and NCP-3b have shown a strong activity against *P. mirabilis* ATCC 14153.

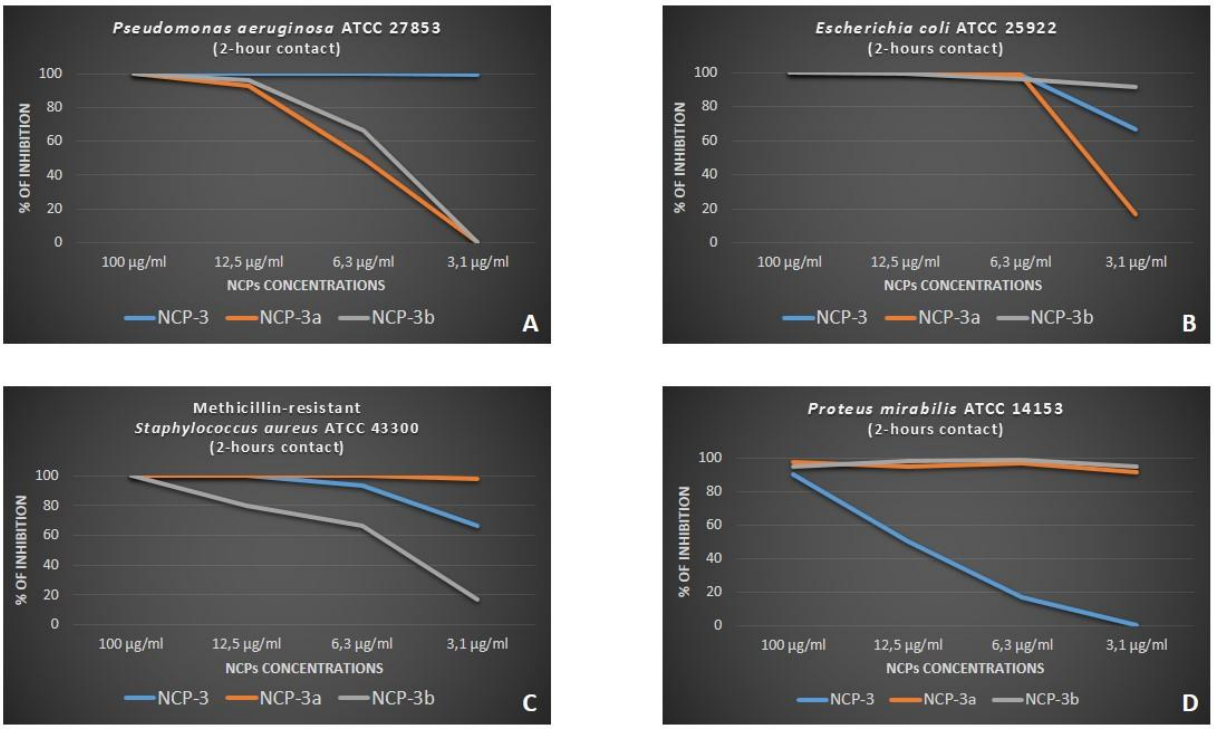

Figure 12: Activity of NCP-3, NCP-3a and NCP-3b against *P. aeruginosa* ATCC 27853 (A), *E. coli* ATCC 25922 (B), MRSA ATCC 43300 (C) and *P. mirabilis* ATCC 14153 (D).

5.1.2 – Time kill assay

In order to evaluate the antimicrobial activity of the NCPs in the course of time, cultures of bacterial strains representative of Gram-negative and Gram-positive species, were incubated with a concentration of 5,4 µM (12.5 µg/ml) for each peptide (NCP-2 and NCP-3). Aliquots of 20 µl were withdrawn at fixed intervals (15', 30', 60' and 120') from every well, and spread onto adequate solid culture medium, as previously indicated.

NCP-2 and NCP-3 confirmed a good activity against *A. baumannii* field strains and *M. catharralis* ATCC 25238, reaching the LD90 and LD99 within 15 minutes (Figg. 13 and 14). NCP-2 have also showed an excellent activity against *E. coli* ATCC 25922, reaching the LD90 within 30 minutes. According to MBCs results, NCP-2 has proven to be weakly

effective only against *Streptococcus agalactiae* ATCC 13813 (LD90 reached in 1 hour) (Fig. 13).

