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Towards the design of peptide antibiotics: new insights from synthetic analogues and from natural sources

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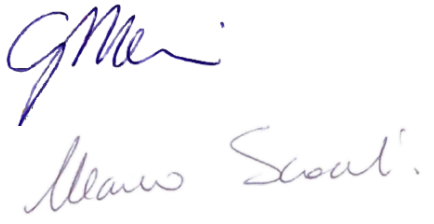

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Abstract

Resistance to antibiotics is spreading worldwide across bacteria generating an increasing number of multi- and even pan-drug resistant pathogens. Antibiotics currently on the market hardly could keep this threat at bay. In order to overcome this problem, novel classes of drugs with different mechanisms of action are extremely necessary. High hopes are pointed towards antimicrobial peptides (AMPs), natural molecules with multiple defensive roles produced by eukaryotes and bacteria. Among AMPs, proline-rich antimicrobial peptides (PrAMPs) share a generally low cytotoxicity thanks to an intracellular mode of action based on inhibition of protein synthesis but also a relatively narrow spectrum of activity. This work was aimed to optimize the antimicrobial activity of two peptides derived from the natural cathelicidin-derived PrAMPs Bac7 and Bac5, to characterize them along with seven novel PrAMPs discovered in cetaceans.

First, we characterized the antimicrobial activity, toxicity and mode of action of eight fragments of Bac5, discovering that Bac5(1-17) was the shortest fragment retaining appreciable antimicrobial and translation-inhibiting activity against *E. coli*. Subsequently, we screened libraries of mutants of Bac5(1-17) and Bac7(1-16), another known PrAMP fragment with antimicrobial activity. This led us to select ten novel (optimized) PrAMPs derived from of Bac7(1-16) and Bac5(1-17), with single or multiple amino acid substitutions, to be further characterized for activity/toxicity. In parallel, we searched and characterized orthologs of the bovine PrAMP Bac7 in cetacean species, finding five novel cetacean PrAMPs (cePrAMPs). These five novel molecules, along with the two recently discovered cePrAMPs Tur1A and Tur1B, and our ten optimized PrAMP fragments, were characterized for antimicrobial activity, mode of action, cytotoxicity and stability in human serum and salty media.

Among the selected optimized PrAMP fragments, some displayed wider activity spectrum or increased activity compared to the original PrAMPs. Two of them showed MIC = 1-8 μM against reference strains of *Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae, and Acinetobacter baumannii,* without cytotoxicity against eukaryotic cell line MEC-1 up to 64 μM. One Bac7 derivative was effective against *E. coli* (MIC ≤ 4μM) also in the presence of 10% human serum, and another peptide retained a MIC = 16 μM in presence of 2.7% NaCl. Analysis of natural cePrAMPs identified two peptides, Bal1 and Lip1, that showed excellent spectrum of activity, with MIC ≤ 2 μM and MIC ≤ 8 μM against reference strains of six Gram-negative and two Gram-positive species, respectively. The two cePrAMPs showed somewhat cell-dependent cytotoxicity but no hemolytic activity and kept their antibacterial activity also in 10% human serum and 2.7% NaCl. Regarding the mechanism of action, cePrAMps may be divided into two

subgroups. Four of them showed scarce translational-inhibiting effectiveness but significant membrane-perturbing activity while the other three peptides displayed excellent translational inhibition, similarly to Bac7(1-35). It is worth noting that some cePrAMP fragments were also found to have a mechanism consisting in both inhibition of protein synthesis and perturbation of *E. coli* membranes.

Importantly, three cePrAMPs and the best optimized peptide share a sequence motif present in other PrAMPs, which was already proposed as consensus for translation inhibition. The best optimized peptides and CePrAMPs were used as starting points to design six chimeric PrAMPs. Preliminary results , however, suggest that no remarkable improvement of the antimicrobial activity have been obtained.

Results of this work let to gain hints for the design of novel peptide antibiotics displaying a wider spectrum of activity and retaining low toxicity but acquiring a dual mode of action not restricted to protein synthesis inhibition.

Introduction

Antimicrobial resistance and threats to healthcare

The discovery of antibiotics has provided modern medicine with outstanding weapons against a great variety of bacterial pathogens, allowing huge improvements in healthcare all over the world. Undoubtedly, since the clinical introduction of antibiotics, some pathogenic microorganisms developed mechanisms of resistance to some of these drugs. However, in the four decades from 1930s to 1970s, a great variety of novel classes of antibiotics reached the market, allowing physicians to fight the novel resistant pathogens with a vast choice of antimicrobials. These four decades were nicknamed the "golden age" of antibiotics, and the majority of antibiotics in use to date belong to classes of compounds developed in those years[1,2]. During the years, bacteria have continued to evolve molecular mechanisms to withstand the drugs they had to face. The threat of antimicrobial resistance (AMR) has always been present since antibiotics were first used in clinical practice, but it could initially be tackled by the copious diversity of molecules available. The AMR became increasingly threatening due to repeated misuse of antibiotics in medical, agricultural and industrial settings. A reckless use of these drugs has heavily increased the selective pressure towards those bacteria able to withstand higher drug concentrations, and multi-drug resistant (MDR) strains arose from a variety of environments, from sewage implants, soil and waters to healthcare facilities[1,3].

As a consequence, there are now numerous extensively-drug resistant (XDR) or even "pan-drug resistant" (PDR) microbes threatening the global public health, and the alarming lack of weapons to tackle these new "superbugs" was also caused by a retreat in investments by the big pharmaceuticals industries and biotechnology companies[4,5].

Nowadays, the MDR pathogens represent one of the most serious threats for the world's healthcare, and infections caused by pathogens with extensive spectra of antimicrobial resistance are reported all over the world. Without antibiotics, previously treatable infections as well as simple routine surgery procedures may easily become fatal. Reports indicated that antimicrobial resistance caused 23'000 deaths per year in the US in 2019 and 33'000 deaths a year in Europe in 2015[6–8]. Moreover, the World Health Organization (WHO) has recently estimated 10 million deaths per year worldwide by 2050 if no action to contrast AMR is taken, with a cost of 1,5 billion € per year only in Europe and millions of people being pushed into extreme poverty[9].

The WHO has also published a list of the most worrisome pathogens of the present years, against which novel drugs are urgently needed[10]. Large economic efforts should be devoted to study the virulence and drug resistance in these microbes and to develop novel therapeutic strategies.

Importantly, for years the biopharma industries have withdrawn many investments from the research and development of novel antimicrobials, deemed not enough profitable[11]. At present, new incentives and initiatives have been launched to foster new research efforts[12 13] and luckily, the clinical antibacterial pipeline is slowly being replenished [14,15], but still the present classes of antibiotics in use are hardly enough to contrast the global antibiotic crisis.

Classes of antibacterial drugs on the market and their targets

Antibiotic compounds have been deployed, more or less consciously, throughout the history of humanity[16]; reports of poultices of organic matter to prevent or treat infections date back to very ancient times[17]. However, the aware use of antibiotics, knowing the chemistry of the deployed molecules, arguably began at the beginning of 1900 with the use of Salvarsan, and the discovery of penicillin[16,18,19]. After that, some microbes were identified as a prolific source of natural antimicrobial compounds[16,20], and an era of fruitful drug discovery started called "the Golden Age of antibiotics". After a few decades, though, it became harder to find entirely novel antibiotics with novel modes of action, and pharmaceutical companies gradually withdrawn their funding from the research in this field. Therefore, as mentioned, most of the antibiotics currently approved for clinical use were discovered in the 30s-70s.

A detailed description of all the classes of antibiotics on the market is beyond the scope of this thesis and can be reviewed elsewhere[21]. However, this section aims to give a brief overview. Broadly speaking, antibiotics can be sorted by grouping them in i) inhibitors of the bacterial cell wall synthesis, ii) inhibitors of protein synthesis, iii) inhibitors of nucleic acid synthesis, iv) inhibitors of crucial metabolic pathways and v) antibiotics damaging the bacterial membrane (Tab. 1.1)[22].

These broad groups include antibiotics with diverse targets but also drugs of different chemical classes directed to very close or even coincident target sites. Nevertheless, even when the binding sites are very close or coincident, the different chemical nature of different compounds can grant different target affinity, pharmacokinetics, or susceptibility to resistance mechanisms.

The cell wall synthesis inhibitors interfere with crucial passages in the synthesis of the peptidoglycan (PG), a polymer that is a main component of the bacterial cell wall, with the exception of mycobacteria[23]. Defects in the peptidoglycan synthesis generate a weak bacterial wall

unable to counter the osmotic pressure, eventually causing the bacterial lysis. There are several classes of antibiotics targeting the cell wall synthesis, of which the beta-lactams, the class that includes penicillin**,** are perhaps the most known. Beta-lactam antibiotics bind and inhibit enzymes called penicillin binding proteins (PBPs), which are responsible of cross-linking together the single filaments of PG into the several layers forming the bacterial wall[24]. Beta lactam drugs can be divided into several subclasses including penicillins (e.g. amoxicillin, piperacillin, carboxicillin), cephalosporins (e.g. cefoxitin, ceftazidime, cefepime), monobactams (e.g. aztreonam, tigemonam) and carbapenems (e.g. doripenem, imipenem, meropenem). To withstand beta-lactam drugs, bacteria have evolved an array of enzymes called beta-lactamases, which can break the beta-lactam ring and thus disrupt the structural motif that enables beta lactams to bind their target. Several classes of beta-lactamases are now spreading across different bacterial species and, collectively, can mediate resistance against almost all beta-lactams on the market[24]. Consequently, some antibiotics maintain their efficacy only when combined with specific beta-lactamase inhibitors which, however, are not equally efficient against all beta-lactamases.

Aside beta-lactams, other cell wall inhibitors have been developed. Phosphomycin and cycloserine inhibit other enzymes involved in peptidoglycan synthesis, (namely MurA for phosphomycin, and *D*-Ala Racemase and *D*-Ala-*D*-Ala Ligase for cycloserine)[25,26]; vancomycin is a glycopeptide which binds a dimer of *D*-alanine present in the peptidoglycan filaments, preventing its access to those enzymes responsible for the cross-linking[27]; bacitracin binds the bactoprenol-prosphate, a phospholipid which eventually mediates the transfer of peptidoglycan units outside of the membrane, and thus bacitracin sequesters 'docking sites' for the cell wall components[28].

Inhibitors of cell wall synthesis are also present among antitubercular drugs, i.e. antibiotics with effective action against the very threatening *Mycobacterium tuberculosis* (MTB). Ethambutol and isoniazid interfere with the synthesis of the mycobacterial cell wall – which is significantly different from that of other bacteria[29] –. Ethambuthol blocks the two proteins embB and embC involved in the synthesis of arabinogalactan, a mycobacterial cell wall component[30]. Isoniazid is actually a prodrug that once converted in its active radical form can release other radicals and damage several pathways, including cell wall, but also DNA synthesis and respiration[31] .

A great number of antibiotics on the market act by interfering with bacterial protein synthesis, thereby arresting bacterial growth or even killing bacteria. Collectively, protein synthesis inhibitors were developed against all the four main stages of the bacterial translational process, i.e. initiation, elongation, termination and ribosomal recycling, but antibiotics on the market act mainly by inhibiting the elongation phase[32 22].

Aminoglycosides, tetracyclines and glycylcyclines are some different classes of antibiotics which bind to the 30S subunit of the bacterial ribosome. Aminoglycosides (e.g. kanamycin, tobramycin, streptomycin) bind to the 16S rRNA, near the so-called mRNA decoding site, where the correctness of the match between mRNA codons and respective tRNAs is verified. Tetracyclines (e.g. tetracycline, minocycline) and glygylcyclines (e.g. tigecycline) prevent the access to the ribosome of the ternary complex aminoacyl-tRNA – elongation factor – GTP [32,33].

The 50S subunit of bacterial ribosome is targeted by amphenicols (e.g. chloramphenicol), macrolides (e.g. erithromycin, azithromycin), ketolides (e.g. telithromycin), streptogramins (e.g. quinupristin and dalfopristin), lincosamides (e.g. clindamycin), oxazolidinones (e.g. linezolid, tedizolid) and pleuromutilins (e.g. retapamulin). All these drugs interact with the 23S ribosomal RNA, which has an essential function for the catalytic activity of the ribosome, but they don't bind all the same target site[32,34,35]. Macrolides and ketolides mainly act by occupying the exit tunnel of the ribosome, preventing the nascent polypeptides from being channeled away, thereby interrupting the translation process. Lincosamides, although partially overlapping their binding site to macrolides, mainly bind and interfere with the peptidyl-transferase center (PTC) of the ribosome, where the formation of the peptide bond is catalyzed. Like lincosamides, amphenicols and streptogramins all bind to the P-site of the ribosome, near the peptidyl-transferase center, impeding the addition of new amino acids to the nascent polypeptide chains[32,34,35]. Oxazolidinones stabilize a distinct conformation of a conserved nucleotide of 23S rRNA, thereby perturbing the correct positioning of tRNAs on the ribosome[33].

A particular protein synthesis inhibitor with a non-conventional mode of action is mupirocin, a pseudomonic acid; this molecule inhibits an enzyme called isoleucine-tRNA synthetase, responsible of transferring isoleucine to its corresponding tRNA, causing accumulation of leucine-devoid tRNAs and arrest of translation at the codons coding for leucine[36].

Antibiotics interfering with the synthesis of nucleic acids include riminofenazines, rifampicin and fluoroquinolones. Riminofenazines (e.g. clofazimine) bind the guanosine on the DNA, hindering the template function of DNA, therefore inhibiting transcription[37], although other mechanisms were also reported[38]. Transcription is also inhibited by rifamycins (e.g. rifampin, rifaximin), which bind and inhibit the bacterial RNA polymerase[39]. The class of fluoroquinolones (e.g. ciprofloxacin, norfloxacin), hampers the DNA synthesis by binding to topoisomerases, which are enzymes responsible to solve structures called supercoiled DNA[40]. Non-resolution of supercoiled DNA results in the arrest of DNA synthesis and eventually DNA rupture and bacterial death.

Some antibiotics on the market interfere with vital biosynthetic pathways. Sulfonamides (e.g. sulfadiazine, sulfanatoxizol) and thrimetoprim target two different enzymes in the biosynthetic pathway of the tetrahydrofolate[41,42], a crucial precursor molecule required for the synthesis of nucleotides and amino acids[43]. The antitubercular drug Bedaquiline inhibits mycobacterial respiration by blocking the ATP-synthase [38].

Finally, membrane-disrupting antibiotics are few and often used as last resort, as most candidate antibiotics with this mode of action failed clinical trials due to their toxicity towards eukaryotic cells. Daptomycin, Polymyxins and Gramicidins are three examples of such molecules on the market. Daptomycin is used as a last-resort antibiotic to treat infections by drug-resistant Gram-positive bacteria; this lipopeptide drug forms complexes with calcium ions, then such complexes form channels across the bacterial membrane leading to cation leakage and a deadly membrane depolarization[44]. Polymyxins are lipopeptides which bind to the lipopolysaccharide of Gram-negative membranes and insert into the lipid bilayer, causing rupture of the membrane and bacterial death[45]. Colistin is a last-resort antibiotic also known as polymyxin E, and because of its mode of action its action is limited to Gram-negative bacteria[46] . Gramicidins are short peptides with an alpha-helical structure, which form ionophoric channels across bacterial membrane, causing loss of vital ion gradients and killing bacteria in different ways[47]. Gramicidins have been shown to kill both Gram-negative and Gram-positive bacteria [47]. Currently, many antimicrobial peptides naturally produced by various sources are regarded as a source of membrane-disrupting drugs or, in some cases, of protein synthesis inhibitors (see further).

Table 1.1. Main classes and modes of action of antibiotics on the market.

Cell wall synthesis inhibitors		
Classes	**Binding target**	**Mechanism**
Beta-lactams	Penicillin-binding proteins (PBPs)	Inhibit the covalent joining of PG filaments.
Phosphomycin	MurA protein	Interference with synthesis of PG units
Cycloserin	d-Ala-d-Ala racemase; d-Ala-d-Ala ligase	
Vancomycin	d-Alanine dimer	
Bacitracin	Bactoprenol phosphate	Inhibit transport of PG building blocks across the membrane
Ethambutol	EmbB, EmbC proteins	Inhib. synthesis of arabinogalactan
Isoniazid	various	Generation of radicals – damage at DNA, lipids, respiration
Protein synthesis inhibitors		
Classes	**Binding target**	**Mechanism**

Tetracyclines/ Glycylcyclines	30S subunit -> 16S rRNA – near mRNA decoding site	Inhibit placement of aatRNA-EF-GTP
Aminoglycosides		Inhibit movement of mRNA-tRNA complex / induce mistranslations
Macrolides/Ketolides, Streptogramins, Lincosamides, Phenicols, Oxazolidinones, Pleuromutilins	50S subunit -> 23S rRNA, ribosomal exit tunnel, near PTC	block of elongation (inhibition of peptide bond formation)
Pseudomonic acids	Bacterial isoleucine tRNA synthetase	block of translation (depletion of aatRNA loaded with leucine)
Nucleic acid synthesis inhibitors		
Classes	**Binding target**	**Mechanism**
Riminofenazines	Guanosine (on DNA)	Inhibition of transcription
Rifamycins	RNA polymerase	Inhibition of transcription
Fluoroquinolones	DNA Gyrase, Topoisomerase IV	Inhib. supercoiled DNA resolution
Inhibitors of metabolic pathways		
Classes	**Binding target**	**Mechanism**
Sulfonamides	dihydropteroate synthase (DHPS)	Inhibition of synthesis of tetra-hydro-folate (THF)
Trimethoprim	dihydrofolate reductase (DHF)	
Bedaquiline	F_0/F_1 ATP synthase	Inhibition of bacterial respiration
Membrane disrupting antibiotics		
Classes	**Binding target**	**Mechanism**
Gramicidins	bacterial membrane (mostly Gram+)	Formation of channel/pores→ depolarization and/or membrane lysis
Daptomycin	outer membrane of Gram+ bacteria	
Polymyxins	LPS on bacterial membranes	

Antibiotic resistance and tolerance in bacteria

The selective pressure imposed by antibiotics caused the diffusion among bacteria of a plethora of molecular strategies to counteract such drugs, collectively referred to as mechanisms of antimicrobial resistance. Antimicrobial resistance is genetically determined. However, such resistance mechanisms are not only acquired via transmission of mutations across generations of the same bacterium. Some resistance genes (sometimes entire operons) coded by plasmids, integrons or transposable elements might be uptaken by bacteria from the environment, or transferred between bacteria, even phylogenetically far apart, through the "horizontal gene transfer" (HGT) mechainsms[48]. There is a multitude of resistance genes, enabling many different resistance mechanisms but, generally speaking, the microbial strategies to withstand current antibiotics mostly

consist in: i) drug inactivation or degradation, ii) protective modification of the drug target; iii) reduced permeability; iv) increased efflux of compounds[49–51] (Fig. 1.1 A-D).

In addition to these strategies, the formation of biofilms is another crucial feature shared by many microbes that contributes to protection from antibiotics (Fig. 1.1E). Biofilms may not fit in the strict definition of 'drug resistance' as the mechanism of biofilm formation has not been selected by drug-mediated evolutionary pressure; nevertheless, it has probably evolved as an inducible mechanism of adaptation to hostile environments and indeed provides bacteria with a strong defense against various drugs[51–53].

Drug inactivation. Many bacteria produce enzymes that degrade or irreversibly modify and inactivate antibiotics; examples include beta-lactamases and aminoglycoside-modifying enzymes[54,55]. Most of the resistance mechanisms to beta-lactam antibiotics involve the destruction of the drug by enzymes called beta-lactamases[50,54]. According to a widely used biochemical classification scheme[56], four main classes of different beta-lactamases are spreading among the most troublesome bacteria. These include penicillinase, cephalosporinases, extended spectrum beta-lactamases (ESBLs), metallo-beta lactamases (MBLs), and oxacillin-hydrolyzing enzymes (OXAs)[54]; each class in turn includes different enzymes with different specificities. Some cause resistance to almost every current beta-lactam drug, and it is not uncommon for ESKAPE pathogens to express many of these enzymes simultaneously. Aminoglycoside-modifying enzymes (AMEs) are able to inactivate aminoglycoside antibiotics by the addition of chemical groups to their structures. AMEs are classified based on the type of chemical reactions they catalyze, which can be phosphorylation, acetylation or adenylation[50] .

Target modification. Besides the drug itself, the target of the antibiotics can also be modified, in order to impede the access of antibiotics. Examples include several different classes of methylases, (such as ermA-B, efmM, rmta-d, cfr, found in a variety of pathogens) which methylate the ribosomal RNA and hamper the binding of several protein synthesis inhibitors, including streptogramins, linezolid and aminoglycosides[49,50]. Alternatively, bacteria can entirely replace the target with a different one, with lower affinity for the drug. The *mecA* gene, for instance, codes for a penicillin-binding protein (PBP) which binds penicillins with much lower affinity compared to other PBPs and is the most expressed PBP in methicillin-resistant *Staphylococcus aureus* (MRSA)[57]. Also, the gene products of the *van* operon cooperate in a pathway that ends with the substitution of a dimer of d-Alanine in the peptidoglycan chain with a d-alanine-d-lactate dimer; while the 'original' d-Ala-d-Ala dimer is the target of vancomycin, the new dimer has no affinity for such antibiotic and confers vancomycin resistance in the dangerous vancomycin-resistant *S.aureus* (VRSA) and vancomycin-resistant *enterococcus* spp. (VRE)[58].

Reduced permeabilization and increase efflux of drugs. Reduced permeability to molecules is another powerful resistance strategy against antibiotics. Hydrophilic antibiotics like beta-lactams and fluoroquinolones often cross the outer membrane of Gram-negative bacteria using water filled membrane channels called porins. Many Gram-negative bacteria can downregulate the synthesis of porins or start synthesizing porins with lower permeability[59]. In addition, bacteria have several systems to actively expel the drug from the cell. Different families of bacterial efflux pumps have been discovered, including the superfamilies of major facilitators (MFS), the ATP-binding cassette (ABC), resistance-nodulation-division (RND), small multidrug resistance (SMR), and multidrug and toxic compound (MATE)[49,54].