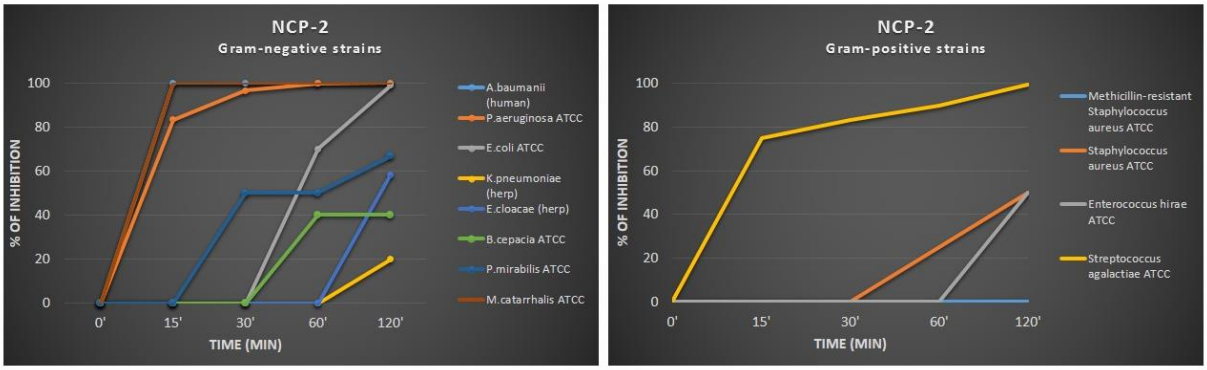

Figure 13: Time kill assay for NCP-2 against Gram-negative (left) and Gram-positive bacteria (right).

Excellent activity has been shown by NCP-3 towards all the tested Gram-positive strains, reaching both LD90 and LD99 within 15 minutes, while towards *E.coli* ATCC 25922 reached the LD90 within 30 minutes (Fig. 14).

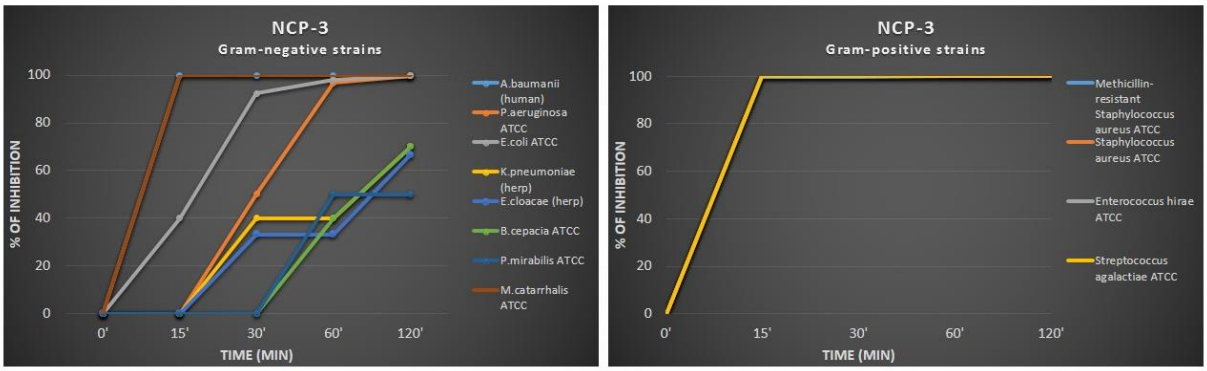

Figure 14: Time kill assay for NCP-3 against Gram-negative (left) and Gram-positive bacteria (right).

5.1.3 – Anti-mycobacterial activity

Results concerning activity of NCP-3 against mycobacteria are reported in Figure 15. NCP-3 at concentration of 100 µg/ml (41,6 µM) has completely inhibited *Mycobacterium fortuitum* DSMZ 46621 and *Mycobacterium smegmatis* DSMZ 43756 growth, with both 50% and 25% of Middlebrook 7H9 medium. To underline the different behaviour on the two strains: while *Mycobacterium smegmatis* DSMZ 43756 growth was completely inhibited with and without the presence of EDTA, *Mycobacterium fortuitum* DSMZ 46621 inhibition occured less quickly, leaving a slight colour change inside the well.

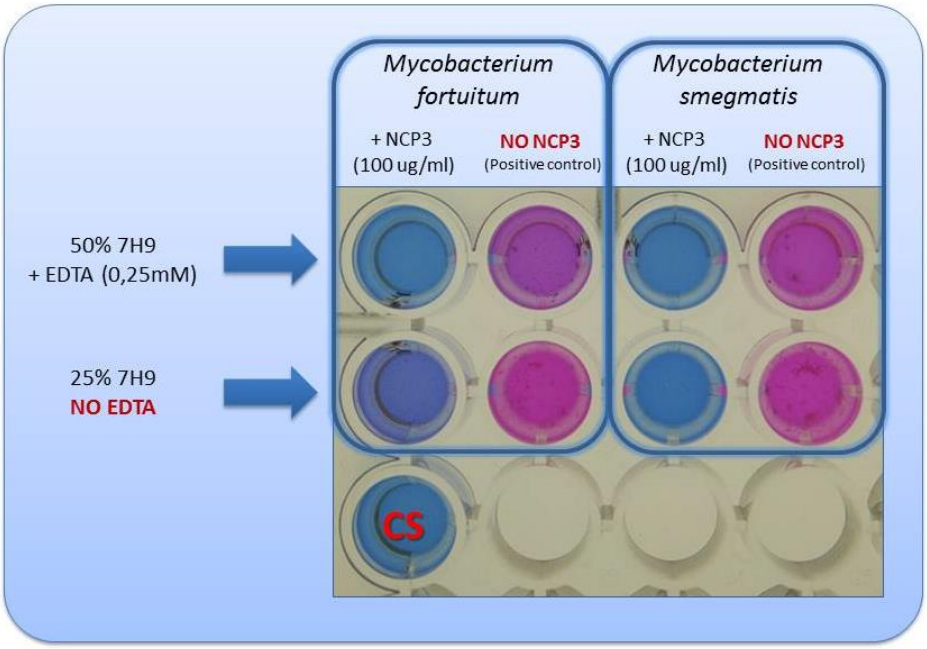

Figure 15: Activity of NCP-3 against *Mycobacterium fortuitum* DSMZ 46621 and *Mycobacterium fortuitum* DSMZ 43756. On the first line are located the wells filled with 50% of Middlebrook 7H9 medium and EDTA 0,25 mM; the wells on the second line were filled with only 25% Middlebrook 7H9. Each strain has been incubated with NCP-3 (first and third column), and without peptide (second and fourth column) as positive control. Bacterial growth is characterized by a color change from blue to pink.

5.1.4 - Antifungal activity

Results of antifungal activities are reported in Table 3.

The reference peptide NCP-0 has shown only a weak activity against all the tested fungal strains (LD90 > 69,2 µM).

NCP-2 has shown instead an excellent activity against *M. pachydermatis* ATCC 14522 (LD99 = 2,7 µM), while higher concentration are required to be effective towards *C. albicans* ATCC 10231 and *C. glabrata* ATCC 2001.

NCP-3 has shown a remarkable activity towards *C. albicans* ATCC 10231 (LD99 = 5,7 µM) and *M. pachydermatis* ATCC 14522 (LD99 = 2,7 µM), while it was found to be less effective against *C. glabrata* ATCC 2001.

Table 3: LD90 and LD99 values for NCP-0, NCP-2 and NCP-3 against fungal strains.

	NCP-0		**NCP-2**		**NCP-3**	
	LD 90	LD 99	LD 90	LD 99	LD 90	LD 99
Candida albicans ATCC 10231	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	56,91 µg/ml (25,6 µM)	58,17 µg/ml (25,1 µM)	12,43 µg/ml (5,2 µM)	13,71 µg/ml (5,7 µM)
Candida glabrata ATCC 2001	>100 µg/ml (>46,3 µM)	>200 µg/ml (92,6 µM)	>200 µg/ml (>68,4 µM)	>200 µg/ml (>68,4 µM)	30,08 µg/ml (0,3 µM)	32,26 µg/ml (13,4 µM)
Malassezia pachydermatis ATCC 14522	150,82 µg/ml (69,8 µM)	151,87 µg/ml (70,3 µM)	2,95 µg/ml (1,3 µM)	6,4 µg/ml (2,8 µM)	4,156 µg/ml (1,7 µM)	6,4 µg/ml (2,7 µM)

5.1.5 – Antiviral activity

Results for antiviral activity are reported in Figure 16. In the virus neutralization assay, noteworthy is the activity of NCP-3 which, starting from the concentration of 25 µg/ml, have completely inhibited cytopathic effect due to the BoHV-1 replication.