Biofilm formation. Biofilms are complex microbial communities which live as a thin layer adherent on biotic or abiotic surfaces, embedded in a complex extracellular matrix, where channels across the matrix allow the passage of water, nutrients and excreted molecules[52,53,60]. The formation of biofilms is a reversible phenotypic trait of bacteria and some fungi, which form biofilms in response to stress signals, and eventually go back to a non-adherent, planktonic state. Therefore, it may not derive from a change in the microbial genetic background selected by the evolutionary pressure of antibiotics. However, in the fight against drug-resistant pathogens, biofilms must surely be minded: microbes into biofilms are 10- to 1000-fold less susceptible to most antibiotics than planktonic microbes, and they are also more resistant to immune cells, disinfectants, phages and other damaging agents, even UV rays[60]. In fact, the vast majority of hard-to-treat infections involve microorganisms in the form of biofilms; plus, drug-resistant microbes cause nosocomial infections mostly because they form biofilms on abiotic surfaces, such as catheters or surgery equipment, that are extremely difficult to eradicate[52,60,61].

The extracellular matrix is a chemically heterogeneous substance, containing polysaccharides, proteins, lipids and nucleic acids, and generates a variety of microenvironments into the biofilm. For instance, the deepest niches of the biofilm may have a lower oxygen concentration and a different pH, and this is reflected in different metabolic states of cells. Bacteria that are deeply embedded into the matrix can enter a state of metabolic quiescence called persistence, where they lose susceptibility to almost all the few antibiotics that can reach them[52]. The biofilm formation occurs in a stepwise process, where the transition to the different phases is mediated by microbial signaling molecules such as the signal molecules of the ‘quorum sensing’ (QS) system[60,62]. The regulation of such transition pathways, therefore, could help in devising therapeutic strategies. However, most of the discovered antibiofilm agents are still in the initial stage of investigation, and several promising anti-biofilm agents have failed clinical trials due to issues of toxicity and scarce efficacy *in vivo*. Therefore, despite the increasing knowledge on biofilms and a long list of anti-

biofilm agents discovered so far, there is no specifically-antibiofilm compound that is currently on the market[63,64].

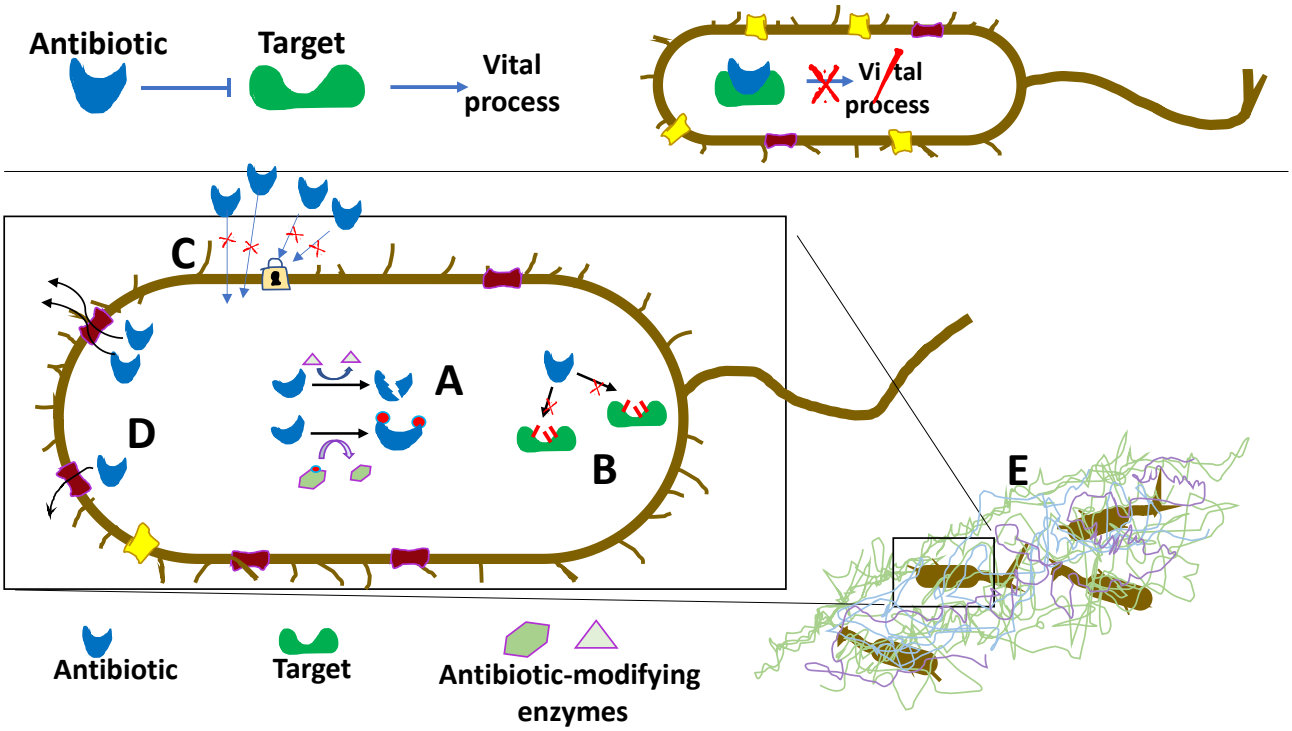

Figure 1.1. Main bacterial strategies to withstand antibiotics. A) enzyme-mediated drug inactivation, B) target modification/replacement, C) decreased permeability to the drug, D) increased drug efflux, E) Biofilm formation.

ESKAPE pathogens and *E. coli*

Among those pathogens listed by the WHO as 'top-priorities' there is a subgroup of bacteria which is causing most of the troublesome infections across the hospitals of the world. Such subgroup, encompassing both Gram-positive and Gram-negative bacteria, was named with the acronym "ESKAPE" in 2008 [65], where "ESKAPE" stands for *Enterococcus faecium*, *Staphylococcus aureus* (Gram-positives) , *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Gram-negatives). This PhD Thesis discusses about the antibacterial action of certain antimicrobial peptides, focusing on their action against ESKAPE bacteria and *E.coli*, which is another most common bacteria causing a variety of infections[65,66].

ESKAPE bacteria collectively became responsible for the majority of nosocomial (i.e. hospital-acquired) infections worldwide, especially in Intensive Care Units, with a significant role in overall global morbidity and mortality. Overall, ESKAPE pathogens can cause infections of the bloodstream, respiratory tract, urinary tract, skin and soft tissues as well as gastrointestinal and

osteoarticular infections, endocarditis and meningitis, with each bacteria preferentially, but not solely, associated to two to six of these infection types[65,66] .

ESKAPE pathogens have drawn the attention and concerns of physicians due to their ability to 'escape' most antimicrobial treatments, thanks to their impressive array of molecular resistance mechanisms. In addition, such resistance is primarily associated with nosocomial environments, but no longer exclusively[65]. According to Louis B. Rice, besides the threat they pose, these bacteria are important because they represent paradigms of pathogenesis, transmission, and resistance. This means that understanding how they infect, damage the host, resist to drugs and transmit their resistance mechanisms, will help devising therapeutic strategies applicable to many other bacteria[65]. All these bacteria can form biofilms which help them to withstand high doses of several drugs and persist on abiotic surfaces such as surgical instruments or catheters, surviving disinfection procedures and eventually causing nosocomial infections. Plus, some species of ESKAPE such as *A. baumannii* and *S. aureus* express a particularly high number of virulence factors, worsening their virulence or pathogenicity [67–69] .

Most of the clinical strains of these bacteria, isolated from patients, express an impressive variety of antibiotic resistance determinants; many of them are intrinsic to the bacterial specie while others were acquired via mutations and horizontal gene transfer[66]. The variety of antibiotic resistances in each of these pathogens is too large to be treated here and is beyond the scope of this thesis. However, each of the resistance determinants mentioned in the previous paragraph is present in at least one of the ESKAPE pathogens, along with many others. As a results, there are clinical isolates of ESKAPE pathogens which can easily survive nearly all the antibiotic treatments available, such as vancomycin-resistant *Enterococci* (VRE)[70], vancomycin-resistant *S. aureus* (VRSA)[71], or carbapenem-resistant *Enterobacteriaceae* (CRE)[72]. For many drug-resistant ESKAPE strains, only few "last resort" treatment options are available, such as colistin (for Gram-negatives) and daptomycin (against Gram-positives); and bacteria with defense mechanisms against these drugs were already found[73–75].

Escherichia coli, instead, is not part of the ESKAPE bacteria but pathogenic *E. coli* strains are among the WHO's priority pathogens, particularly its carbapenem-resistant strains[76]. Different *E. coli* strains are capable of forming biofilms and can cause gastrointestinal infections, urinary tract infections and even cause neonatal meningitis[77,78]. Moreover, there are some concerning virotypes of *E. coli* expressing strong enterotoxins, such as the enterohemorrhagic O157 strains, implicated in some outbreaks of hemorrhagic diarrhea across the world[79]. In addition, the *mcr-1* gene determining colistin resistance was already found in *E. coli* isolates[80]All these characteristics have made *E. coli*

a pathogen worth of attention, for which new antibacterial therapies are needed. For this reason, and because it is a long-studied model organism, susceptible to some AMPs that are studied in our lab, *E. coli* was the organism mainly used in this thesis work.

New strategies to fight AMR

As mentioned, to address the problem of XDR and PDR microbes, novel classes of antibacterial agents are seriously needed. The clinical pipeline of antibiotics in 2019 mostly contained derivatives of established drug classes, with only a small proportion of novel-class drugs, represented by monoclonal antibodies and phage-derived products[14]. Luckily, according to a recent analysis, the preclinical pipeline contains a more encouraging variety of approaches (Fig. 1.2), including direct agents – most of which directed towards new targets –, but also vaccines, antibodies, antibody-drug conjugates, immune system modulators, microbiome modulators, phages and phage-derived products and anti-toxin compounds directed to neutralize virulence factors[15].

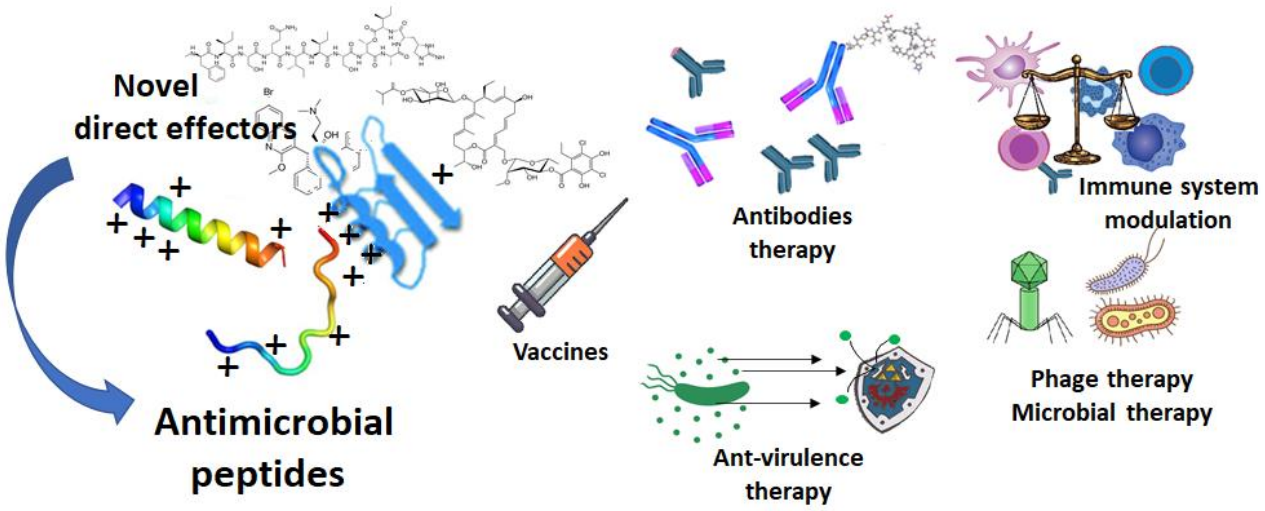

Figure 1.2. Overview of the therapeutic approaches currently under preclinical studies to tackle antimicrobial resistance.

Representatives of both the broad categories of direct antimicrobial agents and immune system modulators, the antimicrobial peptides (AMPs) have raised increasing interests. Within this heterogenous group of molecules, studies have found peptides inhibiting the growth of drug-resistant bacteria[81,82], biofilm inhibitors [83,84] and modulators of the immune systems[85,86]. In addition, some AMPs have shown synergistic activities with conventional antibiotics [87,88] . Due to such promising findings in the last years, AMPs have been raising increasing research efforts.

The potential of antimicrobial peptides

Nature itself could harbor a variety of solutions to the antibiotic resistance crisis. Such variety of ecosystems and organisms surely harbors undiscovered antimicrobial molecules, and some may circumvent the mechanisms of drug resistance currently spreading across bacteria. Antimicrobial peptides (AMPs) are natural molecules attracting great research efforts all over the world. These molecules were shown to be crucial components of the innate immune system of numerous organisms - hence their other denomination 'host defence peptides' (HDPs) - and novel AMPs are continuously discovered in living organisms. During the past decades, AMPs were discovered in animals, fungi, plants and bacteria, exerting their protective function with different modes of action and target specificities, spanning across bacterial and fungal pathogens but also enveloped viruses, and multicellular parasites[81,89–91] Besides their direct antimicrobial function, HDPs of multicellular organisms were often shown to modulate innate immunity in several ways; plus, some AMPs showed anti-cancer and wound-healing properties[92,93] , and perhaps further functions of AMPs are yet to be discovered. A great number of studies have highlighted the potential of AMPs to be employed as antibiotics or as adjuvants for current antibiotic therapies[89] . However, only very few peptides are currently being used in humans, such as vancomycin (actually a glycopeptide), colistin, daptomycin (lipopeptides), bacitracin, and gramicidin. Interestingly, these are produced by different bacteria, not simply translated from a messenger RNA, but instead synthesized as secondary metabolites through other biosynthetic pathways[94–96] . AMPs often fail to pass – or even enter – clinical trials, mostly due to issues related to toxicity, bioavailability or stability in biological fluids. Furthermore, the large-scale production of some peptide antibiotics can be quite complex [97,98]. However, AMPs still raise great hopes for the researchers of new classes of antibiotics, especially those AMPs that are ribosomally synthesized, which can be more suitable for industrial-scale recombinant production. Extensive research efforts are dedicated to discover and study natural AMPs as well as to design their chemically-optimized variants with better pharmaceutical properties[99–102]. The clinical pipeline is now beginning to contain some AMPs[103] and more are surely to come.

Antimicrobial peptides are for the large majority cationic amphipathic molecules, generally 12 to 100 amino acids in size, with several basic residues across their sequence (mostly arginine or lysine), and a significant proportion of hydrophobic residues [81,104]. Anionic antimicrobial peptides also exist[105] but are much less common and less studied, and actually go beyond the scope of this thesis. Most AMPs get close to bacterial membranes via electrostatic interactions with the

negatively charged phospholipids on bacterial membranes, then most of them kill bacteria by perturbing the membrane structure, causing its rupture and bacterial lysis or lethal leakage of ions [91]. A number of AMPs have intracellular targets but also in this case, their cell uptake begins with their approaching to the membrane via electrostatic interactions, which are also partially responsible for their target binding. The lower negative charge of eukaryotic membranes compared to prokaryotic membranes is partly responsible for the preferential activity of AMPs towards bacteria. However, toxicity towards eukaryotic cells is not uncommon and is generally higher for those AMPs with a membranolytic mechanism[106,107] .

Structure of antimicrobial peptides

Thousands of AMPs have been discovered nowadays, and can be found in several bioinformatic databases[80,108]. As the numbers of discovered peptides and relative studies increases, several databases appear where the antimicrobial peptides are classified according to different parameters.

Perhaps the main structural classification scheme according to their secondary structure (Fig. 1.3A), sorts antimicrobial peptides in i) alpha-helical peptides, ii) peptides with beta-sheet structures, iii) peptides with both alpha-helices and beta-sheets structures and iv) peptides with extended conformation (without estensive alpha-or beta- structures) . However, more categories will likely be adopted to sort cyclic peptides or other peptides with different topological properties. Also, different classification schemes were already been proposed, based on the covalent connections between the residues[109 110].

Alpha-helical peptides

Peptides with alpha-helical conformations (Fig. 1.3A) represent the largest and best-studied group of AMPs [111]. Examples include the long-studied melittin from honeybee[112], magainins from the frogs[113], the human peptide LL-37[114] and hundreds of others notable members. Most alpha helical peptides mainly kill bacteria by damaging bacterial membranes[111]. While some alpha-helical peptides have a more rigid structure, other peptides assume an alpha helical structure only upon interaction with hydrophobic or amphipathic structures like bacterial membranes; such inducible alpha-helix structure relates to a generally lower toxicity compared to more rigid alpha helices[91] . The combination of helix length and orientation of charges and hydrophobic residues forms a variety of helical structures with a consequent range of different activities[110].

Beta-sheet peptides

Beta-sheet containing peptides (Fig. 1.3A) often have more than two beta-sheets and generally two to five disulfide bonds to stabilize their structure. They also have a mainly membranolytic

mechanism, although an increasing number of peptides has recently been shown to have also an intracellular target[91,110]. Examples of beta-sheet peptides include human alpha-defensins[115], some of which bind the peptidoglycan precursor lipid II[116], tachyplesin from horseshoe crab[117] which can also bind the minor groove of DNA and may interfere with DNA–protein interactions, and the lactoferricin peptide, derived from the protein lactoferrin[118]. Examples of peptides containing both alpha-helices and beta-sheets include many plant and invertebrate defensins[119,120].

Extended peptides

Extended AMPs (Fig. 1.3A), which mostly do not fold into regular secondary structures, nor in water nor in membranous environment[91], often contain a high proportion of determined residues, like proline (e.g. bactenecins, apidaecins, drosocin[121]), histidine (e.g. histatins[122]), glycine (e.g. serrulin[123]) or tryptophan (e.g. tritripticin, indolicidin[124,125]). This class of AMPs includes peptides mainly displaying intracellular, non-membranolytic mechanism of action. For example, proline-rich AMPs (e.g. bactenecins, apidaecins) act by targeting the ribosome and interfering with protein synthesis [126] (see further); the porcine peptide PR-39 was shown to target the proteasome[127,128]; and indolicidin, besides having a mechanism at the membrane level, can also covalently bind DNA, likely perturbing its replication or transcription[129,130].

However, despite their mainly intracellular action, some peptides can also perturb the bacterial membranes[131,132], and membranolytic peptides are also included in this category.

Peptides with complex topologies

In the aforementioned categories, the backbone of AMPs has a linear topology, albeit arranged in different 3D structures. However, several peptides were discovered whose backbone have circular or more complex topologies (Fig. 1.3B); these include, for instance, daptomycin, polymyxins, theta-defensins**,** 'lasso' peptides (e.g. microcin J25)**,** the 'cycline knot peptide' cyclotides (e.g. kalata B1) lantibiotics (e.g. nisin A) and other peptides - many of which have excellent antimicrobial properties[110,133].

Structural classifications and structure-activity relationships (SAR) studies can help to elucidate the main mechanisms of action of AMPs and provide a precious help in the rational design of synthetic antimicrobial peptides, a goal towards which many laboratories have already pointed with promising results[134,135].

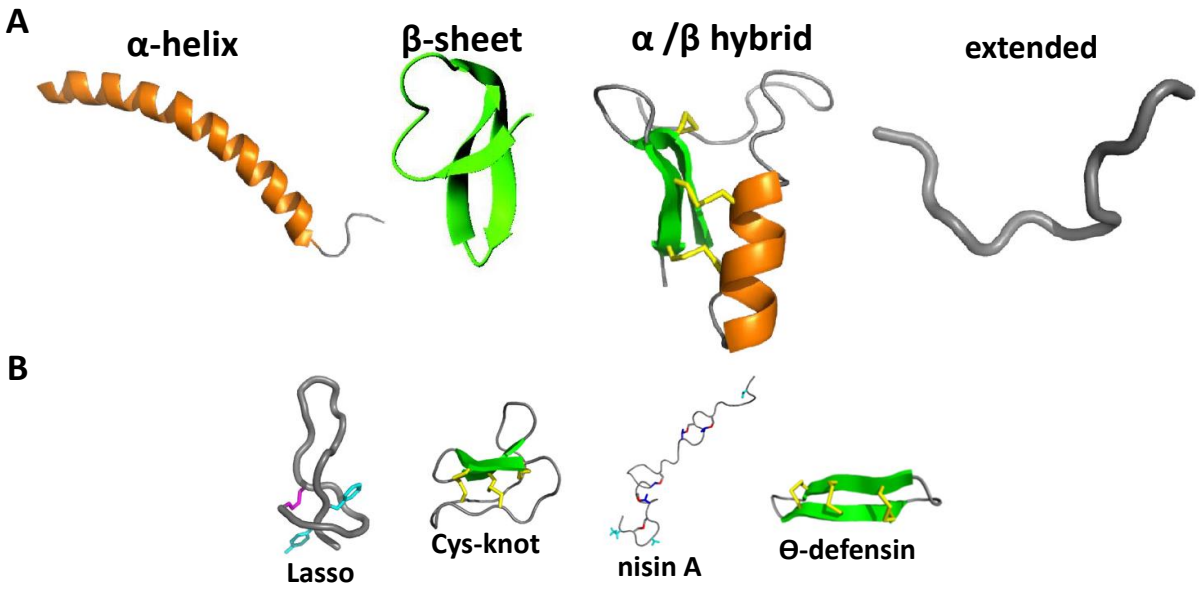

Figure 1.3. Structural diversity of antimicrobial peptides. Representative examples of the four 'classic' structural classes (A) and some of the more complex topologies (B). A) On top, from the left, the α-helical, β-sheet, α/β- and extended conformations are exemplified by the peptides LL-37, human α-defensin, phormicin and indolicidin, respectively. B) On bottom, from the left: the lasso peptide as microcin J25, with a head-to-side cycle; the 'cystine-knot' peptide kalata B1; the lantibiotic nisin A with five thioether bonds; a Θ–defensin, cyclic β–sheet peptide with parallel disulfide bridges. *Image adapted from J.Koehbach & D.J.Craik, (2019) and from G. Wang, (2014).*

Mechanisms of action of AMPs

Disruption of microbial membrane

The membrane-permabilizing antimicrobial activity of AMPs has been traditionally explained via three main models (Fig. 1.4). In general, peptides initially bind to the membrane through electrostatic interactions and lie parallelly to the bilayer until they reach a threshold concentration, after which the peptides start to behave in different ways, which have been explained for many years via three mechanistic models, i.e. the 'barrel stave', the 'toroidal pore' and the 'carpet-like' model[91]. In the barrel-stave model, peptides multimerize together, then enter into the membrane as a multimer, perpendicularly to the bilayer, forming a transmembrane channel with their hydrophilic sides facing the channel lumen[91]. In the toroidal pore model, peptides start to bend within the bilayer, with their hydrophilic moieties 'dragging' the charged phospholipid heads of outer and inner membranes to fold inward, and ultimately form a transmembrane pore whose lumen is surrounded by the hydrophilic AMP residues alternated to the phosphates of phospholipids [91,136]. In the carpet model, peptide first lay parallel to the membranes, with their hydrophilic moieties mainly towards the solution, then cause local weakness and then disrupt the membrane by forming AMP-

coated micelles, more like in a detergent-like fashion[90,91] . However, the great variety of AMPs did not always fit well in these traditional schemes andfurther mechanisms have recently been proposed (Fig. 1.4). These include the "disordered toroidal pore" , the "interfacial activity model" for those peptides with a so called "imperfect amphipathicity", or the "anion carrier" mechanism for some peptides interacting with the membrane causing its depolarization [91] .