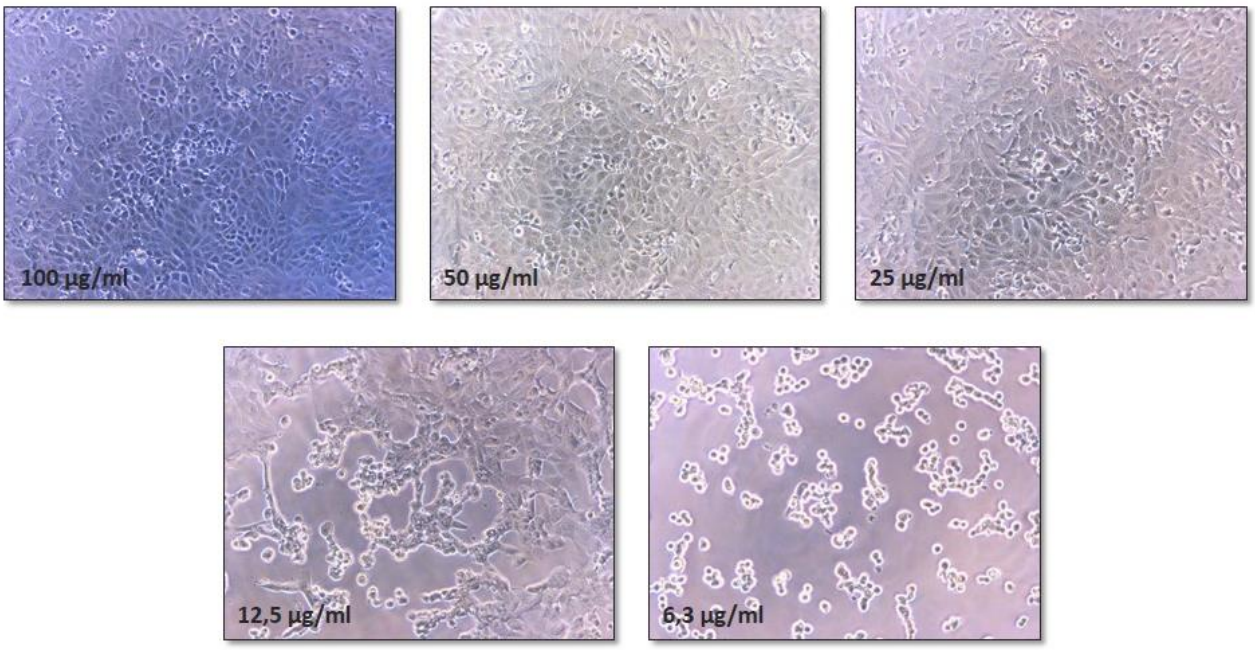

Figure 16: Virus neutralization assay on MDBK cells. The presence of NCP-3 concentrations up to 25 µg/ml completely protect from cytopathic effect.

5.1.6 – Antimicrobial activity in presence of NaCl

Inhibition percentage of bacterial growth in presence of scalar concentration of NaCl are reported in Tables 4, 5 and 6. Each peptide was tested at concentration of 100 µg/ml (45 µM) and 12,5 µg/ml (5,4 µM). As reported on Tab. 4, results with NaCl 10 mM agree with those of MBCs evaluation without showing any disturbing effect on NCPs antimicrobial activity. At NaCl 125 mM concentration (Tab. 5), NCP-3 maintains the maximal antimicrobial activity and NCP-2 shows a good activity against Gram-negative bacteria. At NaCl 250 mM concentration, achievable *in vivo* only in a few pathological states, NCP-3 maintained excellent activity against all tested bacteria, while NCP-2 showed a good antimicrobial activity only against Gram-negative bacteria (Tab. 6). In general, the activity of NCP-2 and particularly that of NCP-3 seems to be very little affected by the presence of NaCl.

Table 4: Growth inhibition percentages of NCP-0, NCP-2 and NCP-3 in presence of sodium chloride 10 mM.

NaCl 10 mM	*Escherichia coli* ATCC 25922		*Pseudomonas aeruginosa* ATCC 27853		Methicillin-resistant *Staphylococcus aureus* ATCC 43300	
Peptide concentration	100 µg/ml	12,5 µg/ml	100 µg/ml	12,5 µg/ml	100 µg/ml	12,5 µg/ml
NCP-0	3%	0%	2%	0%	2%	0%
NCP-2	95%	73%	98%	95%	0%	0%
NCP-3	100%	100%	100%	100%	100%	100%

Table 5: Growth inhibition percentages of NCP-0, NCP-2 and NCP-3 in presence of sodium chloride 125 mM.

NaCl 125 mM	*Escherichia coli* ATCC 25922		*Pseudomonas aeruginosa* ATCC 27853		Methicillin-resistant *Staphylococcus aureus* ATCC 43300	
Peptide concentration	100 µg/ml	12,5 µg/ml	100 µg/ml	12,5 µg/ml	100 µg/ml	12,5 µg/ml
NCP-0	0%	0%	0%	0%	0%	0%
NCP-2	70%	51%	90%	86%	0%	0%
NCP-3	100%	100%	100%	100%	100%	96,75%

Table 6: Growth inhibition percentages of NCP-0, NCP-2 and NCP-3 in presence of sodium chloride 250 mM.