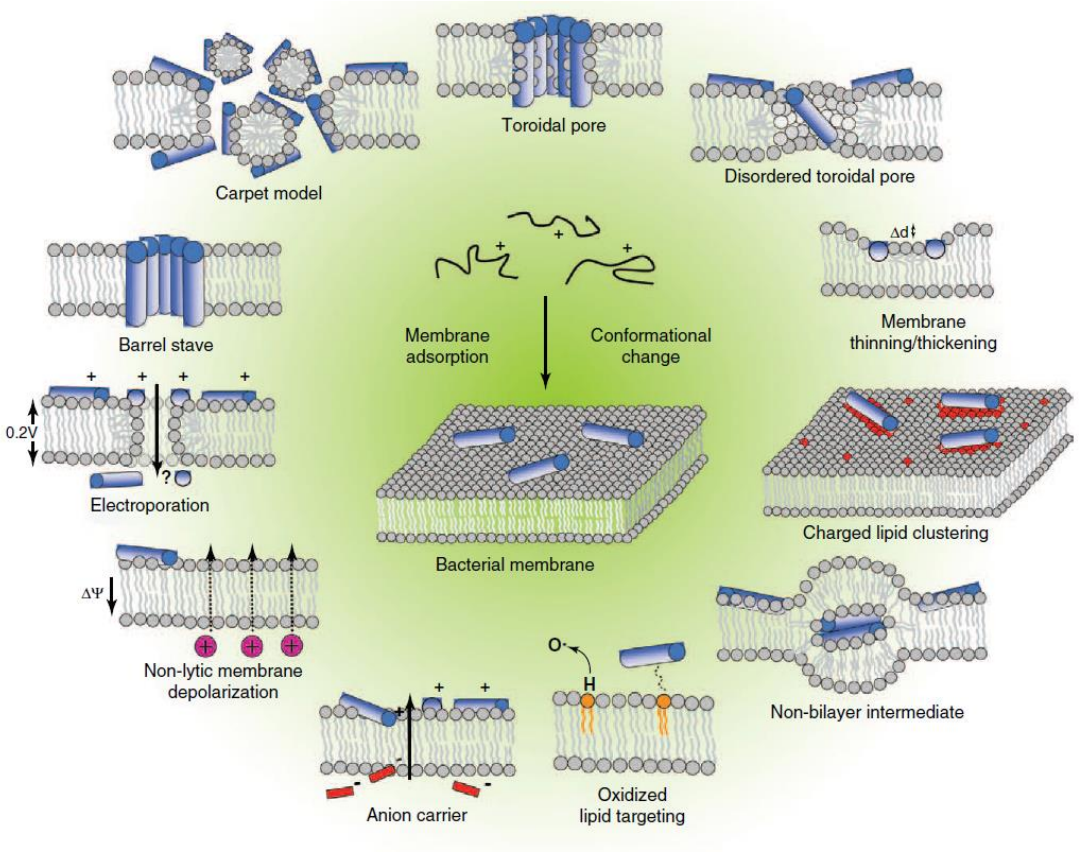

Figure 1.4. Overview of the diversity of bactericidal mechanisms of AMPs targeting the bacterial membrane. Image from L.T. Nguyen et al (2011).

Intracellular mechanisms

Although most AMPs were found to kill bacteria via perturbation/disruption of bacterial membrane, an increasing body of evidences has demonstrated that some AMPs can exert an intracellular action as primary or secondary mechanisms[107,136]. These peptides likely access to the cytoplasm in a 'self-promoted uptake' – e.g. by forming transient pores on the membrane, too short-lived to cause lethal damage[137] –, or by being uptaken by membrane transporters[126,138,139]. Perhaps most of the intracellular AMPs discovered so far interfere with protein synthesis or DNA synthesis, but some studies have discovered AMPs hindering other processes, like cell wall-synthesis, protein folding or protein degradation[107,136,140]. For instance, buforin II, a peptide derived from a species of toad, binds

bacterial DNA and RNA, interfering with their metabolism[141] and the tachyplesin I from the japanese horseshoe crab was shown to bind the minor groove of DNA with no membrane disruption[117]; the proline-rich peptides apidaecin from honeybee and Bac7(1-16) - a N-terminal fragment of the bovine peptide Bac7 - , were shown to inhibit translation by binding in two different sites on the ribosomes[142,143], and the porcine cathelicidin-derived peptide PR39 was shown to inhibit protein synthesis and perhaps cause protein degradation, thereby indirectly disrupting also DNA and RNA synthesis[144]. Additional examples include the lantibiotic mersacidin produced by *bacillus* spp., which was shown to inhibit bacterial cell wall synthesis by binding the cell wall precursor lipid II[145]; or microcin J25, which disrupts transcription by binding to the biggest subunit of RNA polymerase[146]**.**

AMP vs Biofilms

Most AMPs have shown efficacy against planktonic microbes but are not as much efficient in the treatment of biofilm infections. Since biofilms are heterogenous in both their extracellular matrix and their cell population (in terms of species and/or metabolic state), it is unlikely that a single agent alone could eradicate biofilm infections. It was shown that some AMPs preferentially damage bacteria in biofilm but cause only mild damage to planktonic microbes[147] ; this further suggests that combinatorial therapy with different AMPs is likely a valuable approach to treat bacterial infections.

Examples of antibiofilm mechanisms of AMPs include i) the degradation of extracellular matrix, ii) penetration into the matrix and action on bacterial membrane, iii) inhibition of bacterial signaling systems such as quorum sensing (QS) systems, and iv) inhibition of expression of genes responsible of biofilm formation[83,84].

The human peptide LL-37, the chicken peptide Cath-2 and the mouse peptide CRAMP were shown to kill *P. aeruginosa* cells within pre-formed biofilm; plus, CRAMP was also able to strongly reduce the whole biofilm biomass, even though it had weak activity against planktonic cells[147]. Nisin, and the frog skin peptide esculentin(1-21) could reduce the viable cells in biofilms of MRSA and *P. aeruginosa*, respectively[148,149]. In addition, the fish peptides piscidin-1 and piscidin-3 act synergistically with Cu^{2+} ions to eradicate extracellular matrix in biofilms of *P. aeruginosa*[150].

Multiple mechanisms

One of the most promising features of antimicrobial peptides is that they exert their antimicrobial action though multiple mechanisms, and bacteria have a harder time in developing molecular mechanisms to counteract every different function[89]. The ribosomally-synthesized peptide Nisin, for

instance, has five different mechanisms involved in bacterial killing, including inhibition of cell wall synthesis, pore formation dependent or independent on the binding to lipid II, and activation of autolytic enzymes[89]. The difficulty for bacteria to develop resistance to nisin can be proved by the fact that nisin has been used for decades as a food preservatives, with very little evidence of stable and transmissible resistance[151]. The extended AMP indolicidin was shown to act at the membrane level, operating as an anion carrier[152,153], but was also shown to make covalent and non-covalent interactions with DNA[130]. Such multiple mechanism was so promising that one synthetic derivative of indolicidin named omiganan has now entered phase II clinical trials[154]. Also, the bacterial lasso peptide Microcin B25 can kill Gram-negative bacteria via inhibition of RNA polymerase[155], and inhibition of bacterial respiration[156,157]**]**.

Besides the action directed on two or more targets, the ability of many AMPs to modulate the host's immune system can also be considered one further mechanism of (indirect) antimicrobial action. Considering the action on the host's immune system, a great number of animal AMPs have a multiple mechanism of action. Several AMPs, including human defensins and cathelicidins, were found to dialogue with innate immune system by acting as chemokines for phagocytes, activating bactericidal mechanisms in phagocytic pathways and inhibiting or enhancing proinflammatory signals[85,158,159]. For instance, the human cathelicidin LL-37 performs several immune functions including attracting leukocytes, mediating the release of cytokines such as IL-1β and IL-8 and binding bacterial lipopolysaccharide (LPS), thereby reducing LPS-mediated inflammation[158,160] . Human beta-defensin 1 (HBD1) induces production of cytokines including TNF-α and IL-6, upregulates costimulatory CD80, CD86, and CD40 receptors and enhances T-cell proliferation mediated by dendritic cells[161]. Interestingly, modulation of immune system is not something limited to animals AMPs; the bacterial lantibiotic nisin, for instance, apparently decreased the levels of TNF-α in mice[162] and was shown to activate human neutrophils by stimulating the formation of neutrophil extracellular traps (NETs)[163] .

Strength and weaknesses of antimicrobial peptides

It is largely believed that some antibacterial drugs of the future may be derived from antimicrobial peptides. AMPs have showed appealing advantages over conventional antibiotics, and many research efforts are devoted to overcome the weaknesses of these new potential drugs of the future. One major advantage of the use of antibiotics based on AMPs may derive from their mode of action which, generally, induces resistance in microbes at a lower rate, compared to traditional antibiotics[164,165]. Two factors can explain this property: firstly, many AMPs interact with the

bacterial membrane instead of a specific protein (or nucleic acid) target, and mutations countering such unspecific membrane lysis are harder to achieve than mutations in a single specific target; secondly, many AMPs have more than one bacterial target.

Another advantage of AMPs is, of course, the interaction of AMPs with the immune system. Some AMPs are able to neutralize LPS[166] whereas some traditional antibiotics can lead to an increase in the release of LPS, increasing the risk of sepsis[167]. Another strength of some AMPs is their broader spectrum of activity usually encompassing Gram-negative and Gram-positive bacteria, and sometimes even viruses or parasites[168]. In some cases, some AMPs might be used to treat co-infections.

AMPs also possess some disadvantages which hamper their development as therapeutic agents[169]. Firstly, several studies reported toxicity of AMPs against eukaryotic cells and several membranolytic AMPs display also significant hemolytic activity[170–172]. This may not be just a problem for eukaryotic cells; in fact, some broad-spectrum AMPs may seriously damage the bacteria of the human microbiota[169]. Secondly, AMPs often face problems of bioavailability, e.g. scarce serum stability, degradation by proteases, rapid clearance in the body, or reduced activity in salty environment[169,173]. A third, very important issue which has been brought to attention is the chance that therapies with AMPs may promote a microbial cross-resistance against many endogenous host defense peptides, severely neutralizing part of the host's innate immunity[174,175]. Finally, another concern is given by difficulties in an industrial-scale AMP production[164,169] .

Countless chemical optimization approaches are being studied to address cytotoxicity and stability issues, including 'fine-tuning' of the amino acid residues,[100,176], peptidomimetic strategies[177] or combination therapies, looking for synergies with in-use antibiotics allowing to keep AMPs at low concentrations[88,178,179]. Some promising results also came from the research in the AMP production technologies [98,180,181], albeit an industrial-scale level seems still far to reach.

While the research in optimization and production of AMPs goes on, laboratories continue also to discover and characterize new peptides, both to find suitable drug hits, and to gain useful hints from their sequence and structures, activity, toxicity and other parameters, in order to increase data available to drive a rational design of peptide antibiotics.

In this continue research of new antimicrobial peptides, a few decades ago a subgroup of AMPs has gained attention of the researchers due to their generally low cytotoxicity profile; these are the proline-rich antimicrobial peptides (PrAMPs), which have been the starting point of this thesis work.

Proline-rich antimicrobial peptides

Proline-rich antimicrobial peptides or PrAMPs represent a subset of AMPs, mainly active against Gram-negative pathogens, which have the appealing property of a reduced cytotoxicity against eukaryotic cells[182–185]. From the discovery of apidaecins and bactenecins in the late '80s[186,187], other peptides with certain similarities were found in several animal species (Tab. 1.2), mainly (but not only) insects, cattle animals, crustaceans and cetaceans [107,126,188,189]. Such peptides, even the ones found in very distant animal species, might have differed significantly in their overall sequences, but they all shared the following features: **i)** a high content of proline, generally 25-30% or more of the peptide's residues, **ii)** an overall positive charge, mainly given by arginine or lysine residues, which often form short sequence motif with proline, and **iii)** the lack of significant permeabilization of bacterial membranes at their active concentrations (MIC) [188]. The recurrence of these elements in such phylogenetically distant animals, despite the differences in the structures of the coding genes and the primary amino acid sequences, suggests that these peptides could be the result of a convergent evolution [188].

Table 1.2. Examples of insect, mammalian and crustacean PrAMPs.

Organism	Name	Sequence
Apis mellifera	Apidaecin 1A	GNN**R**P**P**VYI**P**Q**P**R**PP**H**P**R**P**I
	Abaecin	YV**P**L**P**NV**P**Q**P**G**RR**P**F**P**TF**P**GQG**P**FN**P**K**P**I**K**W**P**QGY
Pyrrhocoris apterus	Pyrrhocoricin	VD**K**GSYL**P**R**P**T**PP**R**P**IYN**R**N
Oncopeltus fasciatus	Oncocin	VD**K**P**P**YL**P**R**P**X**PP**R**R**IYNN**R**
Palomena prasina	Metalnikowin-I	VD**K**P**P**DY**R**P**R**P**R**P**P**NM
Drosophila melanogaster	Drosocin	G**K**P**R**P**P**YS**P**R**P**TS**H**P**R**P**I**R**V**R**R**EALAIED**H**LTQAAI**R**P**PP**ILP**A**
Sus scrofa	PR-39	**RRR**P**R**P**P**YL**P**R**P**R**PPP**FF**PP**R**L**PP**R**I**PP**GF**PP**R**F**PP**R**F**P**
Bos Taurus	Bac5	**R**F**R**P**P**I**RR**P**P**I**R**P**P**FY**P**P**F**R**P**P**I**R**P**P**IF**P**P**I**R**P**P**F**R**P**P**LG**P**F**P**
	Bac7	**RR**I**R**P**R**P**P**R**L**P**R**P**R**P**R**P**L**P**F**P**R**P**G**P**R**P**I**P**R**P**L**P**F**P**L**P**F**P**R**P**G**P**R**P**I**P**R**P**L
Hyas araneus	Arasin 1	S**R**W**P**S**P**G**R**P**R**P**F**P**G**R**P**K**P**I**F**R**P**R**P**CNCYA**PP**C**P**CD**R**W**RH**

Proline residues and cationic residues are highlighted in bald black and bald red, respectively. The eleventh residue of oncocin is not clearly determined and is indicated as an 'X'.

Most data about their mode of action comes from studies on insects and mammalian PrAMPs, particularly from the insect PrAMPs apidaecin, drosocin, pyrrhocoricin and oncocin (from the

insects *Apis mellifera*, *Drosophila melanogaster*, *Pyrrhocoris apterus* and *Oncopeltus fasciatus*, respectively), and from the mammalian cathelicidin-derived Bac5, Bac7 (from *Bos taurus*) and PR-39 (*Sus scrofa*) peptides [126,188].

Some of the first experiments with PrAMPs suggested that their mechanism of action did not involve membrane disruption but rather a non-permeabilizing action directed towards cytoplasmic bacterial targets. The first permeabilization assays with apidaecin suggested that it did not permeabilize the membrane of *E.coli* and its D-stereoisomer did not have inhibiting activity on bacteria, consistently with the presence of an intracellular target[190]. This hypothesis has now been confirmed for several PrAMPs, and it is now known that PrAMPs exert their action mainly into the cytoplasm [131,191,192], slowly diffusing through outer membrane of Gram-negative bacteria and being internalized in to the cells by the bacterial membrane transporter SbmA/BacA[138] and, to a lesser extent, by the transport system Yjil/MdtM [139]. The exploitation of these transporters partly explained the more prominent effect of these PrAMPs towards those Gram-negative bacteria which express these peptide transporters

Nowadays, the main intracellular targets of many PrAMPs are known. In one of the first experiments to find apidaecin's intracellular targets, metabolic labeling assays to detect the incorporation of radioactive thymidine, suggested an inhibition of protein synthesis[191]. Few years later, co-immunoprecipitation assays and structural studies suggested an interaction with the chaperone DnaK [193,194]. However, as bacterial mutants lacking functional DnaK were still susceptible to oncocin, apidaecin and the Bac7(1-35) peptides, DnaK was considered a secondary target not mainly responsible for bacterial killing [193,195].

Subsequently, the groups of Scocchi and Hoffmann, independently and through different experimental procedures, showed that inhibition of protein synthesis was the main mode of action adopted by both insect apidaecin and oncocin and mammalian Bac7 peptides[195–197] (Fig. 1.5). Further confirmations and structural insights of this mechanism came from crystallographic studies, confirming the validity of the proposed mechanism[142,143,198,199].

Structural data reveal that most PrAMPs insert into the ribosomal exit tunnel, the space through which the nascent polypeptides are extruded, with their N-terminal residues inserted deeper into the tunnel and their C-termini located closer to the cytoplasm (Fig. 1.5A). The position of PrAMPs into the tunnel is stabilized by several hydrogen bonds and stacking interactions, many of which are with bases of the 23S rRNA[126,143,198,200]. X-ray crystallography data also revealed that some of such interactions are conserved across PrAMPs. For instance, the stacking interactions between Arg9 and Tyr6 with two different cytosines of the 23S rRNA are conserved across three insect PrAMPs

[198,199]; in addition, there are equivalent interactions in the PrAMP fragment Bac7(1-16) made by the residues Arg9 and Arg 12[143,198].

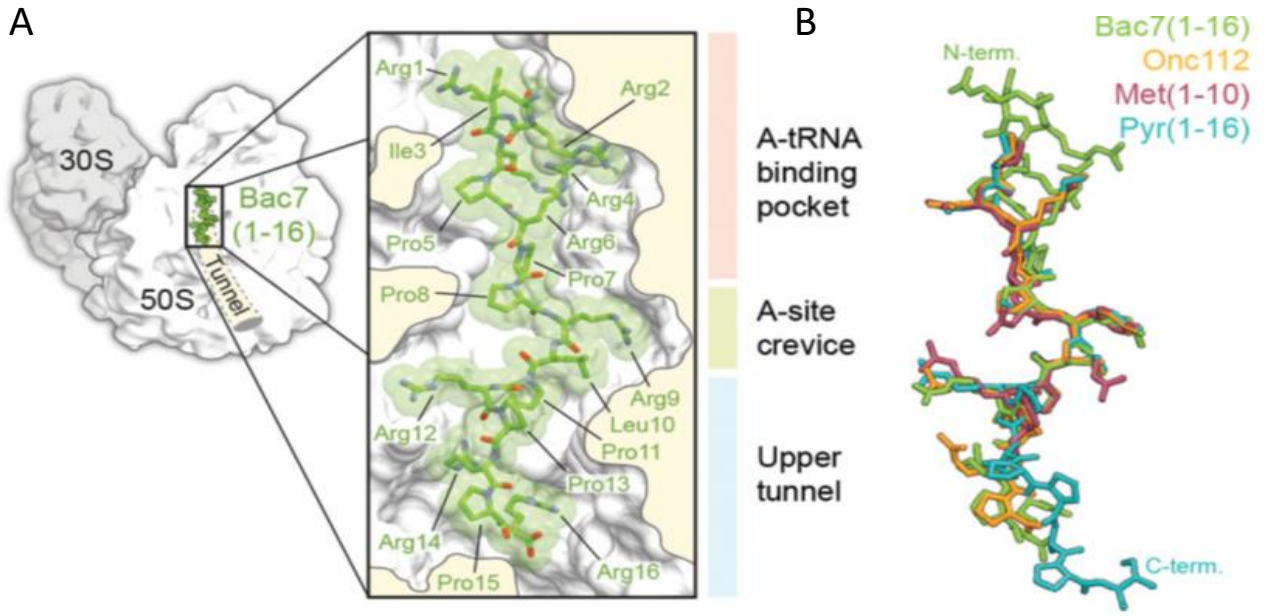

Figure 1.5. Structural details of the binding site of four known PrAMPs. A) Structure of Bac7(1-16) in the ribosomal exit tunnel. B) superimposition of the structures of Bac7(1-16) Oncocin112, Metalnikowin.-1 and Pyrrhocoricin into the exit tunnel. Image from Seefeldt et al., 2015.

Structural data and biochemical assays suggest that these PrAMPs allow the accommodation of the aminoacylated initiator tRNA into the initiation site for the translation, but prevent the accommodation of the second aminoacyl-tRNA in the correct position due to a steric clash. In this way, PrAMPs prevent the transition to the elongation phase of bacterial translation[126]. A different mechanism of inhibition was highlighted for Api137, a derivative of Apidaecin. The groups of Rodnina and Mankin together showed that Api137 binds to the exit tunnel, but acts by trapping and blocking the release factor RF1, thereby impeding the release of the polypeptide chain and halting the translational machinery [142].

Besides the structural data, functional assays comparing similar PrAMP sequences and systematic mutagenesis approaches such as the 'Ala-scan' have greatly helped in defining those residues that are crucial for the action of PrAMPs [186,195,201–205]. These works allowed, for instance, to determine that the N-terminal fragment Bac7(1-35) retained much of the antibacterial activity of the whole Bac7, and that removal of its first four N-terminal residues dramatically decreased its activity[204]. Studies on apidaecin-type peptides in different insect species identified a conserved core motif 'R/KPxxxPxxPRPPHPRI/L' at the peptides' C-terminal[206] .