NaCl 250 mM	*Escherichia coli* ATCC 25922		*Pseudomonas aeruginosa* ATCC 27853		Methicillin-resistant *Staphylococcus aureus* ATCC 43300	
Peptide concentration	100 µg/ml	12,5 µg/ml	100 µg/ml	12,5 µg/ml	100 µg/ml	12,5 µg/ml
NCP-0	0%	0%	0%	0%	0%	0%
NCP-2	68%	43%	78%	65%	0%	0%
NCP-3	100%	96,75%	100%	100%	100%	37,5%

5.1.7 – Antimicrobial activity in presence of 20% of Mueller-Hinton broth

Data reported in Tables 7 and 8, show that NCP-3, in presence of 20% of Mueller-Hinton broth has maintained a good antimicrobial activity against the tested Gram-negative bacteria, while the activity against Gram-positive has shown to be a little more affected by the presence of this medium. NCP-2 has maintained a good activity towards *E. coli* ATCC 25922 rather than *P. aeruginosa* ATCC 27853, while the reference peptide NCP-0 has shown absence of antimicrobial activity. Peptides structural characteristics (in particular distribution of charge and hydrophobic regions) allow to maintain at least partially the antimicrobial activity, even in this unfavourable environment.

Table 7: Growth inhibition percentages of NCP-0, NCP-2 and NCP-3 against Gram-negative bacteria, in presence of 20% of Mueller-Hinton broth.

	Escherichia coli ATCC 25922				*Pseudomonas aeruginosa* ATCC 27853			
Peptide concentration	100 µg/ml	12,5 µg/ml	6,3 µg/ml	3,1 µg/ml	100 µg/ml	12,5 µg/ml	6,3 µg/ml	3,1 µg/ml
NCP-0	0%	0%	0%	0%	0%	0%	0%	0%
NCP-2	100%	100%	99,1%	91%	86,7%	0%	0%	0%
NCP-3	100%	100%	100%	100%	100%	100%	98%	66,7%
NCP-3 (PB*)	100%	100%	100%	100%	100%	100%	98%	93,8%

Table 8: Growth inhibition percentages of NCP-0, NCP-2 and NCP-3 against Gram-positive bacteria, in presence of 20% of Mueller-Hinton broth.

	Staphylococcus aureus ATCC 22953				Methicillin-resistant *Staphylococcus aureus* ATCC 43300			
Peptide concentration	100 µg/ml	12,5 µg/ml	6,3 µg/ml	3,1 µg/ml	100 µg/ml	12,5 µg/ml	6,3 µg/ml	3,1 µg/ml
NCP-0	0%	0%	0%	0%	0%	0%	0%	0%
NCP-2	0%	0%	0%	0%	0%	0%	0%	0%
NCP-3	100%	100%	94,3%	58,3%	100%	97%	5%	0%
NCP-3 (PB*)	100%	100%	100%	98,3%	100%	100%	100%	82,8%

5.2 - Mechanism of action

5.2.1 - Permeation of the bacterial internal membrane by propidium iodide (PI) assay

As shown in Figure 17, the mechanism of action was investigated on *P. aeruginosa* ATCC 27853. The stain-dead assay highlighted the fast permeabilization of the inner membrane, with the initial occurrence of red fluorescence within 15 min after contact with NCP-3. Intriguingly, these results are perfectly superimposable to that previously observed in the time kill assay for NCP-3 against *P. aeruginosa* ATCC 27853.

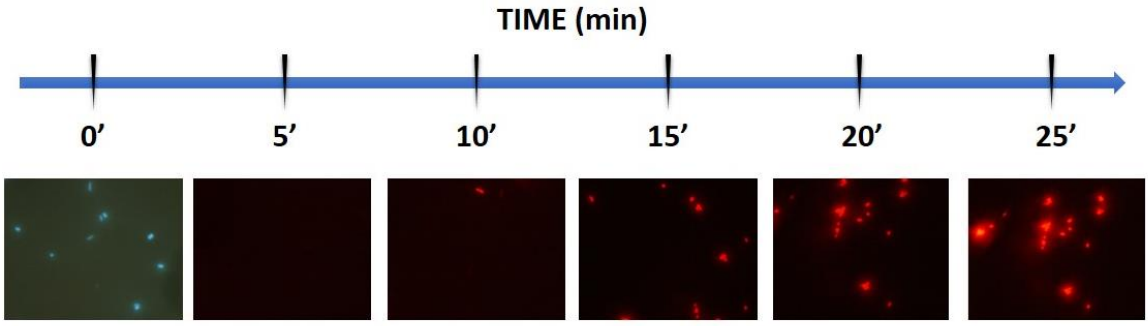

Figure 17: Propidium iodide dead-cell stain assay: permeabilization of the inner membrane of *P. aeruginosa* ATCC 27853 as a function of contact time (min) with the peptides.

5.2.2 - Permeation of bacterial membranes assay

In Figure 18 the permeabilization curves of the 2 tested peptides are shown.

NCP-2 showed the ability to penetrate the outer membrane without destruction of the membrane itself, as demonstrated by the oscillation of the optical density around the base value during the entire test. However, the same peptide has shown a destructive action to the inner membrane, with a linear increase of the optical density.

NCP-3 showed a time-dependent increase of the optical density, which may suggest a lithic action on both the inner and outer membranes. This action complies with what is reported in literature about antimicrobial peptides.

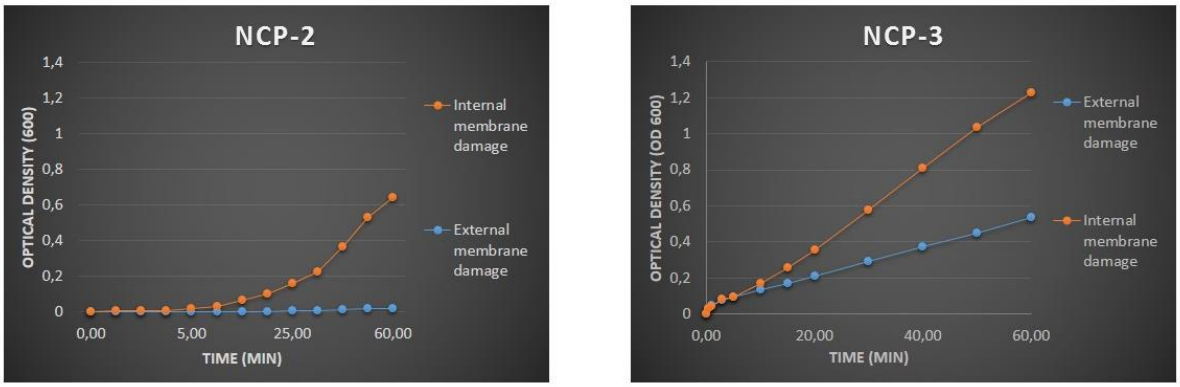

Figure 18: Permeabilization of bacterial membranes assay results for NCP-2 and NCP-3.

5.3 - Cytotoxicity assessment

5.3.1 - Haemolysis test

The haemolytic activities of the tested peptides are shown on Figure 19. NCP-2 and NCP-3 have shown <10% haemolytic effect at maximum concentration 45 µM (100 µg/ml).

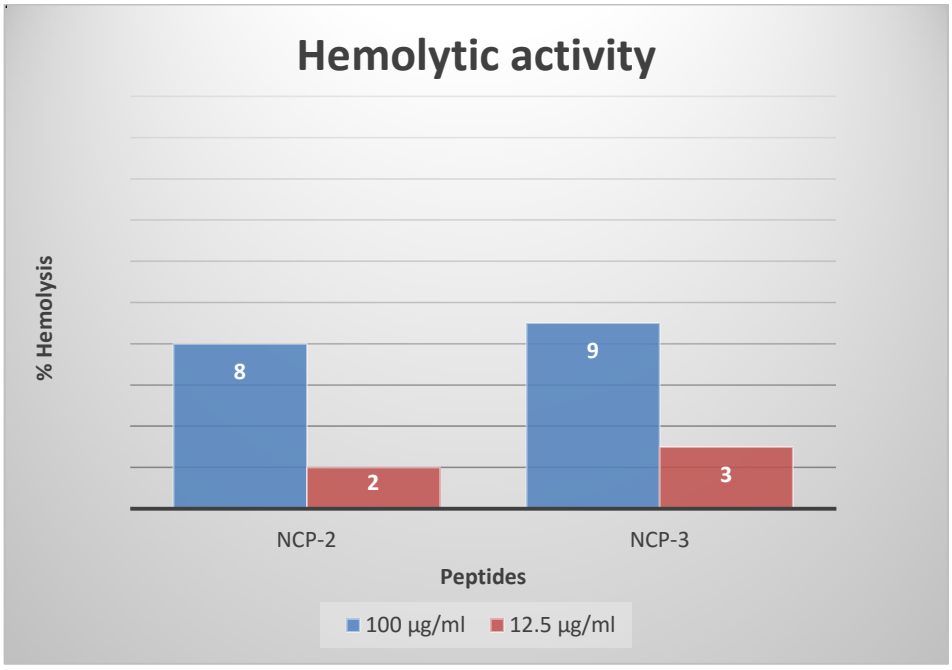

Figure 19: Hemolysis assay of NCP-2 and NCP-3.

5.3.2 - Cytotoxicity test

The cytotoxic activities of the tested peptides against MDBK cells are shown in Tab. 9. All peptides have shown <10% cytotoxicity at 45 µM (100 µg/ml). These data confirm little or no peptides cytotoxicity towards eukaryotic cells.

Table 9: Cytotoxicity assay of NCP-2 and NCP-3 against MDBK cells. Percentages reported are referred to the cytopathic effect scale, where 0% = all intact cells and 100% = cytopathic effect extended to all cells.