Furthermore, these data on the crucial and disposable residues have enabled researchers to obtain optimized PrAMP derivatives, with increased serum stability and/or antimicrobial activities, or

lower toxicity profile. An apidaecin-1b derivative, generated by substituting the N-terminal glycine with a non-natural amino acid, showed better serum stability[207] . Amino acid substitutions with non-proteinogenic amino acids improving activity and stability were also devised for Oncocin[203,208] .

Two drawbacks of the characterized PrAMPs, with respect to other AMPs, may be their quite limited spectrum of activity and their dependence of membranes transporters such as SbmA. In fact, the spectrum of activity of most PrAMPs seems limited to only Gram-negative pathogens and some fungal strains[188] . Plus, the main responsible for PrAMPs' uptake is the inner membrane transporter SbmA, whose physiologic function is still unknown and which appears disposable for *in vitro* bacterial growth[209]. Although some studies suggest a role in host invasion and establishment of chronic infections[210,211] , if some bacteria can survive without SbmA, perhaps they might easily mutate it or lose it - thereby lowering their susceptibility to PrAMPs. Additionally, some mutations in the ribosomal tunnel increasing the MIC of oncocin were already found[198] , showing that ribosomal mutations to elude PrAMPs may not have a high fitness cost.

However, these weaknesses may be counterbalanced by a higher biocompatibility of PrAMPs compared to membranolytic peptides, and some PrAMPs at higher concentrations can switch to a membranolytic mechanisms[131]. In addition, numerous findings have already demonstrated that there's much room for optimization of these peptides and, provided the optimizations will maintain their low cytotoxicity, PrAMPs can be a good starting base for the design of peptide therapeutics. Some Proline-rich AMPs have already been tested in mouse models of infections, demonstrating high efficacy albeit a high clearance rate[212,213]**,** and these findings are strongly motivating for the research on PrAMPs.

Promising PrAMPs derived from the mammalian Bac7 and Bac5

Our laboratory has been studying PrAMPs for many years, focusing on the ones produced in mammalian species. In our lab, Romeo and colleagues have isolated from the neutrophil granules of *Bos taurus* two proline-rich AMPs, which have been extensively studied and characterized [187]. These peptides, collectively named 'bactenecins' (from bacterium + 'necare', latin for 'to kill') weighted 7 and 5 kDa and were named Bac7 and Bac5, respectively, and are the PrAMP that were most studied in our Lab.

Bac5 and Bac7, along with all the mammalian PrAMPs discovered so far, belong to the wider group of cathelicidins, which together with defensins is one of the two largest families of antimicrobial

peptides found in animals. More specifically, the proline-rich peptides are actually the C-terminal part of the pre-pro-peptides coded by the cathelicidin genes [214,215].

The cathelicidins family includes a great number of cationic and usually amphipatic multifunctional peptides, which have a heterogenous set of antimicrobial and immunomodulatory functions. Members of this family were found in mammals, fish, birds, and other phylogenetically distant species[216,217]. The cathelicidin genes share a well conserved 'four exons-three introns' structure. Three exons code for the pre-prosequence, comprising an N-terminal signal sequence and a so called 'cathelin-like domain' of approximately 100 residues, well-conserved across cathelicidins, while the fourth exon code for the antimicrobial peptide moiety. Such antimicrobial peptide moieties are extremely variable in their sequence and structure (and thus their spectrum of activity), encompassing linear, circular, alpha helical, β-sheet or extended structures[214].

Bac7 and Bac5, as well as the other mammalian cathelicidins, are synthesized as inactive pre-propeptides in the progenitor cells of neutrophil granules[218,219]. The pre-sequence ensures their targeting to the secretory granules; once into the granules, the pre-sequence is lost while the pro-sequence keeps the C-terminal antimicrobial moiety inactivated. Then, when the granules are secreted or fuse with azurophil granules, the C-terminal antimicrobial peptides is cleaved away from its pro-sequence by specific proteases such as elastase [126,219]. Although the term 'cathelicidin' was originally used only to indicate the entire pre-pro-peptide, it is now often used also to refer to the corresponding antimicrobial moiety. Likewise, 'Bac7' and 'Bac5' will hereafter indicate only the C-terminal PrAMPs. Plus, although orthologs of Bac5 and Bac7 were found in other species such as goat and sheep[220], this thesis will always refer to the bovine orthologs.

Bac7 is a PrAMP of 60 amino acids, with numerous -PRP- motifs repeated across its sequences; Bac5 instead consists of 43 residues, with many sequence motif -X_1PPX_2- tandemly repeated, where X_1 is generally arginine and X_2 in a hydrophobic residue (Ile, Phe or Leu). Both these peptides showed good antimicrobial activity against *E. coli, S. typhimurium, K. pneumoniae* and *E. cloacae*[187].

However, most studies about Bac5 and Bac7 were actually focused on their N-terminal fragments. In fact, studies have shown that these N-terminal fragments retain a good antimicrobial activity and their shorter size than the original molecule may imply a lower cost of synthesis.

Studies on Bac7 fragments revealed that the first 35 N-terminal residues of Bac7 are necessary and sufficient to have the same antimicrobial activity of the entire bactenecin. The Bac7(1-35) fragment displayed excellent activity (MIC $\leq$ 1 μM) versus *E. coli*, *Salmonella typhimurium* and *K.*

pneumoniae, and very good activity (MIC ≤ 8 μM) against some multidrug resistant clinical isolates of *A. baumannii* and *P.aeruginosa*, and strains of the fungus *Cryptococcus neoformans* [204]. Significant antimicrobial activity against *E. coli*, *S. typhimurium* and *K. pneumoniae* was retained also by the shorter Bac7(1-16), albeit with more variable potencies in the different strains. Instead, the '5-16' and '5-35' fragments decreased their potency of 16 to 128 fold, compared to Bac7(1-16) and Bac7(1-35), highlighting the crucial importance of these five N-terminal residues[204].

The group of Scocchi and colleagues was the first to confirm the SbmA-mediated intracellular uptake of Bac7(1-16) and Bac7(1-35)[138], and X-ray structures and biochemical assays have confirmed that the N-terminal Bac7 fragments mainly acted by inhibiting protein synthesis[143,196]. However, Bac7(1-35) at its MIC was shown to be able to permeabilize the bacterial membrane of *P. aeruginosa* – which does not express the transporter SbmA –, suggesting a possible double mechanism of action [132]. Bac7(1-35) also showed a strain-dependent anti-biofilm effect at sub-MIC concentrations against clinical isolates of *A. baumannii*[221]. Moreover, Bac7(1-35) neutralized LPS and showed a low hemolytic activity[184,222]. Bac7(1-35) was also promising *in vivo*, significantly reducing the mortality of mouse models of *S. typhimurium* infections, although it was rapidly cleared from the mice body and its *in vitro* activity seemed to suffer from the presence of serum[212] .

Bac5 fragments have not been extensively studied yet. However, Mardirossian et al., assessed the antimicrobial activity and the inhibition of protein synthesis of three N-terminal Bac5 fragments [223], confirming a bactericidal action against *E. coli* for Bac5(1-25) and Bac5(1-31). These two fragments were also shown to inhibit bacterial protein synthesis *in vitro*. An X-ray structure of Bac5 into the ribosomal tunnel is not yet available, but biochemical assays suggested that Bac5(1-25) and Bac5(1-31) prevented the elongation steps of translation, as other PrAMPs do. The 31-residue fragment displayed significant cytotoxicity already at 4 fold its MIC, and both fragments were shown to partially inhibit also eukaryotic translation *in vitro*[223] .

All these data considered, our laboratory has deemed that the two proline-rich peptides Bac7(1-16) and Bac5(1-25) have a sufficiently promising set of properties. Therefore, we decided to use these peptides as a starting point on which to try a series of optimizations, in order to develop novel peptides with significant antimicrobial activity, low toxicity towards eukaryotic cells and a good level of stability in biological fluids.

In addition, prompted by the promising properties of Bac7(1-35), we have looked for orthologs of this PrAMP in other mammalian species. Until 2018, artiodactyls were the only group of mammals known to express more than one cathelicidin. Many orthologs of Bac5 and Bac7 were already found in artiodactyl species such as cows, pigs, goats or sheeps or deers[220,224,225], and are currently being

studied by other research groups. Recently, orthologs of Bac7(1-35) were searched in the dolphin *Tursiops truncatus*, because cetaceans and artiodactyls had been fused in the same taxonomic order of '*cetardiodactyla*', based on their close phylogenetic relation [226,227]. This has led to discover two novel PrAMPs, which were Named Tur1A and Tur1B. Surprisingly, only Tur1A inhibited bacterial translation [189].

We decided to further explore the cetacean repertoire of orthologs of Bac7(1-35), looking for novel potent cathelicidins to characterize, in order to find novel antibacterial agents and data on structures and sequence that could be useful for the future optimization of antimicrobial peptides.

Aim of this thesis

The rate of discovery of novel classes of antibiotics is not keeping pace with the emergence of multidrug resistance in bacteria, and novel classes of drugs are urgently needed. Antimicrobial peptides (AMPs) harbor a variety of potential drug candidates with promising antibacterial and immune-modulating properties. Unlike most AMPs, the group of proline-rich antimicrobial peptides (PrAMPs) mainly act through intracellular mechanism without significant membrane permeabilization. This intracellular, non -lytic mode of action suggested a relatively low toxicity towards eukaryotic cells, which has been confirmed experimentally. Thus, optimization of PrAMPs could be a suitable strategy to develop new non-toxic drug candidates.

In this thesis work, I pursued the following aims:

- Design and characterize short optimized fragments of Bac7 and Bac5: this was accomplished by i) testing several mutant fragments for their antimicrobial activity and toxicity and ii) designing novel peptides combining the favorable mutations
- Characterize the optimized Bac fragments, assessing their antimicrobial activity, mode of action, cytotoxicity and activity in biological fluids.
- Identify novel cetacean proline-rich cathelicidins and characterize their properties, similarly to what was done for Bac5 and Bac7 derivatives.
- Hopefully, gain insights on the relationships between certain sequence motifs and antimicrobial properties of optimized bactenecin fragments and cetacean cathelicidins, and design novel peptides hoping to devise molecules encompassing all the best features of the molecules they derive from.

Materials and Methods

Bacterial cultures

The microbial reference strains that were used during this project were: *Escherichia coli* BW25113, *E. coli* BW25113*ΔSbmA* (JW0368-1, KEIO collection42), *E. coli* ML-35, *E. coli* ATCC 25922, *Enterococcus faecium* ATCC 19434, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 700603, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter cloacae* ATCC 13047, *Salmonella enterica* serovar *typhimurium* ATCC 14028, *Stenotrophomonas maltophilia* ATCC 13637 and the yeast *Candida albicans* ATCC 90029. The other *E. coli* pathogenic strains indicated in Tab. 3.7 were generously provided by the European Union Reference Laboratory for *E. coli*, Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità, Rome, Italy.

Both liquid and solid bacterial cultures were grown in Müller-Hinton Broth (MHB) and MHB supplemented with 1.5% (w/v) Agar, respectively. *Candida albicans* was grown in Sabouraud medium, supplemented with 1.5 % (w/v) agar in case of solid cultures. (MHB, Sabouraud broth and Agar were purchased by Difco Inc.). Before every experiment, overnight microbial cultures were made. Bacterial strains were grown at 37°C with the exception of *E. cloacae*, grown at 30°C. *C. albicans* overnight (o/n) cultures were grown at 30°C. Then, the next day, the overnight cultures were diluted 1:30 - 1:40 in fresh culture medium and incubated at the same temperature of the o/n cultures, shaking at 140 rpm, and let grown until they reached mid-log phase and an optical density at 600 nm (OD600) between 0.3 and 0.5. Kanamycin (50 μg/mL) was added to the medium for *E. coli* BW25113*ΔSbmA*. Upon reaching the desired range of OD600, bacteria were removed from the incubation, diluted to the desired concentration and utilized for the experiments.

Peptide synthesis

Solid phase FMOC chemistry

Bac5 fragments, Bac5 and Bac7 selected derivatives and the cetacean PrAMPs were purchased by NovoPro Bioscience (Shanghai, China), where they were synthesized via solid-phase FMOC chemistry, purified by reversed-phase HPLC to a purity of ≥95% and checked for their sequence via mass spectrometry. All the peptides were shipped in a lyophilized form with trifluoroacetate (TFA) as a counterion. The TFA ion was replaced with the more biocompatible chloride (Cl-) counter-ion by resuspending the peptides in ca 500 μl of 10 mM HCl and freeze-drying the peptide solution, for

a total of three times. Then the peptides were finally resuspended in sterile milli-Q water. To quantify the peptides, the absorbance of peptide solutions at 214 nm was quantified with a NanoDrop 2000 spectrophotometer; then, the molar extinction coefficient at that wavelength was calculated according to the guidelines from a work of Kuipers and Gruppen[228], and the peptides concentration was then calculated with the Lambert-Beer law. All the peptides were stored frozen, at -20°C.

SPOT synthesis

The PrAMPs of the libraries of Bac substituted derivatives were synthesized via SPOT synthesis in the laboratory of Kai Hilpert and colleagues (St. George hospital, London). Details about the SPOT synthesis protocol can be found in the references[229,230] . Briefly, before automated synthesis, cellulose membranes of 10×15 cm were functionalised overnight with 0.2 M Fmoc-Gly-OH (Aldrich), 0.24 M N,N'-diisopropylcarbodiimide (DIC, Fluka) and 0.4 M N-methylimidazole (NMI, Aldrich) in dimethylformamide (DMF, VWR); then, glycine was de-protected in 20% piperidine (v/v, Acros Organics) in DMF (20 min+10 min). Peptide synthesis was automatically performed at discrete spots using 9-fluorenyl-methoxycarbonyl/ tert-butyl (Fmoc/tBu) strategy. Once completed the synthesis, peptides underwent a final deprotection step and were incubated overnight in saturated ammonia gas atmosphere to cleave the peptide amides from the solid support. In order to check the yield and quality of the spot synthesis, **HPLC/MS analysis** was performed on an internally standardized control peptide and individually chosen peptides from that synthesis.

Determination of minimum inhibiting concentrations (MIC)

The minimum inhibitory concentration was determined by the broth microdilution method according to the CLSI (former NCCLS) protocol. Briefly, an overnight bacterial or fungal culture was diluted in fresh MH or Sabouraud broth, respectively (300 µl of O/N culture in 10 ml MHB/Sabouraud), and the culture was let grow at 37°C or 30°C, to reach an optical density at 600 nm (OD_{600}) comprised between 0.3 and 0.5. During bacterial growth, two-fold serial dilutions of AMPs in culture medium (Sabouraud, MHB or 20% MHB in PBS) were prepared in a final volume of 50 µl, in the wells of a round bottom 96-wells microtiter plate (Sarstedt). Then, the bacterial culture was diluted to a bacterial load of 5×10^5 CFU/ml, and 50 µl of such suspension were added to each well of the plate -except the negative control wells-, halving the final concentration of bacteria and peptides. The plate was incubated at 37°C (all bacteria except *Enterobacter* species) or 30°C (*Enterobacter* species and *Candida albicans*), and the MIC was determined after 18h by

visual inspection as the first clear well. Every MIC was determined through at least three independent experiments ($n \geq 3$).

When assessing the MIC of peptides in presence of serum or high salt concentrations, the bacterial culture was diluted (to 5×10^5 cfu/ml) in presence of 20% (v/v) human serum, or MHB with 5,4% (w/v) NaCl, respectively. When adding such bacterial suspensions to the plate, the serum concentration was halved to 10% and the NaCl concentration was decreased to 2.7% (w/v).

Before the beta-galactosidase assays, the MIC of PrAMPs and colistin against *E. coli* ML35p was determined using a more concentrated bacterial suspension, with ~2×10^7 CFU/mL (=1×10^6 CFU/well), in order to assess the MIC at the same bacterial density used in the beta-galactosidase assays.

Circular Dichroism

Peptides were dissolved to final 20 μM concentration in 10 mM sodium-phosphate buffer (SPB) Ph7.2, 10 mM SPB supplemented with 10 mM sodium dodecyl sulphate (SDS) pH 7.2, and 10 mM SPB pH 7.2 supplemented with 50%(v/v) tri-fluoro ethanol (TFE). The CD spectra of the peptide solutions were acquired in quartz cuvettes (optical path 0.2 cm) scanning at the wavelengths from 190 to 250 nm at the speed of 50 nm/min, data pitch 0.5 nm, band width 1.0 nm. All displayed CD spectra were the accumulation of three scans, acquired with a J-715 nm spectropolarimeter (Jasco).

Permeabilization assays

Propidium iodide (PI) uptake assay.

Mid-log phase cultures of *E. coli* BW25113 were diluted to 10^6 cfu/ml in MHB with 10 μg/ml propidium iodide (PI), then exposed to the peptides for 30 min at 37°C. PI-mediated fluorescence was measured using a Cytomics FC 500 instrument (Beckman-Coulter Inc., Fullerton, CA, USA). The instrument evaluated 10^4 bacterial cells for each measurement. In the PI uptake assays of Bac7-Bac5 derivatives, an almost identical protocol was adopted but the mid-log *E. coli* BW25113 cultures were diluted to 5×10^5 cfu/mL (in MHB, with a final PI concentration of 10 μg/ml), and the PI uptake was measured with a MACSQuant Analyzer 10 (Milteny Biotec) flow cytometer. In the flow cytometry assays, membrane permeabilization was calculated as the % of PI-positive cells, and the entity of membrane damage was quantified measuring the mean fluorescence intensity

(MFI). In the permeabilization control samples, bacteria were treated with the membrane-disrupting peptides Polymyxin B or Colistin, while in the negative control samples water was added to the cell suspension instead of peptides.

β-galactosidase assay.

The β-galactosidase assay was adapted from a protocol of Lehrer et al [231]. Peptides were diluted in PBS in a final volume of 50 µl in the wells of a transparent, flat-bottom 96 wells-microtiter plate. Then a mid-log phase *E. coli* ML35p culture was diluted in PBS and O-nitrophenyl-β-d-galactopyranoside (ONPG), to get a bacterial density of 2×10^7 CFU/mL, and ONPG concentration of 3 mM. Subsequently, 50 µl of the bacterial suspension in PBS+ONPG were added to each well of the a microtiter plate, halving the final concentrations of bacteria, ONPG and peptides, to final peptide concentrations equal to ½ × , 1×, and 2× their MIC values against that *E. coli* strain at that bacterial density. Immediately after the addition of bacteria, the plate was read with a Nanoquant infinite M200 pro plate reader (Tecan) pre-heated at 37°C, and the absorbance at 405 (absorbance of O-nitrophenol) was measured every 10 minutes, right after a 10 s – pre-shaking of the plate (57 rpm -orbital shaking, 2mm diameter). Bacteria treated with colistin were assayed as permeabilization control, while bacteria not treated with peptides were the negative control.

In Vitro Transcription/Translation Assays

Bac5 fragments. The transcriptional/translational inhibiting activity of Bac5 fragments was determined using the S30 T7 High-Yield Protein Expression System (Promega). To prevent RNase-mediated degradation, RNase inhibitor (0.1 µl, RNasin®, 20- 40 U/µl, Promega) was added to the master mix.

Reaction tubes contained the lysate of *E.coli* and every tube minus the 'No DNA' control was supplemented with a plasmid coding for a luciferase. Then, with the exception of the 'No peptide' and 'No DNA' control samples, 2.5 µL of peptide or antibiotic (erythromycin) solution were added to each reaction, to obtain the desired final concentrations (of 1, 10, or 100 µM), in a final volume of 12.5 µL.

Erythromicin was used as a positive control for translation inhibition. In the 'No peptide' control samples, the required volumes of AMPs were replaced with nuclease-free water. In the 'No DNA' control sample, nuclease-free water was added to the reaction instead of peptides and DNA template.

These reactions occurred in nuclease-free PCR tubes. The samples were incubated for 1 h at 37°C with vigorous shaking (1200 rpm), the reaction was stopped by transferring the samples on ice and adding immediately to each sample 12.5 µl of ice-cold RNase-free water. The luminescence produced by the *Renilla reniformis* luciferase was used as reporter signal, which was quantified using the commercial kit Renilla- Glo Luciferase Assay System (Promega). 20 µl of the aforementioned reaction mix were added to 20 µl of the provided buffer (+substrate diluted), waiting 10 min before performing the luminescence measurement. Black flat-bottom 96-well plates were used in a luminometer Plate Camaleon (Hidex; software Mikrowin 2000). In all the luminescence measurements, the relative values were calculated as a percentage of the untreated control.

Bac7 and Bac5 selected derivatives and cePrAMPs. Transcription/translation reactions were set up using lysates of the RTS 100 *E. coli* HY kit from Biotechrabbit (Berlin, Germany), following the instructions of the manual. Briefly, reaction tubes contained the lysate of *E.coli* and every tube minus the negative control was supplemented with a plasmid coding for a luciferase; then volumes of peptide solution were added to each reaction tube, to reach the desired final concentration (0.5, 1, 5, 10 and 25 µM) in a final volume of 6 µL. Samples were incubated at 30°C for 1 h with shaking (600 rpm), then 2 µL of each reaction were extracted and stopped using 8 µL kanamycin solution (50 µg/mL) before being combined with 40 µL of Luciferase Assay substrate (Promega, Madison, WI, USA) in a 96-well white flat bottom microtiter plate (Sarstedt, Milan, Italy). The presence of luciferase reporter protein was quantified by measuring its luminescence using a Infinite M200 plate reader (Tecan). The relative luminescence values for all the assays were calculated as a percentage of the positive control (reactions with nuclease-free water instead of the peptides) using three independent replicates. In the negative control, nuclease-free water was added instead of peptides and DNA template.

Human serum and red blood cells

Human serum was collected and pooled from the blood samples of six healthy donors, kindly provided by the Blood Bank of the University Hospital “Ospedale Maggiore” of Trieste, Italy. Briefly, to collect the serum, six blood samples were pooled together and incubated at room temperature (~22°C) to allow the coagulation to take place.

Subsequently, the blood clot was separated via centrifugation (2000 × g for 10 min) and the remaining serum was filtered in two subsequent passages, with a Filtropur device (Sarstedt) with

0,4 and 0,22 µm pore diameter in the first and second passage, respectively. Filtered serum was aliquoted aseptically and stored at -20°C. Human Red Blood Cells (hRBCs) were collected from single donor-human whole blood (Cambridge Bioscience, UK), being isolated by centrifugation at 800 × *g* for 20 min at 4 °C and discarding the supernatant. Then, the pellet of hRBCs was washed three times by resuspending it in PBS (PBS volume doubling the volume of the RBCs pellet) and centrifuging 500 × *g* for 10 min at 4 °C, and finally resuspended to either 8% or 0.8% (v/v) in PBS, ready for the hemolytic assay.

Toxicity on eukaryotic cells

Hemolysis assay

After the isolation, RBCs were isolated and resuspended in PBS at 8% or 0,8% (see above, 'human serum and red blood cells'). Serial two-fold peptide dilutions in PBS were made in a total volume of 100 µl, in the wells a 96-well flat bottom microtiter plate (Sarstedt). Then 100 µl of hRBCs suspension was added to the wells (thus halving the RBCs concentrations to 4% or 0,4%), and the plate was incubated for 1 h at 37°C. After the incubation, the plate was centrifuged at 1000 × *g* for 10 min to pellet the non-lysated erythrocytes and 100 µl of the supernatant were collected into the clean wells of a new 96-well flat bottom microtiter plate (Sarstedt). Finally, the release of hemoglobin was assessed by measuring the absorbance of the supernatants at 540 nm with a Nanoquant infinite M200pro plate reader (Tecan). Samples of hRBCs treated with 1% v/v Triton X-100 or PBS were prepared as positive and negative control of hemolysys, respectively.

MTT assays

In the MTT assay against MEC-1 cells, serial two-fold dilutions of the peptides were prepared in final 50 µl of complete RPMI 1640 medium, in the wells of a transparent, 96-wells, flat bottom microtiter plate, treated for tissue-culture (Euroclone). Cells were counted in a Burker-Türk Chamber, then diluted in RPMI 1640 to 2×10^6 cell/ml and finally, 50 µl of cell suspension were aliquoted to the wells of a microtiter plate, halving the peptide concentrations and cell density. The plate was incubated for 20 h at 37 °C in 5% CO_2. Then, 25 µl of an MTT/PBS stock solution were added to each well, reaching a final MTT concentration of 1 mg/ml and the plate was incubated in the dark for 4 h at 37°C, 5% CO_2. Subsequently, 100 µl of a solution of 10% Igepal (w/v) in 10 mM HCl were added to each well, and the plate was incubated overnight at 37°C, 5% CO_2. The formazan absorbance at 570 nm was measured in a Nanoquant Infinite-M200 Pro plate reader (Tecan).

Before the MTT assay against HaCat cells, HaCat cells were first counted in a Burker-Türk Chamber and diluted in complete DMEM medium to 2,2 × 10^5 cells/ml. 90 µl of such cell suspensions were seeded in a 96-wells transparent flat bottom microtiter plate treated for tissue-culture (Euroclone), reaching a cell density of 2 × 10^4 cells/well, then the plate was incubated 24 h at 37°C, 5% CO_2. After this incubation, two-fold serial dilutions of 10X peptides were prepared in PBS and then 10 µl of peptide solutions were added to the wells - reaching the desired 1X concentrations in a final total volume of 100 µl/well - and the plate was incubated for 21 h at 37 °C, 5% CO_2. At the end of the incubation, 25 µl of an MTT/PBS stock solution were added to each well, reaching a final concentration of 1 mg/ml/well, and the plate was incubated in the dark for 3 h at 37°C with 5% CO_2. Subsequently, the plate was centrifuged at 2000 × *g* for 10 min, and the supernatant from each well was then substituted with 125 µl of PBS + 100 µl of 10% Igepal (w/v) in 10 mM HCl. The plate was incubated overnight in the dark at 37 °C, 5% CO_2 and the next day the absorbance at 570 nm was measured in a Nanoquant Infinite-M200Pro plate reader. Student's T-test was performed as statistical analysis.

Results

Characterization of short Bac5 fragments

One of our first goals was to optimize the activities of Bac5 and Bac7 fragments starting from the shortest ones which retained substantial antimicrobial activity. The goal of maintaining the shortest possible sequence was mainly because the shorter the peptides is, the lower synthesis cost would be. Since Bac7 fragments as short as Bac7(1-16) were already found to retain good antimicrobial potency[204], we started this study by seeking the shortest fragments of Bac5 endowed with appreciable antimicrobial activity. A previous work from our laboratory, [223] revealed that Bac5(1-25) was active against a strain of *E. coli* while the shorter Bac5(1-15) was not. Therefore, we characterized the antimicrobial potential and mode of action of eight Bac5 fragments spanning between 15 and 25 residues (Tab. 3.1).

Table 3.1. Sequences and charge of the Bac 5 fragments that were characterized.

Bac5 fragment	Sequence	Charge
Bac5 (1-25)	RFRPPIRRPPIRPPFYPPFRPPIRP	+7
Bac5 (1-23)	RFRPPIRRPPIRPPFYPPFRPPI	+6
Bac5 (1-21)	RFRPPIRRPPIRPPFYPPFRP	+6
Bac5 (1-19)	RFRPPIRRPPIRPPFYPPF	+5
Bac5 (1-17)	RFRPPIRRPPIRPPFYP	+5
Bac5 (1-15)	RFRPPIRRPPIRPPF	+5
Bac5 (3-25)	RPPIRRPPIRPPFYPPFRPPIRP	+6
Bac5 (5-25)	PIRRPPIRPPFYPPFRPPIRP	+5

Antimicrobial activity of Bac5 fragments

We tested the spectrum of antimicrobial activity of Bac5 fragments by determining their minimal inhibiting concentration (MIC) against six different bacterial species (Tab. 3.2). Overall, the antimicrobial activity of these fragments appeared proportional to their length, with the fragments 1-23 and 1-25 showing the most potent action and broadest spectrum of activity. However, each active fragment significantly decreased its efficacy in the absence of the membrane transporter SbmA. First, the peptides were tested against *Escherichia coli* BW25113, challenging both the wild type strain and the same strain knocked out for the membrane transporter (BW15113 Δ*SbmA*); as seen with other PrAMPs, the MIC of the active Bac5 fragments significantly decreased in the Δ*SbmA* strain, compared to the wild type counterpart. Plus, none of the fragments could inhibit the growth of *P. aeruginosa* or *S. aureus*, neither of which expresses the membrane transporter. Appreciable antimicrobial activity of some fragments was shown against *E. coli*, *Salmonella*

enterica and *Acinetobacter baumannii*. Conversely, the 1-15 and 5-25 fragments showed no activity against any of the strains tested. The dramatic decrease in activity observed in Bac5(3-25) and the drop of activity of Bac5(5-25) appeared to confirm the importance of the first N-terminal residues, as shown for other PrAMPs (including the other bovine bactenecin Bac7).

Table 3.2. Antimicrobial activity of Bac5 fragments against a panel of reference strains of bacterial pathogens.

	MIC (μM)							
	1-15	**1-17**	**1-19**	**1-21**	**1-23**	**1-25**	**3-25**	**5-25**
E. coli BW 25113	64	8	8	2	2	1	4	>64
E. coli BW 25113*ΔSbmA*	>64	64	32	16	32	16	>64	>64
E. coli ATCC 25922	64	16	12	4	2	1	8	>64
S. enterica ATCC 14028	>64	32	32	8	4	8	>64	>64
K. pneumoniae ATCC 700603	>64	>64	>64	32	32	32	>64	>64
A. baumannii ATCC 19606	>64	64	32	16	1	1	8	>64
P. aeruginosa ATCC 27853	>64	>64	>64	>64	>64	>64	>64	>64
S. aureus ATCC 25923	>64	>64	>64	>64	>64	>64	>64	>64

The minimum inhibiting concentration (MIC) of Bac5 fragments was measured after 18 h incubation of bacteria with the peptides. Results are the median of at least three independent experiments (n ≥ 3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Circular dichroism spectra in polar and amphypathic environments

We investigated the structure of these fragments by analyzing their circular dichroism (CD) spectra in aqueous and amphipathic environments (Fig 3.1). We diluted each peptide in sodium phosphate buffer (SPB) alone or supplemented with sodium dodecyl sulphate (SDS) or 50% (v/v) trifluoroethanol (TFE). The two supplements served to increase the amphipathicity of the environment and possibly to promote peptide's structure induction. Despite some minor changes in the CD spectra we did not find any notable secondary structures for any of the peptides under different conditions. The CD spectra of all the peptides showed a minimum peak between 200 and 210 nm, which is a feature found in CD spectra of poly-proline II structures [232], which could fit well in the proline-rich sequence of Bac5 fragments. However, the rest of the CD spectra move a bit away from the prototypical scheme of poly-proline-II proposed by Woody et al.[232] . In addition, the nearly identical CD spectra in the three conditions seems to exclude the transition to a defined secondary structure, neither in amphipathic environment, in agreement with the non-lytic mode of action for these PrAMPs.

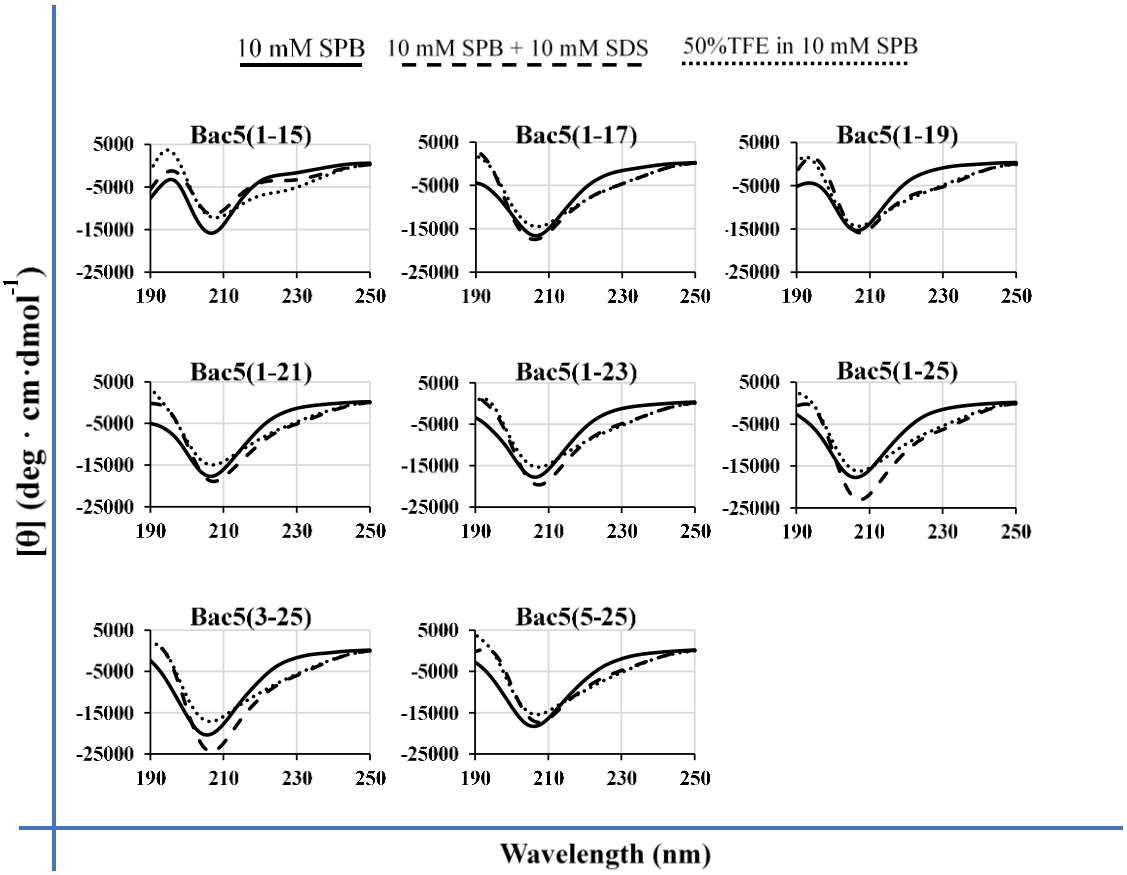

Figure 3.1. Circular dichroism (CD) spectra of Bac5 fragments in aqueous and amphipathic environment. CD spectra of Bac5 fragments (20μM) were taken in 10 mM sodium phosphate buffer alone (SPB, continuous line), and SPB additioned with 10 mM sodium dodecyl sulphate (10 mM SDS in 10 mM SPB, dashed line) or SPB with 50% Tri-fluoroethanol (50% TFE in 10 mM SPB, dotted line). Results are the average of three scans (n = 3).

Effects of Bac5 fragments on the bacterial membrane

The reduced activity in absence of SbmA as well as the lack of peptide structure induction in amphipathic environment suggested the absence of a membrane-disrupting mechanism for these peptides. To confirm this hypothesis, we assessed any membrane-permeabilizing capability by measuring the uptake of propidium iodide (PI) by *E.coli* BW25113 cells treated with the peptides. The PI probe emits fluorescence only when it is intercalated into the DNA, after crossing breaches in the bacterial membrane. Hence, the PI-mediated fluorescence indicates membrane permeabilization and/or disruption. Bacteria were incubated with Bac5 fragments at 8 μM, a concentration equal to, or higher than the MIC of the fragments. Results of the flow cytometry analyses (Tab. 3.3) indicated that none of the peptides caused PI uptake in less than 0.3% of analyzed cells, while polymyxin B, used as positive control, permeabilized 98% of bacterial cells.

Table 3.3. Permeabilizing activity of Bac5 fragments on *E.coli* membrane.

	Bac 5 fragments (8 µM)								PxB (1µM)	Untr.
	1-15	1-17	1-19	1-21	1-23	1-25	3-25	5-25		
% PI-positives	0	0,01	0,02	0,08	0,24	0,19	0,07	0,04	98,84	0

Fluorescence cytometry analysis of bacterial uptake of the propidium iodide (PI) probe, after 30 min incubation with the peptides. Results are indicated as the percentage of PI-positive bacteria in the sample and are the average of three independent experiments (n = 3).

Inhibition of bacterial translation

Once determined that the fragments acted by a non-lytic mode of action, we evaluated their inhibitory activity on bacterial translation, in line with previous investigations on longer Bac5 fragments[223]. Using a commercial transcription/translation assay kit, lysates of *E. coli* BW25113 were exposed to the peptides and the synthesis of a reporter luciferase was evaluated (Fig. 3.2). Although the assay could not discriminate between inhibition of transcription or translation, the transcriptional inhibition had been previously excluded for Bac5(1-25)[223], demonstrating that its inhibiting activity was limited to the translational machinery. Thus, we confidently agreed that the same mechanism could be attributed to the other fragments. Excluding Bac5(1-15) and Bac5(5-25), the other fragments significantly inhibited protein synthesis *in vitro* already at 10 µM. The most potent inhibitor was Bac5(1-23), almost completely inhibiting luciferase expression (>97% inhibition), followed by Bac5(1-25) and Bac5(3-25) both inhibiting ca 86% of luciferase synthesis. Fragments 1-25 and 3-25 showed nearly identical inhibitory activity on the transcription/translation *in vitro* but their MIC were different. This may be because Bac5(1-25) gets more easily uptaken into *E. coli*, suggesting that the first N- terminal of these PrAMPs have a role in their intracellular uptake.

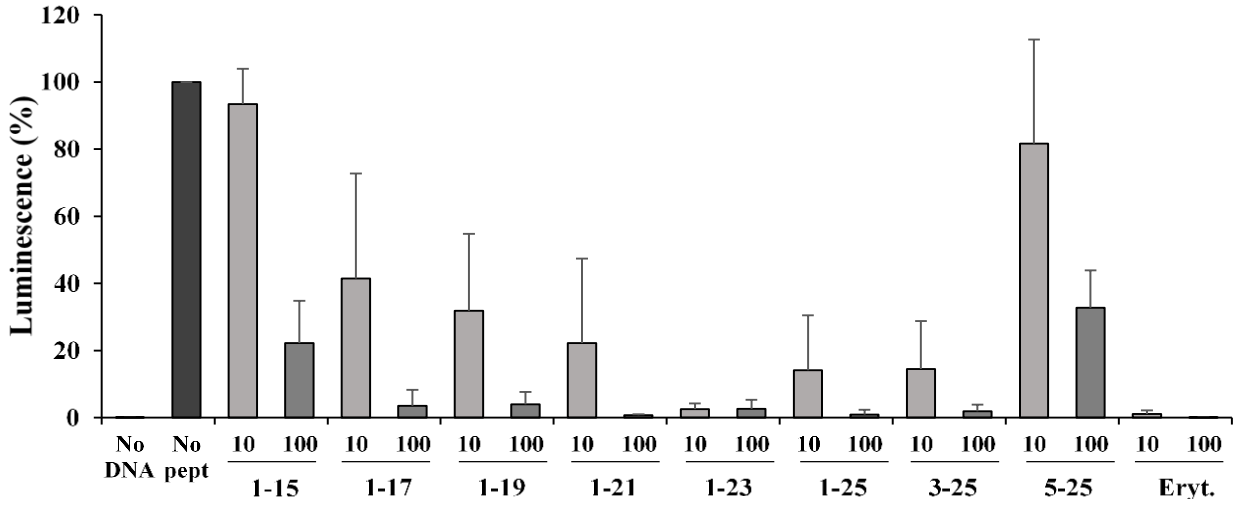

Figure 3.2. **Inhibition of *in vitro* transcription/translation reactions mediated by Bac5 fragments**. Luminescence of a reporter luciferase produced after 1h incubation of an *E. coli* lysate with the Bac5 fragments. Luminescence is expressed as percentages with respect to the negative control of inhibition, where RNAse-free water was added instead of the peptides. As a positive control, the translation-inhibiting antibiotic erythromycin (Eryt) was used. Results are the average and respective standard deviations of four different experiments (n = 4).

Cytotoxicity on eukaryotic cells

The scarce effect of Bac5 fragments on bacterial membranes suggested a low cytoxicity for eukaryotic cells, as was observed for other PrAMPs. This aspect was investigated by MTT assays on the B-chronic lymphocytic leukaemia MEC-1 cells (Fig. 3.3), which revealed that Bac5 fragments were not detrimental for cell viability. Bac5(1-25) was the only one decreasing cell viability of approximately 15% at 64 µM.

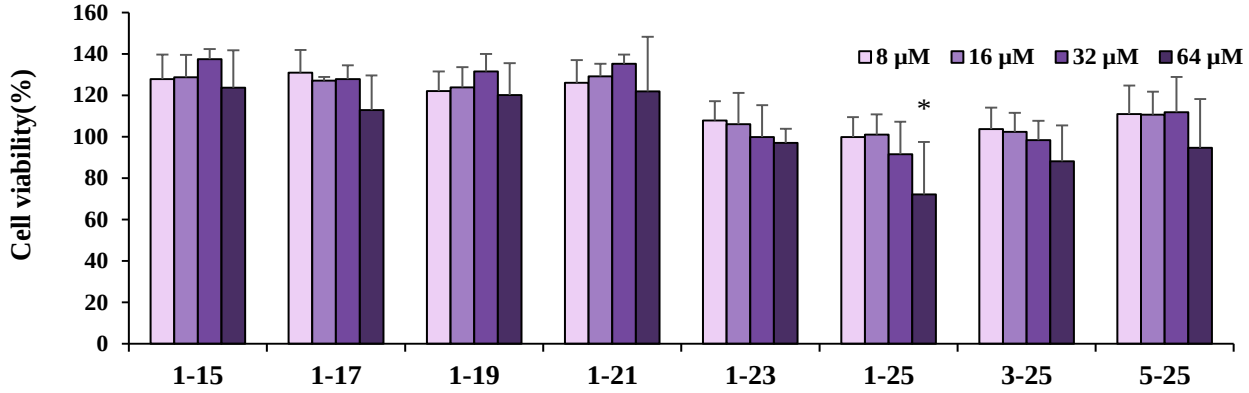

Figure 3.3. Effects of Bac5 fragments on cell viability. MTT assay on MEC-1 cells after treatment with Bac5 fragments. The cell viability was measured as absorbance at 570 nm after 24 h of exposure to the peptides. Results are reported as percentages of viable cells with respect to the untreated control cells (set as 100% of viability). Error bars represent the standard deviation calculated on the average of three independent experiments, each with an internal duplicate (n = 6). * = $p < 0,05$ (T-test, comparing treated samples versus untreated controls).

Overall, Bac5 fragments shorter than the fragment 1-25 retained antimicrobial activity and acted via inhibition of translation rather than membrane permeabilization. None of these fragments appeared to be cytotoxic. Bac5(1-17) was the shortest fragment retaining significant antimicrobial activity against *E. coli* and *S. typhimurium*, although its activity was lower than that of the 25-residues peptide. We decided to use the 1-17 Bac5 derivative to design libraries of peptides with single amino acid modification (mutant peptides).

Libraries of substituted derivatives of Bac5 and Bac7 fragments

One of our aims was to modify PrAMPs studied in our laboratory, to get hints on their crucial and replaceable residues and the most advantageous amino acid substitutions. For this reason we decided to test a number of derivatives starting from the original fragment of Bac7(1-16), which has been previously characterized, and of Bac5(1-17), described above, introducing single amino acid substitutions in each position of both peptides. We used the high-throughput technique of the SPOT synthesis that allows to obtain in parallel a high number of mutants peptides[233]. Bac5(1-17), and Bac7(1-16) were short enough to allow the use of the SPOT synthesis protocol.

Ala-scans of Bac7(1-16) and Bac5(1-17)

The first libraries contained mono-substituted derivatives of Bac7(1-16) and Bac5(1-17), each peptide with a single alanine substitution in a different position of the peptide's sequence. Collectively, these small peptide libraries allowed us to perform the Ala-scan of Bac5(1-17) and Bac7(1-16). We, together with collaborators, used Ala-scans to identify crucial residues for the antimicrobial activity of Bac5(1-17) and Bac7(1-16), measuring the MIC of each mono-substituted derivative as well as evaluating their ability to inhibit protein synthesis *in vitro*, with the same transcription/translation assay used for the Bac5 fragments.