Peptide concentration	100 µg/ml	12,5 µg/ml
NCP-0	6%	4%
NCP-2	5%	3%
NCP-3	7%	3%

Discussion

Toxins found in venoms are the result of recruitment events in which an ordinary gene, encoding for a secreted protein, is duplicated and selectively expressed in the venom gland. The large family of genes encoding for AMPs (such as those encoding for defensines) is one of the many that have undergone to this process of gene recruitment (Fry, Roelants et al. 2009). Because of evolutionary needs, the selective pressure in many venomous animal species has brought to the development of toxins starting from the original secreted protein recruited. These molecules share many common features with their ancestors, such as the three-dimensional structure. These AMPs-derived toxins, in addition to their constitutive toxic activities, intriguingly maintain a certain amount of their original antimicrobial activity (Fry, Roelants et al. 2009; Yount, Kupferwasser et al. 2009). Moreover, even some toxins not AMPs-derived have shown antimicrobial activity. It has been noted in the case of elapid snakes cardiotoxins (CTXs), belonging to the family of three-finger toxins (3FTxs), that this activity is correlated, similarly to AMPs, with their ability to interact with phospholipid membranes (Chen, Kao et al. 2011).

Taking into account the considerations made so far, using an *in silico* approach, I have designed and developed a new class of antimicrobial peptides, starting from the primary structure of cardiotoxin 1 (CTX-1) of Chinese cobra (*Naja atra*). Of these novel AMPs, the antimicrobial activities against several bacterial strains (clinical isolates and reference strains), fungi and an enveloped virus (BoHV-1) have been examined during my PhD course. Finally, I have evaluated the mechanism of action and the possible cytotoxicity against eukaryotic cells.

CTX-1 of *Naja atra*, as others S-type cardiotoxins, is able to binds phospholipid membranes only with its loop I. There is no surprise in the fact that the original derived peptide sequence,

with the best predictive indexes, has been obtained from this portion of the protein that, similarly with AMPs, can interact with membranes.

These novel peptides have showed a wide and diversified spectrum of antimicrobial activity depending on the microbial strain and the peptide sequence considered. While the effects of NCP-0 against almost all the tested bacteria were limited, NCP-2 and NCP-3 have shown a broad spectrum of antimicrobial activity. In particular, of interest is the good activity against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. These data are encouraging because the treatment of infections caused by these pathogens is often complicated by their ability to develop resistance against a number of antibiotics (Tommasi, Brown et al. 2015), not only in domestic animals (Markey, Leonard et al. 2013), but also in humans (Pendleton, Gorman et al. 2013). While most of AMPs report a best activity against Gram-negative bacteria, I have obtained at least 2 NCPs (NCP-3 and its variant, NCP-3a) very active against Gram-positive bacteria by a single residue substitution in the original sequence.

The mutants NCP-3a and NCP-3b differ from the NCP-3 for only a single residue on position 4. Those with an aromatic residue on that position (Trp in NCP-3 and Phe in NCP-3a) showed to be more effective against Gram-positive strains than NCPs presenting non-aromatic residues on that position (Pro in NCP-2 and Leu in NCP-3b). Intriguingly, the presence of an aromatic amino acid at position 4 does not seem to be so relevant for the antimicrobial activity against Gram-negative bacteria as for Gram-positive bacteria. The importance of the role played by aromatic residues (in particular from tryptophan and phenylalanine) in determining the affinity for phospholipid membranes, resulting in widening of the antimicrobial activity of a peptide, is well documented in literature (Sato and Feix 2006; Bi, Wang et al. 2013). This finding is supported in this study by the activity shown by NCP-3 even against mycobacteria, fungi and an enveloped viral strain.

One of the great advantages of AMPs, compared to conventional antimicrobial molecules, is the great speed of action (Papagianni 2003; Brogden 2005). NCPs have reported bactericidal activity within 15-60 min of contact for most tested bacteria. These results encourage a potential use of NCP-3 in the treatment of bacterial infections.

In order to explain the mechanism of action, I have explored the ability of NCPs to establish a membrane damage.

Previous studies have suggested that the bactericidal activity of cationic peptides depends on their own ability to bind, through electrostatic interaction, to the anionic elements on the outer membrane of pathogens, and then penetrate into the membrane using hydrophobic type interactions. This suggests that in addition to the structural components, the peptides should possess a positive charge density and an excellent balance between hydrophilic and hydrophobic surface. In addition, it is believed that the amphiphilic conformation confers a bactericidal activity, which is undertaken through the destruction of bacterial membranes. The target of many cationic peptides is represented by the cytoplasmic membrane of bacteria, and the depolarization of this membrane by the peptides leads to the destruction of the gradient of the electric potential ($\Delta\Psi$) with consequent cell death, probably due to loss of membrane integrity (Shai 2002; Sato and Feix 2006).