Among all the alanine-substituted derivatives of Bac7(1-16), only the peptide with the R1A substitution showed a slightly lower MIC than the wild-type sequence, changing the MIC from 8 µM (wild-type fragment) to 4 µM (R1A mutant peptide) (Fig. 3.4 upper panel). However, this derivative did not show a higher level of protein synthesis inhibition , indicating that the substitution perhaps increases the bacterial uptake, rather than improving the ribosome binding. The most detrimental substitutions were located in the residues 9 to 11, suggesting a crucial function of the -R_9LP_{11}- core motif.

In Bac5(1-17), the alanine introduction in P5 and in the sequence interval from R8 to F15 appeared to nullify the peptides' inhibition of transcription/translation. Apparently, the stretch of crucial amino acids results more extended for Bac5(1-17) than for Bac7(1-16). (Fig. 3.4 lower panel).

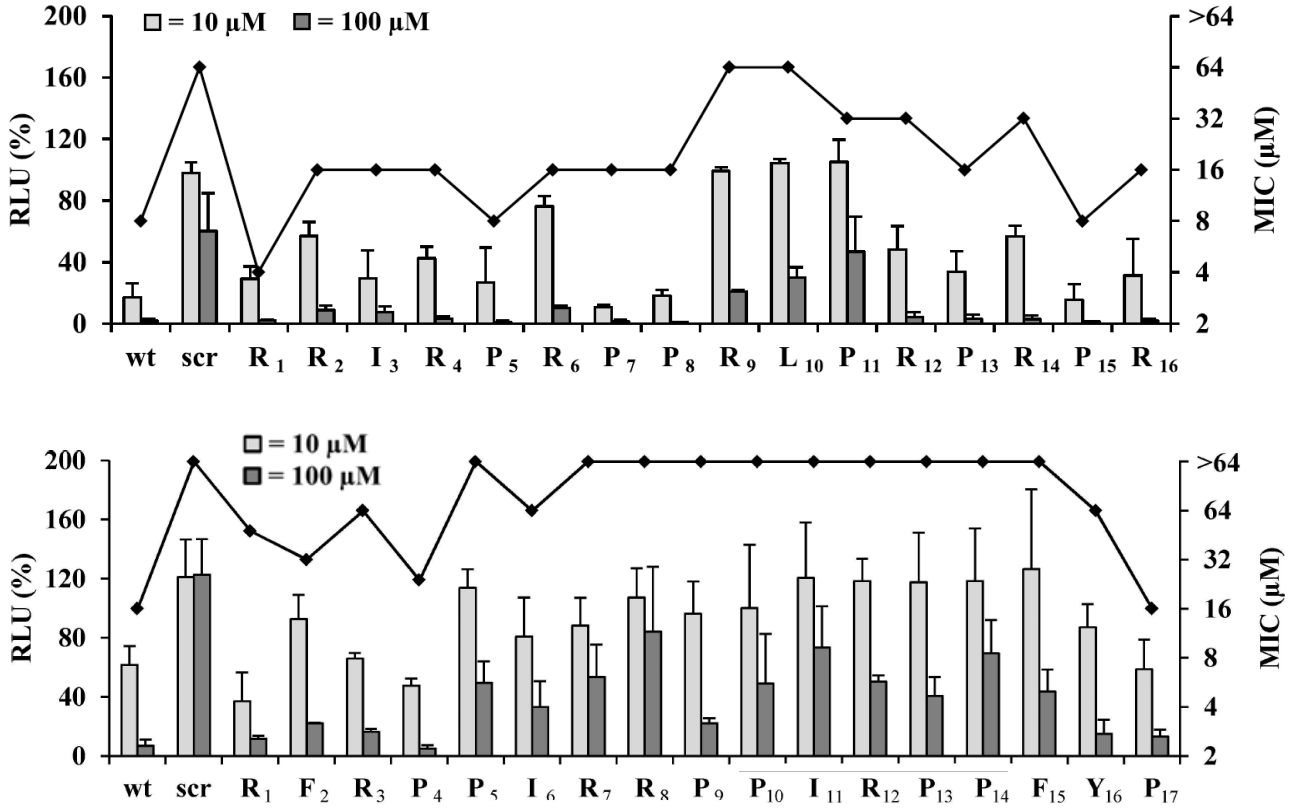

Figure 3.4. Ala-scans on Bac7(1-16) and Bac5(1-17). Effects of alanine substitutions on antimicrobial and translation-inhibiting activities of Bac7(1-16) (top) and Bac5(1-17) (bottom). Letters on the x axis represent the mono-substituted peptides, each of which harbors the alanine substitution in place of the indicated residue. The relative luminescence units (RLU, left y-axis) refer to a reporter luciferase produced in *E.coli* lysates treated with the peptides, and are expressed as percentages with respect to the control samples, not treated with peptides. Results of the MIC assay (diamonds, right y-axis) and the *in vitro* transcription/translation assay are the median and the average of at least 3 independent experiments ($n \geq 3$), respectively. Unmodified wild type (**wt**) and scrambled (**scr**) Bac5(1–17) peptides were also used as controls for both assays.

Scans of the Bac fragments introducing different residues

The Ala-scans gave us useful insights about the importance of the single residues in the Bac7(1-16) and Bac5(1-17) sequences. Subsequently, we have tested seven other scans for each Bac fragment, each scan with a different amino acid substituent. The substituents that have been used were glycine (absence of-side chain), then phenylalanine or tryptophan for their bulky aromatic side chains, proline to test its secondary structure-disrupting effect, serine as an example of short polar side chain, and glutamate or arginine to test the introduction of negatively or positively charged side chains, respectively.

Table 3.4. Activity against *E. coli* BW25113 (MIC, μM) of eight different scans of Bac7(1-16) and Bac5(1-17), with eight different substituent residues.

	Ala	Gly	Ser	Arg	Pro	Glu	Phe	Trp
R_1	**4**	8	16	-	8	64	**4**	**2**
R_2	16	16	16	-	32	64	16	**8**
I_3	16	16	16	8	16	64	16	**4**
R_4	16	16	32	-	16	>64	16	16
P_5	8	8	16	8	-	32	8	**4**
R_6	16	32	32	-	32	>64	16	8
P_7	16	16	16	8	-	32	8	**4**
R_8	16	16	16	16	-	64	16	**4**
L_9	64	64	32	-	16	>64	16	**4**
P_{10}	64	64	32	32	64	>64	32	16
P_{11}	32	32	32	16	-	64	32	8
R_{12}	32	32	16	-	32	>64	16	8
P_{13}	16	16	16	8	-	64	8	**2**
R_{14}	32	32	32	-	32	64	8	**4**
P_{15}	8	16	16	16	-	32	8	**4**
R_{16}	16	16	16	-	32	64	8	8
wt	8 μM							
scr	> 64 μM							

	Ala	Gly	Ser	Arg	Pro	Glu	Phe	Trp
R_1	32	32	>64	-	64	>64	64	32
F_2	32	16	64	16	64	>64	-	**8**
R_3	64	64	64	-	>64	>64	64	32
P_4	16	**8**	16	**4**	-	64	**8**	**8**
P_5	>64	>64	64	16	-	>64	32	16
I_6	64	64	64	16	>64	>64	16	**8**
R_7	>64	>64	>64	-	>64	>64	32	64
R_8	>64	>64	>64	-	>64	>64	>64	32
P_9	>64	>64	64	**8**	-	>64	16	16
P_{10}	>64	>64	>64	>64	-	>64	64	32
I_{11}	>64	>64	>64	>64	64	>64	>64	32
R_{12}	>64	>64	>64	-	>64	>64	>64	>64
P_{13}	>64	>64	>64	64	-	>64	32	16
P_{14}	>64	>64	>64	32	-	>64	32	16
F_{15}	>64	>64	64	32	64	>64	-	**8**
Y_{16}	64	32	64	16	>64	>64	16	**8**
P_{17}	16	16	16	8	-	>64	16	**8**
wt	16 μM							
scr	> 64 μM							

MIC was determined after 18 h incubation with the peptides. The MIC of the wild type (**wt**) and scrambled (**scr**) peptides (also produced via spot synthesis) are reported on bottom. Grey shades indicate the substitutions which improved the MIC with respect to the original fragments. Results are the median of at least 3 independent experiments (n ≥ 3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Among the Bac7(1-16) derivatives, tryptophan substitutions were nearly the only ones lowering the MIC, apart from R1A and R1F (Tab. 3.4, left panel). The most effective substitutions were R1W and P13W, both decreasing the MIC by four-fold, while the other favorable mutations only halved the MIC. Arginine substitutions, albeit giving a higher positive charge to the peptide, did not increase peptide activity.

In the libraries of Bac5(1-17) derivatives (Tab. 3.4, right panel), the MIC was lowered two-fold by arginine and tryptophan substitutions in more than one position. The MIC improved also with substitutions in the fourth residue with phenylalanine or glycine. P4R was the most effective substitution, lowering the MIC by four-fold. Overall, in both Bac5(1-17) and Bac7(1-16), the introduction of a single Trp in different positions of the sequence improved the peptides' antimicrobial activity against *E. coli* even when Trp replaced those residues identified as important in the Ala-scans.

Mono – and multi-substituted Bac7 and Bac5 derivatives

After the screening of the libraries of mono-substituted peptides, we designed new peptides harboring combinations of the most favorable mutations identified above. In order to avoid excessively hydrophobic peptides, likely causing membrane disruption and cytotoxic effects, we did not incorporate more than three tryptophans in the same peptide sequences. We followed these considerations to design the derivatives of both Bac7(1-16) and Bac5(1-17), with the aim to select multi-substituted derivatives of our PrAMPs to be studied in the next experiments. However, we chose different approaches for the two groups of derivatives. While multi-substituted derivatives of Bac7(1-16) were designed based on the previous screening (Tab. 3.4), multi-substituted derivatives of Bac5(1-17) were selected after the screening of a second peptide library (Tab. 3.5),

We designed a library of 32 multi-substituted Bac5 derivatives (Tab. 3.5), which were SPOT-synthesized by the laboratory of Kai Hilpert and colleagues. Along with them, the original Bac5(1-17) and 6 of its mono-substituted mutants were included in the library, to be used as controls. Bac5(1-17) derivatives were screened for their antimicrobial activity against *E. coli* BW25113 and their cytotoxicity against MEC-1 cells (Tab. 3.5). This time, the MIC of Bac5(1-17) was four-fold higher than it resulted from our first experiment. We attributed such discrepancies to the lower purity of the SPOT-synthesis technique. Most of the substituted derivatives, anyway, displayed a better antimicrobial activity than the wild-type peptide fragments, with many having a four-fold improvement in the MIC. In the MTT assays, most peptides with few exceptions generally displayed a low cytotoxicity at up to 32 µM (Tab. 3.5) not decreasing cell viability of more than 20%, – with respect to the untreated controls –.

From the second screening of Bac5(1-17) mutants, we selected four multi-substituted peptides. Collectively, for the subsequent experiments we selected five mutants of Bac7(1-16) and five mutants of Bac5(1-17), encompassing from one to five substitutions (Tab. 3.6).

Table 3.5. MIC and cytotoxicity of the SPOT-synthesized substituted derivatives of Bac5(1-17).

Name	Sequence	MIC (µM)	Cell viab. (%)	Name	Sequence	MIC (µM)	Cell viab. (%)
B5$_{(1-17)}$	**RFRPPIRRPPIRPPFYP**	32	106	**277**	RWRRPIRRRPIRPPFYP	8	99
258	RFRPPIRRPPIRPPFYR	8	95	**278**	RWRWPIRRPPIRPPFYR	4	86
259	RFRRPIRRRPIRPPFYP	8	105	**279**	RFRWPIRRRPIRPPFYR	8	84
260	RFRRPIRRPPIRPPFYR	8	97	**280**	RFRWPIRRRPIRPPFYW	4	78
261	RFRRPIRRRPIRPPFYR	16	91	**281**	RWRRPIRRRPIRPPFYW	4	81
262	RWRPPIRRPPIRPPFYP	16	90	**282**	RWRRPIRRRPIRPPFYR	4	99

263	RFR**W**PIRRPPIRPPFYP	8	86	**283**	R**W**R**R**PIRR**R**PIRPP**W**YP	4	91
264	RFRPPIRRPPIRPP**W**YP	32	86	**284**	RFR**W**PIRR**R**PIRPP**W**Y**R**	4	72
265	RFRPPIRRPPIRPPF**W**P	16	81	**285**	RFR**R**PIRR**R**PIRPPF**WR**	8	90
266	RFRPPIRRPPIRPPFY**W**	16	94	**286**	RFR**W**PIRR**R**PIRPPF**WR**	4	80
267	R**W**RPPIRRPPIRPP**W**YP	16	73	**287**	RFR**R**PIRR**R**PIRPP**W**Y**R**	8	95
268	RFR**W**PIRRPPIRPP**W**YP	8	92	**288**	R**R**R**R**PIRR**R**PIRPPFY**R**	16	102
269	R**W**R**W**PIRRPPIRPPFYP	8	90	**289**	R**R**R**R**PIRR**R**PIRPP**W**YP	8	95
270	R**W**R**W**PIRRPPIRPP**W**YP	8	90	**290**	R**W**R**R**PIRR**R**PIRPP**W**Y**R**	8	90
271	RFR**W**PIRR**R**PIRPPFYP	4	98	**291**	R**W**R**R**PIRR**R**PIRPPF**WR**	4	96
272	RFR**W**PIRRPPIRPPFY**R**	4	105	**292**	R**W**R**R**P**W**RR**R**PIRPPFY**R**	8	76
273	RFR**R**PIRRPPIRPP**W**YP	12	68	**293**	R**W**R**W**PIRR**R**PIRPP**W**Y**R**	4	59
274	R**W**R**R**PIRRPPIRPPFYP	8	84	**294**	R**W**R**W**PIRR**R**PIRPPF**WR**	4	73
275	R**W**R**R**PIRRPPIRPPFY**R**	8	89	**295**	R**R**R**R**P**W**RR**R**PIRPPFY**W**	8	77
276	R**W**R**R**PIRRPPIRPP**W**YP	8	83	**296**	R**R**R**R**PIRR**R**PIRPP**W**Y**R**	16	96

Substitutions are in bald and red. The MIC of the peptides was determined against *E. coli* BW25113 after 18 h of incubation, and the cytotoxicity was assessed against MEC-1 cells. Toxicity is reported as percentage of viable cells after exposure to the peptides, and is reported as percentage with respect to the untreated control cells. Light blue shades indicate the peptides that were chosen for further analyses. Results are reported as the median of at least 3 independent experiments (n ≥ 3); in the single experiments, the MIC differed from the median value of a factor 2, at most. Peptides in the table are indicated only with the number without the 'B5'prefix (e.g. 258 instead of B5_258).

Among the Bac5(1-17) derivatives, we selected the peptides called B5_258, B5_272, B5_278 and B5_291 for the mono-, di-, tri- and penta-substituted peptides, respectively (best combination of MIC and cytotoxicity among the peptides with an equal number of substituents); B5_281 was chosen for the tetra-substituted, not only due to its MIC and relatively low toxicity but also for the position of the Trp residues at the two extremities of the peptides (Tab. 3.6). Differently, as mentioned above, Bac7(1-16) multi-substituted derivatives were chosen without a previous screening. We designed one derivative by combining the two most favorable tryptophan substitutions resulting from the previous screening, (peptide B7_003) and other two by combining an arginine substitution with two or three tryptophan substitutions (peptides B7_004 and B7_005). Along with these three peptides, we included in the next experiments also the two mono-substituted derivatives B7_001 and B7_002.

Table 3.6. Selected derivatives of Bac7(1-16) and Bac5(1-17) to be characterized in the following experiments.

Bac7(1-16) derivatives				Bac5(1-17) derivatives			
Name	**Sequence**	**q**	**H(GI)**	**Name**	**Sequence**	**q**	**H(GI)**
B7$_{(1-16)}$	RRIRPRPPRLPRPRPR	+8	-2.33	**B5$_{(1-17)}$**	RFRPPIRRPPIRPPFYP	+5	-1.20
B7_001	**W**RIRPRPPRLPRPRPR	+7	-2.11	**258**	RFRPPIRRPPIRPPFY**R**	+6	-1.37
B7_002	RRIRPRPPRLPRPR**W**R	+8	-2.29	**272**	RFR**W**PIRRPPIRPPFY**R**	+6	-1.33
B7_003	**W**RIRPRPPRLPR**W**RPR	+7	-2.06	**278**	R**W**R**W**PIRRPPIRPPFY**R**	+6	-1.55

B7_004	**W**RIR**R**RPPRLPRPR**W**R	+8	-2.24	**281**	R**W**R**R**PIRR**R**PIRPPFY**W**	+7	-1.72
B7_005	**W**RIR**R**R**W**PRLPRPR**W**R	+8	-2.2	**291**	R**W**R**R**PIRR**R**PIRPPF**WR**	+8	-1.91

Substituting resisdues are in bald and red. 'q' = net charge of the peptide. H(GI) Average hydropathy (GRAVY index).

Characterization of the Bac5 and Bac7 selected derivatives

The selected derivatives of Bac5(1-17) and Bac7(1-16), hereafter collectively referred to as 'Bac selected derivatives', were then synthesized via solid-phase FMOC chemistry, to ensure a higher purity of the preparation, before proceeding to assess their spectrum of activity, cytotoxicity on cell lines, mode of action and other properties.

Antimicrobial activity

The Bac selected derivatives were first tested for their activity against *E. coli*. Specifically, they were tested against *E. coli BW25113* and the mutant BW25113 *ΔSbmA*, lacking the SbmA transporter, and six pathogenic *E. coli* strains, including verotoxigenic (VTEC), enteropathogenic (EPEC), enteroinvasive (EIEC) and enteroaggregative (EAEG) *E. coli* strains (Tab. 3.7). The most substituted peptides generally showed little changes in MIC between the wild type and the *ΔSbmA* strain, suggesting to be less dependent from the membrane transporter (Tab. 3.7). Such difference between Bac7(1-16) and the other derivatives was also visible in the case of *E. coli* STEC:O157 strain. Derivatives of Bac7(1-16) were equally effective (MIC ≤ 2 µM) in comparison with Bac7(1-16) being nearly as efficient against all the other *E. coli* strains.

Different results were observed for Bac5 derivatives. The multi-substituted variants, having MIC ≤ 8 µM, were significantly more efficient than Bac5(1-17) and the mono-substituted B5_258, (Tab. 3.7). The highest difference was observed against Δ*SbmA* and STEC:O157 strains. The tri- and tetra-substituted B5_281 and B5_291 were the most effective with MIC ≤ 2 µM against all strains except BW25113*ΔSbmA*, (MIC = 4 µM) (Tab. 3.7).

Table 3.7. Antimicrobial activity (MIC) of Bac7 and Bac5 selected derivatives against different *E. coli* strains.

	MIC (µM)											
***E. coli* strains**	**Bac7 (1-16)**	**001**	**002**	**003**	**004**	**005**	**Bac5 (1-17)**	**258**	**272**	**278**	**281**	**291**
BW25113	3	1	1	2	2	2	16	8	4	2	2	2
BW25113*ΔSbmA*	16	12	4	16	3	4	> 64	64	8	8	4	4

EURL-VTEC A07 EPEC:O111	2	1	1	1	1	1	16	8	2	1	2	2
EURL-VTEC C07 STEC:O157	>64	32	4	8	4	2	>64	>64	8	8	2	2
SSI-NN14 ETEC	1	1	1	1	1	1	2	2	1	1	2	1
EA22 ETEC	1	1	1	1	1	1	2	2	1	1	2	1
SSI-OO15 EIEC	1	1	1	1	1	1	16	4	1	1	1	1
C679-12 EAEC:0104	1	1	1	1	1	1	16	8	2	2	2	1

MIC was determined after at least 18 h incubation of bacteria with the peptides. Results indicated are the median of at least 3 independent experiments (n ≥ 3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

These results validated the screening of the SPOT-synthesized libraries on different *E. coli* strains. We then explored the spectrum of activity of Bac selected derivatives, by testing them against reference strains of four ESKAPE pathogens and *S. typhimurium* (Tab 3.8). Again, the new Bac selected derivatives, and especially the multi-substituted ones, displayed higher antimicrobial potency than the original peptides (Bac5-derivatives) or a broader activity spectrum (Bac7-derivatives). All the Bac7 peptides were highly active against *S. typhimurium* (MIC = 0,5 - 4 µM) and *K. pneumoniae* (MIC = 2 - 8 µM, except B7_001). Notably, B7_005, B5_272, B5_278, B5_281 and B5_291 inhibited the growth of *A. baumannii* at 2-8 µM. Interestingly, B7_005 had MIC = 8 µM against the Gram-positive *S. aureus*, while PrAMPs are usually less efficient against this pathogen. On the contrary, none of the PrAMPs was particularly effective against *P. aeruginosa*. B5_278 resulted the most active with a MIC of 16 µM (Table 3.8).

Table 3.8. Antimicrobial activity of Bac7 and Bac5 selected derivatives against reference bacterial pathogens.

	MIC (µM)											
Bacterial strain	**Bac7 (1-16)**	**001**	**002**	**003**	**004**	**005**	**Bac5 (1-17)**	**258**	**272**	**278**	**281**	**291**
S. aureus ATCC 25923	>64	>64	>64	>64	32	8	>64	>64	>64	>64	32	64
K. pneumoniae ATCC 700603	4	16	2	8	4	2	>64	>64	24	16	64	6
A. baumannii ATCC 19606	48	32	12	32	32	4	>64	64	8	4	2	8
P. aeruginosa ATCC27853	> 64	>64	>64	>64	64	32	>64	>64	64	16	32	32
S. typhimurium ATCC 14028	1	2	0,5	4	1	1	64	32	8	4	2	4

MIC was determined after at least 18 h incubation of bacteria with the peptides. Results indicated are the median of at least 3 independent experiments (n ≥ 3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Effects of peptides on the bacterial membrane

The type and position of the amino acids substitutions in Bac selected derivatives and the lower influence of SbmA on the effectiveness of some peptides suggested that they could have changed their mechanisms of action. Therefore, we tested their ability to permeabilize the membranes of *E. coli* BW25113 (Fig. 3.5). Bacterial cells were incubated with peptides at 4 and 16 µM, so as to encompass concentrations in the range of 1- to 16-fold the MIC of the peptides. The mean fluorescence intensity of propidium iodide was measured. Bac7(1-16), B7_001, and B7_003 caused no detectable membrane permeabilization (Fig. 3.5A). B7_002 and B7_004 caused some detectable membrane permeabilization only at 16 µM, 8-16 fold their MICs. Differently, B7_005 already at 4 µM, twice its MIC, caused 10% fluorescence elicited by colistin, used as a positive control for membrane permeabilization. Fluorescence caused by 16 µM B7_005 was 29% of that of colistin (Fig. 3.5). A similar behavior was detected also with the Bac5-derivatives. The peptides B5_281 and B5_291 displayed permeabilizing effects already at 4 µM, twice their MIC (Fig. 3.5B).

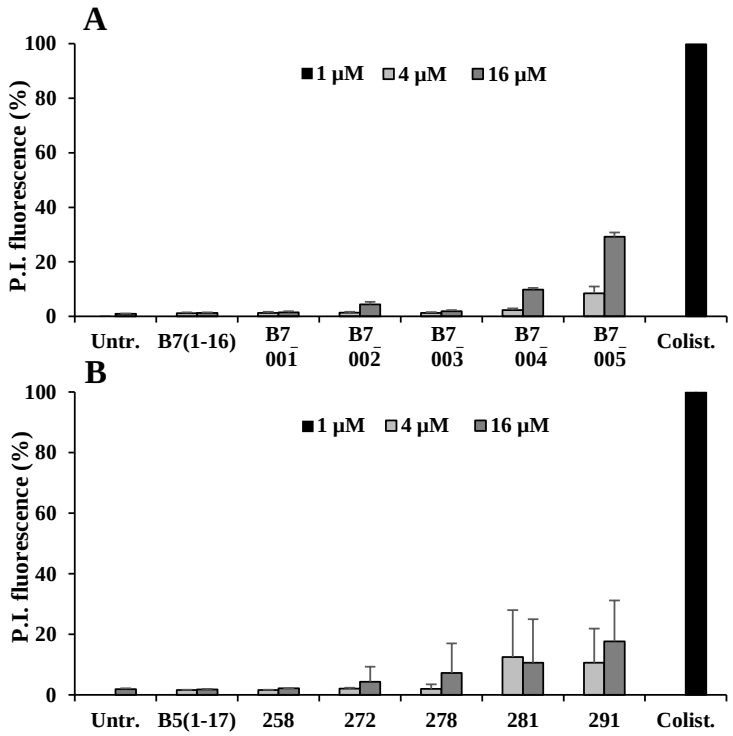

Figure 3.5. Membrane-permeabilizing activity of the selected derivatives of Bac7(1-16) (A) and Bac5(1-17) (B) on the membranes of *E. coli* BW 25113. Propidium iodide (PI) fluorescence of *E. coli* BW 25113 cells incubated 30 min with the peptides. Results are the average of three indepepdent experiments (n=3) and are reported as percentage with respect of the positive control (Colistin 1µM). Measurements were taken with two different fluorescence cytometers for Bac5 and Bac7 derivatives.

However, none of the PrAMPs, including the most permeabilizing ones at 16 µM, reached even a third of the fluorescence caused by 1 µM colistin.

Inhibition of bacterial translation

Investigation of the mechanism of action of Bac selected derivatives included transcription/translation assays. These assays have been carried out by collaborators, working in the lab of Prof. Daniel Wilson (Fig. 3.6) who also obtained X-ray structures of Bac7(1-16) and the peptides B7_001 and B7_002 complexed with the ribosomes of *Thermus thermophilus* (not shown)[229,230]. The transcription/translation assays were made as described above for Bac5 fragments of different length. The assays confirmed the strong inhibiting activity of Bac7(1-16) and Bac5(1-17), suppressing more than 90% translation at 1 μM and 80% translation at 5 μM, respectively. Among Bac7(1-16) derived PrAMPs, the three peptides B7_001, B7_002 and B7_004 retained excellent inhibiting activity, reducing translation of more than 70% at 1 μM. peptide B7_005 was less effective at 1 μM but still caused a > 90% decrease in luciferase expression at 5 μM. On the contrary, B7_003 lost much of the translation-inhibiting power of the original fragment, reducing luciferase expression of ca 50% and 65% at 5 μM and 10 μM, respectively. Among the derivatives of Bac5(1-17), peptides B5_258, B5_272 and B5_278 at 5 μM reduced translation of 80% or more, while 5 μM peptide B5_291 had a slightly lower but still significant effect. B5_281 was the worst inhibitor yielding only a 60% reduction in luciferase at 25 μM. It is worth noting that B7_005 and B5_291 besides retaining a translation-inhibiting activity, also resulted to significantly permeabilize *E. coli* membranes, thus suggesting a double mechanism of action for these peptides. A double mode of action can explain also their wider activity spectrum, and perhaps this feature may make it harder for bacteria to develop efficient mechanisms of antimicrobial resistance.

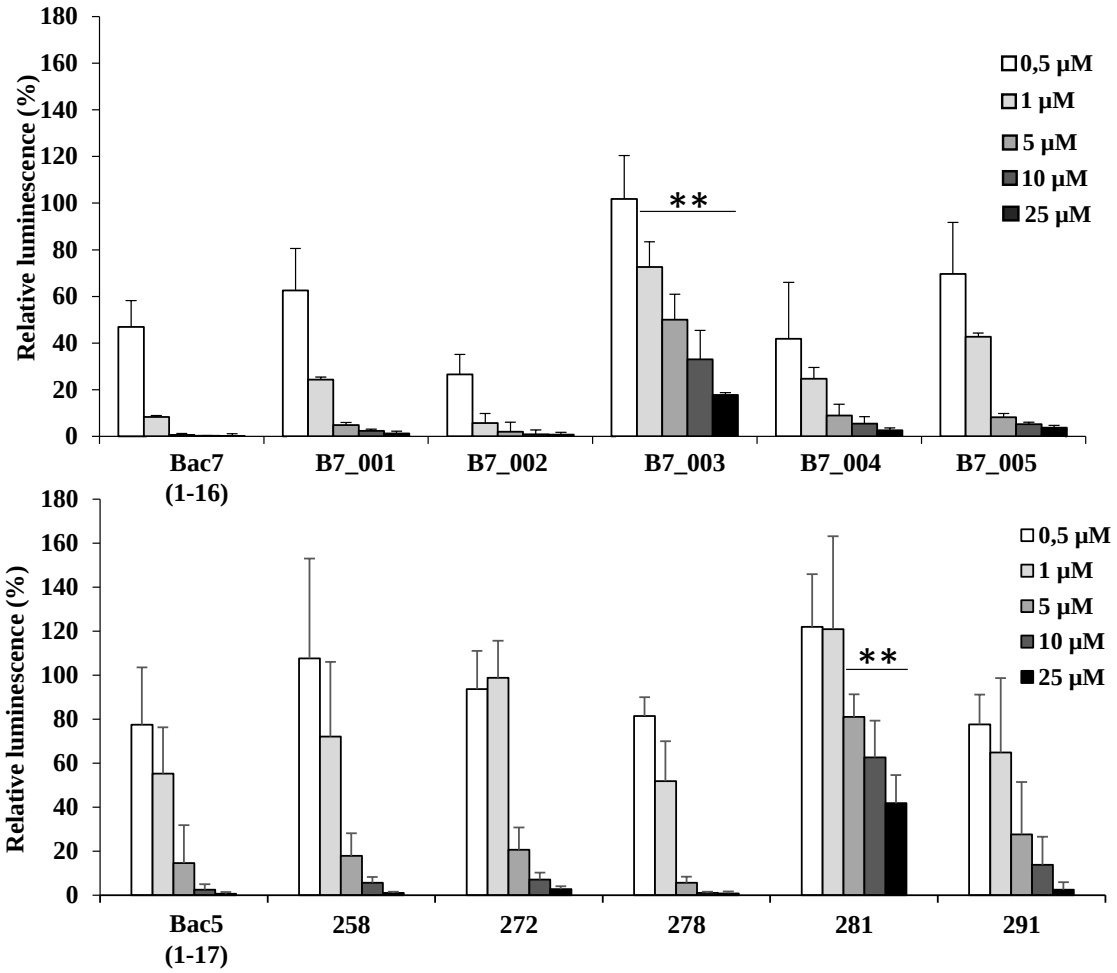

Figure 3.6. ***In vitro*** **Inhibition of transcription/translation mediated by selected derivatives of Bac7 (top) and Bac5 (bottom)**. Luminescence of a reporter luciferase produced after 1 h incubation of *E. coli* lysates with the peptides.. Luminescence is expressed as the percentages with respect to the negative control of inhibition, where RNAse-free water was added instead of the peptides. 'wt' = wild-type fragments Bac7(1-16) on top and Bac5(1-17) on bottom. Results with Bac7 derivatives are reported the average of three independent experiments each with internal duplicate (n=6), while results with Bac5 derivatives are the average of five indepentend experiments (n=5). ** = $p < 0,01$ (T-test, comparing treated sample versus the untreated samples, i.e. negative control of inhibition)

Cytotoxicity on eukaryotic cells

We determined the possible cytotoxic effects of Bac selected derivatives on the lymphocytic leukaemia MEC-1 cells, already used (see above) to test the Bac5 fragments. Bac7(1-16) and its derivatives showed basically no significant effects on the viability of MEC-1 cells(Fig. 3.7). Bac5(1-17) derivatives B5_258, B5_272 and B5_278 reduced cell viability of 20% at 16-64 µM, resulting slightly more cytotoxic. Apparently, it is not a dose-dependent effect. Peptide B5_281 reduced viability of a striking 80% when used at 64 µM, while B5_291 did not significantly affect cell vitality at any concentration (Fig. 3.7).

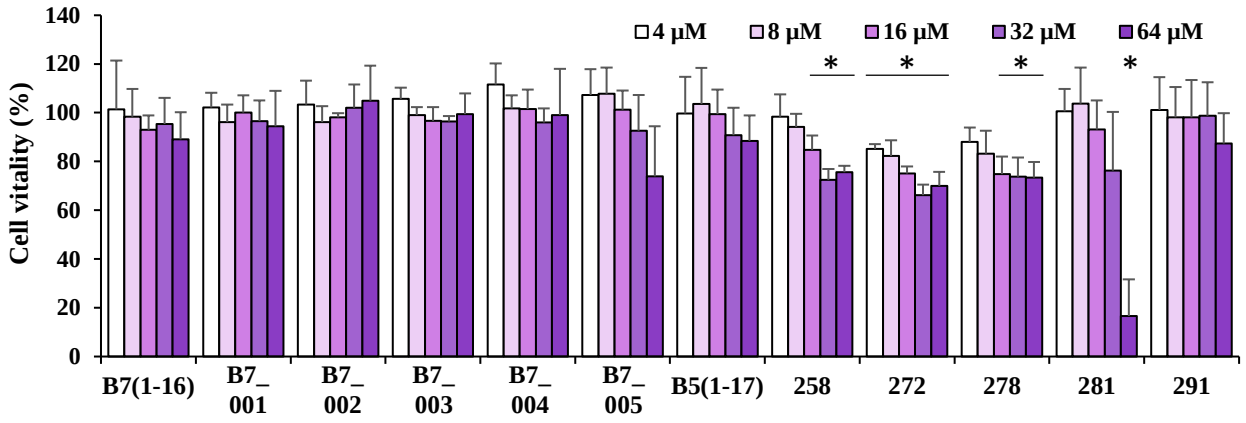

Figure 3.7. Effects of Bac7 and Bac5 selected derivatives on cell viability. Results of MTT assays on MEC-1 cells exposed for 24 h to the peptides. Cell viability (measured as absorbance at 570 nm) is reported as a percentage with respect to the untreated controls. Averages and standard deviations of three independent experiments (n=3). * = $p < 0,05$ (T-test, comparing treated samples versus untreated controls).

Antimicrobial activity in presence of serum and high salinity

Stability in presence of serum would be important in case of parenteral injection of peptides, while the stability in saline concentration would be a crucial requirement to include PrAMPs in aerosols with highly salty water; these latter preparations are used as a therapy to treat of infections in cystic fibrosis[234]. For these reasons, stability of Bac7 and Bac5 derivatives were assayed testing their antimicrobial activity in MH medium supplemented with 10% of human serum (HS) and in MH with a high saline concentration (2.7% NaCl, w/v) (Tab 3.9). Preliminary data indicated that Bac7 derivatives could withstand the presence of serum, increasing their MICs of no more than four-fold. Notably, the increase in MIC was higher when the serum was previously inactivated by a pre-heating of 1h at 60° C. This suggested that the inhibition by human serum was not due to the cleavage of peptides by some protease (which would be inactivated by the pre-heating step); instead, it may be due to some non-covalent interactions between peptides and serum proteins that could sequester the peptides, and could still take place on a denatured protein exposing previously hidden portions of its sequence. Presence of high saline concentrations represented for Bac7 derivatives a higher hindrance than the serum. All Bac7 derivatives increased their MIC by four-fold or more, with the most potent derivatives B7_004 and B7_005 showing an 8-fold and a 16-fold increase in their MIC. Importantly, however, the high saline concentration yielded quite variable results in the MIC assays, and it was not uncommon to see bacterial growth at one peptide concentration, with no growth at both higher and lower concentrations. This made it difficult to

clearly interpret these data. Nevertheless, these data suggested that some peptides were less affected by high salinity than others. We found it hard to relate salt and serum sensitivity to precise features of the peptides like sequence motifs, mode of action or other properties. Bac5 derivatives were significantly more affected by both conditions, with the three most efficient derivatives increasing their MIC of 16 to 32-fold in presence of 10% HS or 2.7% NaCl. The less marked change in MIC was seen for Bac5(1-17), which in presence of 10% HS doubled its MIC from 16 to 32 µM..

Table 3.9. Variations of antimicrobial activity of Bac7 and Bac5 derivatives against *E. coli* ATCC 25922 in presence of human serum or high saline concentration.

	MIC (µM)*											
	$Bac7_{(1-16)}$	**001**	**002**	**003**	**004**	**005**	**$Bac5_{(1-17)}$**	**258**	**272**	**278**	**281**	**291**
MH	1	1	1	1	2	1	16	16	4	2	2	1
+10%HS	1	1	1	2	4	2	32	>32	>32	32	32	32
+10%HS (h.i.)	2	2	2	4	8	4	>32	>32	>32	>32	>32	>32
2,7% NaCl	4	32	4	>32	16	16	>32	>32	>32	>32	>32	16

*MIC (µM) of Bac5 and Bac7 selected derivatives after 18 h of incubation in Mueller-Hinton broth alone (MH, on top), supplemented with 10% human serum (+10%HS) without or with previous heat-inactivation (h.i.), or with 2,7% (v/w) NaCl. Results are the median of at least three independent experiments (n=3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Characterization of novel cetacean PrAMPs (cePrAMPs)

The second part of this thesis was aimed to characterize novel PrAMPs, to search for novel alternatives to the peptides currently under study. The research of new PrAMPs started by looking for orthologs of Bac7(1-35) because this peptide was long studied in our laboratory, and several aspects of its activity, mode of action, structure and indirect antimicrobial action had been investigated.

Discovery of the cePrAMPs

Using the complete protein sequence of the cathelicidin Bac7 – including the conserved pre-pro-sequence – as a query, we performed a 'protein-BLAST' (pBLAST) search in the genome of cetacean species (taxid: 9721) in the 'Non-redundant protein sequence' database of NCBI. Then we observed the C-terminal domains of the cathelicidins displayed as search output, looking for sequences with significant similarity with the PrAMP Bac7(1-35). This allowed us to find the two dolphin PrAMPs called Tur1A and Tur1B discovered by Mardirossian et al. (2018), along with

other five PrAMPs coded by the genomes of killer whale (*Orcinus orca*), beluga (*Delphinapterus leucas*), minke whale (*Balaenoptera acutorostrata scammonii*), chinese freshwater dolphin (*Lipotes vexillifer*), and narrow-ridged finless porpoise (*Neophocaena asiaeorientalis*). The C-terminal proline-rich AMP moiety of these peptides was clearly recognizable after the tripeptidic 'QSV' sequence motif, which is the site recognized and cleaved by elastases in order to release the C-terminal AMP of cathelicidins from their pro-sequence[126].

We named these cetacean PrAMPs (Tab 3.10) – hereafter also referred to as cePrAMPs – with the first letters of the genus in which they were found, followed by the number 1. All such sequences show a significant degree of similarity with Bac7(1-35), with a great number of residues being conserved across all the cePrAMPs and the bovine peptide. All the cePrAMPs sized 32 residues in length, with the exception of Neo1 which has 19 residues. However, the shorter size of Neo1 seems due to a premature stop codon, as the triplets of the Neo1 gene beyond the stop codon code for a stretch of twelve residues highly homologous to the C-terminus of cePrAMPs.

Table 3.10. Sequences, animal of origin and physico-chemical properties of the cetacean PrAMPs of this work, compared to the bovine PrAMP Bac7(1-35).

Species	peptide	sequence	q	H(GI)
Orcinus orca	Orc1	RRIPFWPPNLPGPRRPPWFLPDFRIPRIPRKR	+8	-1.1
Delphinapterus leucas	Del1	RRIPFWPIPLRWQWPPPWFPPSFPIPRISRKR	+7	-0.8
Neophocaena asiaeorientalis	Neo1	RRIRFPFPPFPWQWPPAGF*ptf**h**ip**r**ip**rk**q*	+3	-0.6
Tursiops truncatus	Tur1A	RRIRFRPPYLPRPGRRPRFPPPFPIPRIPRIP	+10	-1.1
	Tur1B	RRIPFWPPNWPGPWLPPWSPPDFRIPRILRKR	+6	-1.0
Balaenoptera acutorostrata	Bal1	RRIRFRPPRLPRPRPRPWIPPRFPFPRIPGKR	+12	-1.5
Lipotes vexillifer	Lip1	RRIRIRPPRLPRPRPRPWFPPRFPIPRIPGKR	+12	-1.4
Bos taurus	Bac7(1-35)	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFP	+11	-1.4

Positively and negatively charged residues are indicated in red and blue, respectively; tryptophan residues are indicated in violet; residues conserved in > 50% of the sequences are shaded in grey. 'q' = charge; H(GI) = average hydropathicity score (GRAVY index). Residues of Neo1 that are coded by the triplets downstream of a premature stop codon are written in lowercase. A highly identical stretch (16 residues on 17) in The N-terminal of Bal1, Lip1 and Bac7(1-35) is underlined.

The seven cePrAMPs have a high proline content (>30%) and a high cationicity; the charge of the six 32 aa-long peptides span from +6 to +12, and the shorter Neo1 has a charge of +3 (although,

were it not for the premature stop codon, it would have a charge of +7). Along with a different total charge, the peptides have a different degree of hydrophobicity (the GRAVY index in Tab 3.10) and different amphipathicity, differing in the number, type and localization of their hydrophobic residues. Peptides Del1, Tur1B and Neo1 had the highest hydrophobicity and the longest hydrophobic portions, with stretches of 7-9 consecutive hydrophobic residues. Tur1A, Bal1 and Lip1 were the most cationic and most similar to Bac7(1-16) in their sequence. The cationicity and hydrophobicity of Orc1 kind of locates in between the two mentioned groups.
Similarly to what we did with Bac5(1-17), Bac7(1-16) and their derivatives, we have characterized the cePrAMPs for their antimicrobial activity, cytotoxicity and mode of action, comparing their properties with Bac7(1-35).

Antimicrobial activity of cePrAMPs

To start the characterization of the cePrAMPs, we have investigated their spectrum of activity by testing them against a wide panel of pathogens, including members of the ESKAPE group, *S. typhimurium, S. maltophilia* and the yeast *Candida albicans* (Tab 3.11A). All the cePrAMPs except Neo1 exhibited MIC = 8 μM against *E. coli, A. baumannii, S. maltophilia* and *C. albicans*. Strikingly, the three cePrAMPs Bal1, Lip1 and Del1 showed a good antimicrobial action against Gram-positives, having MIC between 4 and 16 μM against *E. faecium* and *S. aureus*. Bal1, Lip1, Tur1A and Bac7 (1-35) showed a significantly broad spectrum of activity, with MIC between 0,5 μM and 2 μM against *E. coli, S. typhimurium, K. pneumoniae, A. baumannii, S. maltophilia, C. albicans* and a clinical strain of *E. agglomerans*. In particular, Bal1 and Lip1 resulted the most potent antimicrobial peptides, with MIC ≤ 2 μM against all the Gram-negative pathogens and *C. albicans*, and MIC of 4-16 μM against the Gram-positives; notably, their MIC was 2 μM also towards *P. aeruginosa*, a bacterium that is rarely susceptible to PrAMPs. The less potent of the tested peptides was Neo1, displaying MIC ≥ 16 μM against almost every pathogen tested. Again, this may be due to the premature stop codon. Interestingly, while the MIC of Bac7(1-35) against the wild type and the *ΔSbmA* variant of *E. coli* BW25113 differed more than eight-fold, the MIC of cePrAMPs did not change much in absence of SbmA, being in some case even lower. These findings suggest that the action of cePrAMPs is less dependent on the uptake via SbmA than Bac5 and Bac7 fragments.

Along with Neo1, the peptides Orc1, Del1 and Tur1B also displayed a generally lower activity compared to other cePrAMPs. Orc1, Del1, Tur1B and Neo1 were also hardly soluble in MHB, forming some visible precipitates, possibly due to their higher hydrophobicity, which likely

hampered their antimicrobial power. Thus, all the cePrAMPs and Bac7(1-35) were tested again against some of the previous bacteria in 20% MHB in PBS (v/v) (Tab 3.11B). However, such diluted MHB did not greatly alter the difference in MIC between those peptides that formed precipitates (Orc1, Del1, Tur1B and Neo1) and the other more hydrophilic peptides, and in some cases unexpectedly exacerbated this difference. This could be because the peptides still formed precipitates in 20% MHB, but we did not try other poorer media so as not to excessively stress bacteria.

Table 3.11A. Antimicrobial activity of cePrAMPs and Bac7(1-35) against a panel of bacterial pathogens.