All the membrane permeabilization assays confirm the kinetic observed for the NCPs in the time kill assays. Similarly to the time kill assay performed on *Pseudomonas aeruginosa* with NCP-3, also with propidium iodide the fluorescence initially occur within 15 minutes. Even more interesting are the data collected through the membrane permeabilization assay performed on *Escherichia coli* ML-35pYC. The curve referred to the inner membrane damage begins to rear later with NCP-2 than it does with NCP-3, thus mimicking the trend shown in the curves related to the kinetics. For what concern the outer membrane damage, it is interesting to observe how in the case of NCP-2, differently to what happens with NCP-3, the

outer bacterial membrane is not damaged in any way by the action of this peptide, which focuses its action on the inner one instead. This difference in activity is possibly provided only by the substitution of the proline located at position 4 of NCP-2, with a tryptophan that characterizes NCP-3. This seems to confirm the importance not only of the residue type, as mentioned before, but also the relevance of this sequence position in determining the activity of this family of AMPs. However, further studies are required to better understand the effects of this and others amino acidic substitutions on the NCPs activities.

Initially, antimicrobial activity has been evaluated under optimal conditions (PB) to avoid environmental interferences. Subsequently tests were performed in complex media (salt rich media and Mueller-Hinton medium) to mimic *in vivo* conditions.

The environment with high concentrations of salt weakens the initial electrostatic interactions between the peptides and the bacterial cells, reducing their bactericidal activity (Zasloff 2002). The inactivation of this activity in Mueller-Hinton Broth medium depends instead on a more complex mixture of salts and peptones. Despite their positive charge is not high as other known AMPs, both NCP-2 and NCP-3 maintain their activity even in presence of high concentrations of sodium chloride or Mueller-Hinton Broth medium. Normally the higher positive charge possessed by some AMPs, is considered a key feature in maintaining the antibacterial activity in the presence of salts. Nevertheless, in this case, another hypothesis, based on the structural distribution of the positive charge as a key factor for the salt sensitivity of the antibacterial peptides, could explain this NCPs behavior. Moreover, this maintained antibacterial activity could be explained taking into account that peptides with many intra-backbone hydrogen bonds (such as NCPs) less suffer the high ionic strengths (Park, Cho et al. 2004).

At last, in order to hypothesize an *in vivo* use, it has been necessary to evaluate a possible cytotoxicity of NCPs. This step is particularly appropriate considering the original protein (CTX-1) from which NCPs derive.

Moreover, given that the killing of the bacteria and the cytotoxic activity are both direct consequences of the binding of peptides with cells membranes, we have demonstrated the selectivity of NCP-3 for different cell types. In general, hemolysis and cytotoxicity values obtained for NCP-3 are low enough to be considered safe for eukaryotic cells.

However, the information derived from *in vitro* tests should be considered carefully as they may not reflect its real *in vivo* cytotoxicity (Yeaman and Yount 2003).

Despite these encouraging *in vitro* results, a possible use of these peptides in the treatment of infectious disease still require further studies in order to overcome some common issues to many AMPs. The *in vivo* application of antimicrobial peptides is limited due to the loss of their function in serum, partially because their short half-life owing to enzymatic degradation and binding to serum components; moreover, there are still few data available on the *in vivo* real toxicities of AMPs and the stability of the peptide/peptide-formulation (Hiemstra and Zaat 2013).

Conclusions

Data reported in this personally conducted work show how we can get antimicrobial peptides starting from known secreted proteins. In literature, there are many studies on mutant peptides, obtained by artificial modification of the primary structure of known AMPs naturally occurring. However, the extrapolation of antimicrobial peptides from sequences of proteins, not related with AMPs, but able to interact with biological membranes, is a completely innovative approach.

It was also shown that highly cytotoxic toxins, such as CTX-1, could be recruited in this development process, and the derived peptide not necessarily represents a risk for its own cytotoxicity.

Through subsequent modifications of the amino acid sequence, it has been possible to greatly enhance its antimicrobial effects. It was possible to observe how substitutions of a single amino acid residue, can lead not only to an increase in broad-spectrum activity, but also to the expression of more targeted activities towards specific groups of microorganisms.

Like other AMPs, through the investigations conducted on the mechanism of action, it was possible to see how these new sequences present antimicrobial activity mainly dependent from damage on the biological membranes.

The use of secreted proteins able to interact with biological membranes for the creation and the development of new AMPs led to the generation of these encouraging results. On the basis of the results so far obtained with NCPs, we consider that this process can represent a new and wide potential resource for the development of new antimicrobial peptide sequences for the treatment of many infectious diseases.

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