Microorganism and strain	MIC (μM)							
	Orc1*	**Del1***	**Bal1**	**Lip1**	**Tur1A**	**Tur1B***	**Neo1***	**Bac7$_{1-35}$**
E. coli ATCC 25922	6	6	1	1	1	8	16	1
***E**. faecium* ATCC 19434	16	4	4	4	64	16	> 64	64
***S.** aureus* ATCC 25923	*32*	8	16	16	> 64	32	16	> 64
***K.** pneumoniae* ATCC 700603	32	> 64	1	1	2	> 64	> 64	2
***A.** baumannii* ATCC 19060	2	4	1	1	1	4	16	2
***P.** aeruginosa* ATCC 27853	32	16	2	2	16	32	64	16
***E.** agglomerans - clinical isolate*	8	8	0,5	0,5	1	16	64	0,5
S. maltophilia ATCC 13637	2	4	1	0,5	1	8	24	1
C. albicans ATCC 90029	4	8	1,5	2	2	8	32	2
S. typhimurium ATCC 14028	32	> 64	1	0,5	0,5	> 64	> 64	0,5
E. coli BW25113	8	8	1	1	0,75	16	24	0,75
E. coli BW25113 - *Δ SbmA*	2	8	2	1,5	1,5	8	16	8

The MIC was measured after 18 h incubation of bacteria with the peptides. Results are the median of at least 3 independent experiments (n ≥ 3). * Peptides Orc1, Del1, Tur1B and Neo1 displayed visible formation of precipitates in MHB. Results are the median of at least 3 independent experiments (n ≥ 3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Table 3.11B. Antimicrobial activity of cePrAMPs and Bac7(1-35) against five bacterial species in 20%(v/v) MHB in PBS.

Microorganism and strain	MIC (μM) – 20%MHB –							
	Orc1	**Del1**	**Bal1**	**Lip1**	**Tur1A**	**Tur1B**	**Neo1**	**Bac7$_{1-35}$**
E. coli BW25113	4	1	0,5	0,5	0,5	4	16	0,5

E. coli BW25113 *ΔSbmA*	2	1	0,5	0,5	1	4	8	2
E. coli ATCC 25922	4	2	0,5	0,5	0,5	4	32	0,5
S. aureus ATCC 25923	64	2	4	4	>64	16	64	>64
K. pneumoniae ATCC 700603	64	8	0,5	0,5	1	>64	>64	1
A. baumannii ATCC 19606	1	1	0,5	0,5	0,5	1	8	0,5
P. aeruginosa ATCC 27853	16	8	1	1	4	16	64	2

Results are the median of at least 3 independent experiments (n ≥ 3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Circular dichroism spectra in polar and amphipathic environments

We tried to get some insights on the secondary structure of cePrAMPs and Bac7(1-35) by determining their CD spectrum in polar and amphipathic environments (Fig. 3.8). The CD spectra were measured in 10 mM SPB without and with the supplement of 10 mM SDS, to mimic the physiological aqueous milieu and the amphipathic environment of bacterial membranes, respectively. Those cePrAMPs with the highest hydrophobicity and the most extended stretches of hydrophobic residues, i.e. Del1, Tur1B and Neo1, showed a marked change in their CD spectrum in presence of SDS micelles. Along with the minimum in the CD function showed by all the peptides, the CD spectrum of these peptides in presence of SDS showed a second minimum at around 230 nm. Although we could not relate this spectrum to any standard secondary structure, these results showed a clear structural rearrangement in presence of SDS micelles, which has often been associated to those AMPs which perturb or disrupt the bacterial membrane. Differently, Orc1, Tur1a and Bac7(1-16) did not show a significant spectrum change in the two environments. Quite unexpectedly, slighter but visible structural rearrangements in presence of SDS micelles occurred to Bal1 and Lip1, despite having the highest positive charge and the lowest hydrophobicity.

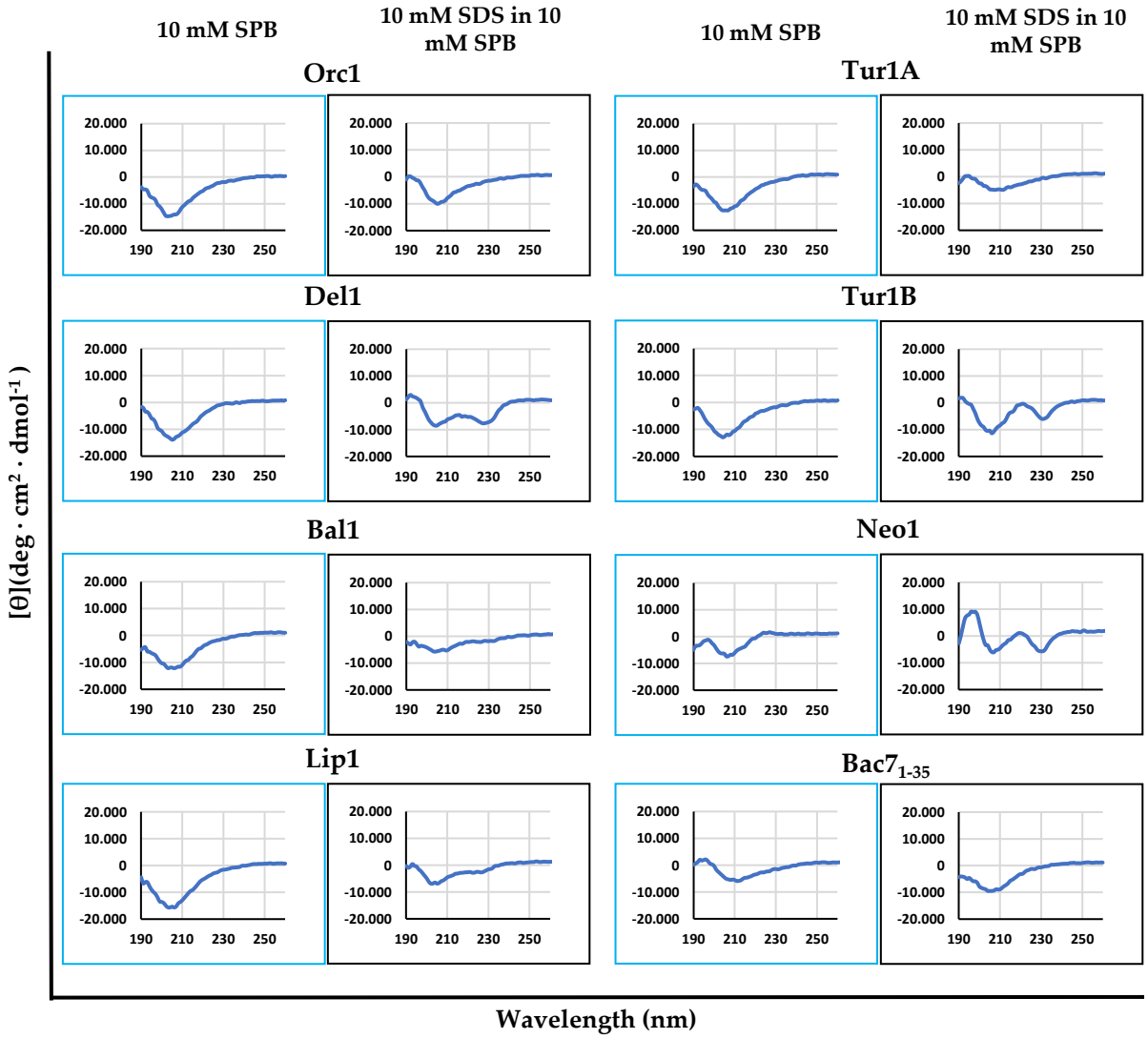

Figure 3.8. Circular Dichroism spectra of cetacean PrAMPs and Bac7(1-35). CD spectra of the peptides (20 μM) in 10 mM sodium phosphate buffer (SPB, first and third columns) and in 10 mM sodium dodecyl sulphate (SDS) in 10 mM SPB (second and fourth columns). Spectra derive from the accumulation of three scans.

Effects of cePrAMPs and Bac7(1-35) on the bacterial membrane

The scarce reliance on SbmA and the structural changes in CD spectra in amphipathic milieu suggested that some cePrAMPs might have the ability to permeabilize bacterial membranes. To shed light on this point, we assessed the peptide-mediated permeabilization of ML35 *E. coli* membranes through the beta-galactosidase assay. Briefly, The ML35p strain of *E. coli* constitutively expresses a beta-galactosidase which can cleave the chromogenic probe dye O-nitrophenyl-beta-d-galactopyranoside (ONPG), releasing the colored compound O-Nitrophenol (ONP). However, since this *E. coli* strain does not express a sugar transporter, ONPG cannot access the bacterial cytoplasm unless passing through breaches in the membrane, and thus the cleavage of

ONPG becomes an indicator of membrane permeabilization [231] . The peptides were tested at concentrations equal to ½-, 1- and 2-fold their MIC, being very different in their permeabilizing ability (Fig. 3.9). Tur1B and Neo1 displayed the most rapid and potent permeabilizing action at all these concentrations, strongly increasing the ONP signal already at half their MIC, showing a faster permeabilization kinetic than the permeabilizing peptide colistin used as positive control. At their MIC, Tur1B and Del1 were more than twice as permeabilizing than colistin. Orc1 also caused a mild permeabilization at ½ × MIC and was the third most permeabilizing cePrAMP when tested at the MIC. Del1 at its MIC caused a mild permeabilization, approximately 55% of the positive control, after one hour of incubation, but when tested at 2 MIC its action was more permeabilizing than colistin and Orc1. Thus, the low action of Del1 at its MIC may be related to a slower permeabilization kinetics. The membrane-permeabilizing action of Del1, Tur1B and Neo1 is quite consistent with their change in CD spectrum in presence of SDS. On the other hand Orc1, not showing changes in CD spectrum, showed permeabilization kinetics which differed from colistin and the other cePrAMPs, with little variation at MIC and 2 MIC. One could speculate that such different kinetics and CD profile may suggest that Orc acts via its own permeabilization mechanism different from the others. On the contrary, Tur1A and Bac7(1-35) showed no permeabilizing activity. Bal1 and Lip1 caused no permeabilization at their MIC but elicited a mild ONP signal (ca 17-19% of positive ctrl) when used at twice their MIC. This result can also be considered consistent with the very mild alterations of their CD profiles in amphipathic environments.

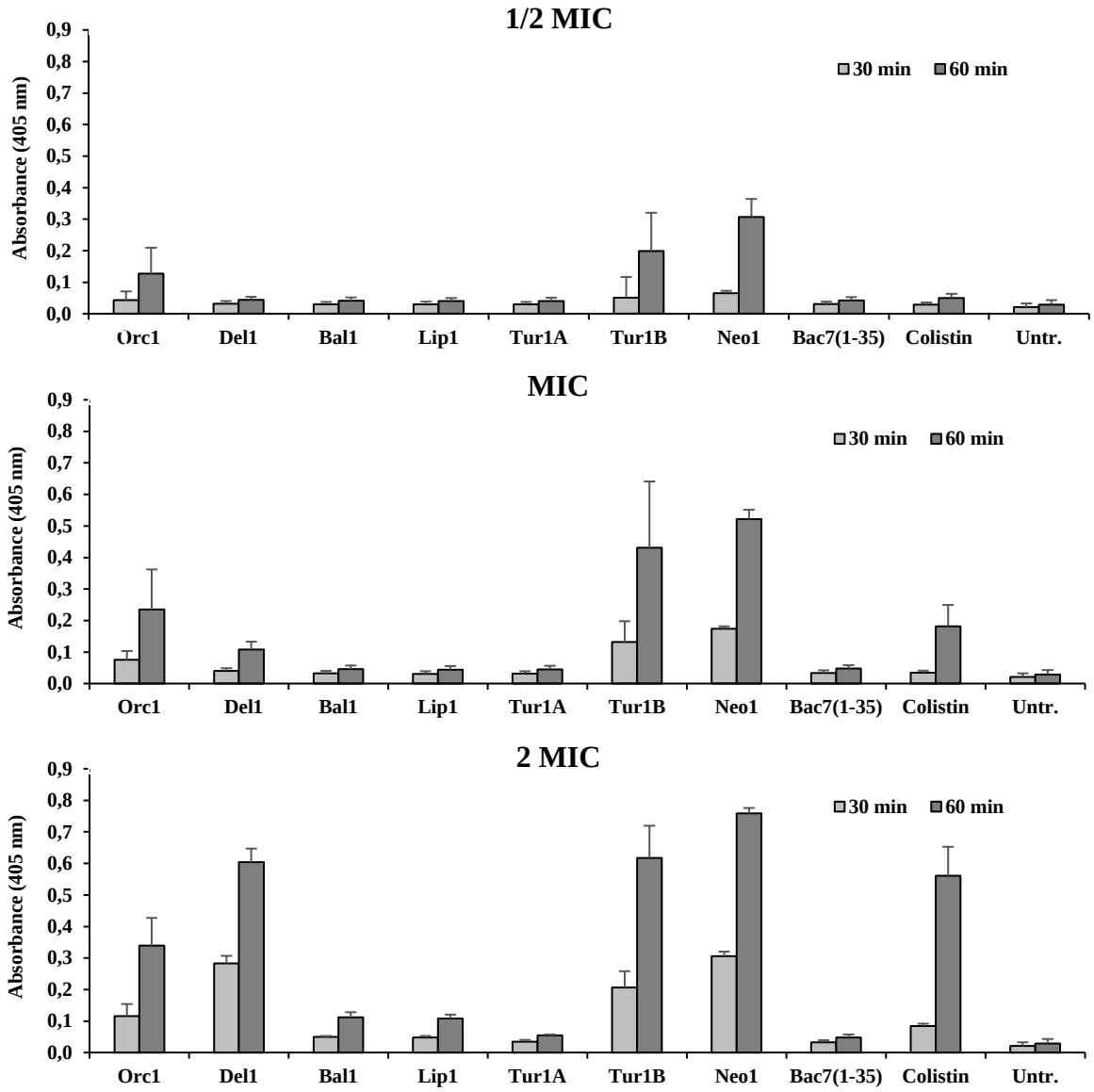

Figure 3.9. Effects of cePrAMPs and Bac7(1-35) on the permeabilization of *E. coli* ML-35p membranes. Measurement of peptide-mediated membrane permeabilization at ½ MIC, MIC and 2 × MIC after 30 min and 60 min of exposure of bacteria to the peptides. Permeabilization was followed by measuring the absorbance at 405 nm of the metabolite o-nitrophenol, produced only in case of membrane permeabilization. 1 μM Colistin was used as permeabilization control. Averages and standard deviations of 3 independent experiments (n = 3).

Inhibition of bacterial translation

Subsequently, thanks to a collaboration with prof. Wilson and colleagues, we assayed the novel peptides with the transcription/translation assay (Fig. 3.10). Bac7(1-35) and Tur1A were used as a control of translation inhibition [143,196] . Bal1 and Lip1 strongly inhibited the luciferase expression at 1 μM, completely nullifying it at 5 μM, similarly to Bac7(1-35) and Tur1A. This was expected,

given the almost complete homology of the N-terminal 17 residues of Bac7, Bal1 and Lip1. Moreover, due to such similarity, we can suppose that they inhibit translation rather than transcription. Conversely, Tur1B and Neo1 displayed almost no inhibitory activity, while Orc1 and Del1 showed only a mild concentration-dependent inhibitory effect.

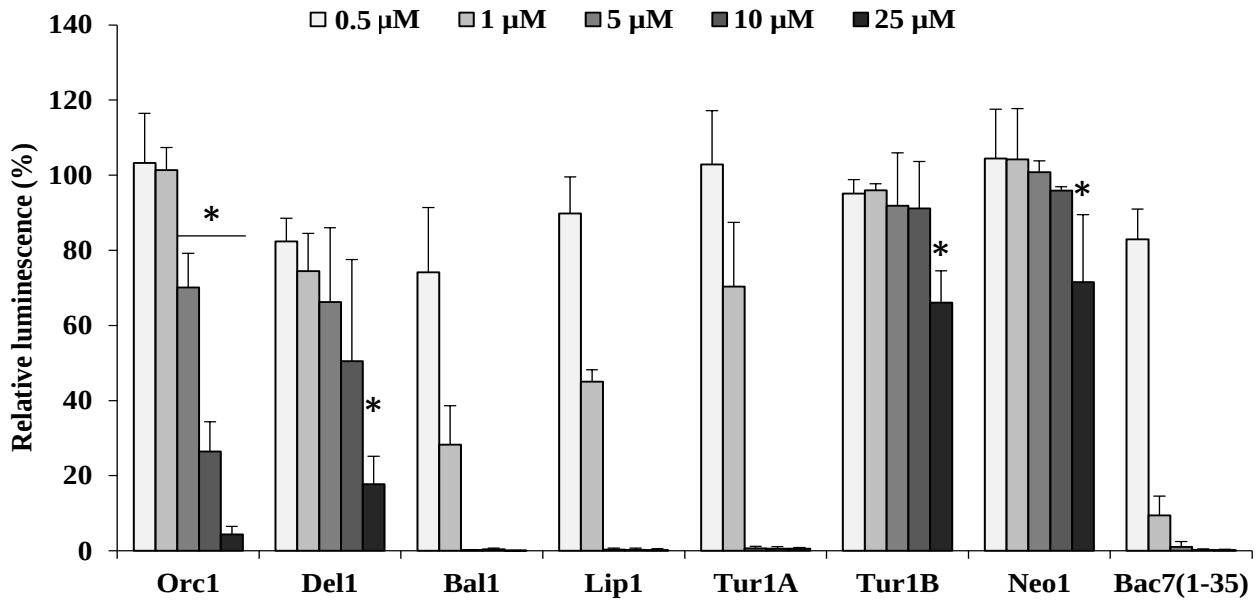

Figure 3.10. In vitro inhibition of transcription/translation mediated by cePrAMPs and Bac7(1-35). Luminescence of a reporter luciferase, produced after 1 h incubation of *E. coli* lysates with the peptides, is expressed as percentages with respect to the negative control of inhibition, where RNAse-free water was added instead of the peptides. Bac7(1-35) was used as positive control of inhibition. Results are the average and standard deviations of three independent experiments (n = 3). * = P< 0,05 (T-test, comparing treated samples versus the untreated samples, i.e. negative control of inhibition).

Cytotoxicity on eukaryotic cells

To broaden the characterization of these cePrAMPs, we have tested their toxicity against human red blood cells (hRBCs) and two human cell lines MEC-1 and HaCat (immortalized human keratinocyte) (Fig. 3.11 and 3.12). In the hemolysis assay, Del1 caused an evident concentration-dependent hemolytic effect; Tur1B caused a 32% hemolysis only at 32 µM, while Bac7(1-35) and five cePrAMPs did not cause any detectable hemolysis. Such results remained basically the same at hRBCs concentrations of 4% (v/v) and 0,4%(v/v) (Fig. 3.11). The MTT assay on HaCat cells revealed that none of the peptides affected cell viability at up to 16 µM while only Tur1A and Orc1 caused a mild damage at 32 µM (Fig. 3.12, upper panel). Differently, The MTT assay on MEC-1

cells revealed a certain degree of susceptibility of these cells to most of our cePrAMPs (Fig. 3.12, lower panel). All the peptides at 32 µM, including Bac7(1-35), decreased cell viability of least 30%. Quite surprisingly, the membrane-permeabilizing peptides were did not show the highest toxicity whereas Bal1 and Lip1 caused a drop in cell viability to less than 50% at 8 µM and to less than 20% at 16 µM, thus showing the most drastic cytotoxic effect.

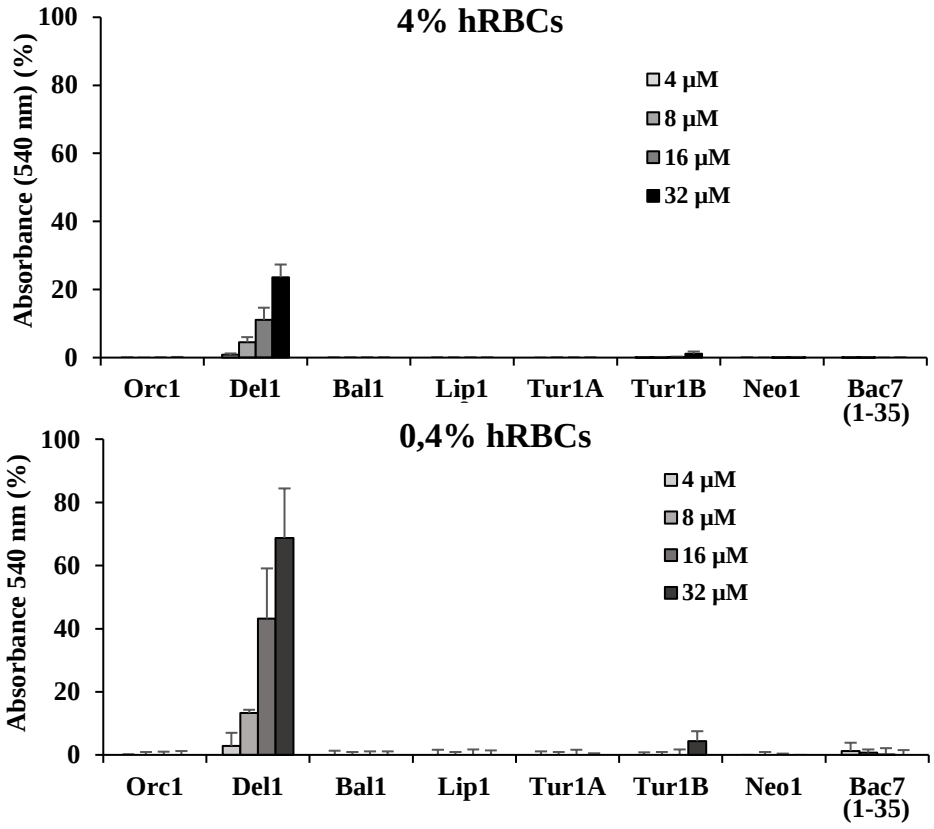

Figure 3.11. ***In vitro*** **hemolytic activity of cePrAMPs and Bac7(1-35) on human red blood cells (hRBCs)**. *In vitro* hemolysis assays with hRBCs resuspended in PBS to the final concentrations of 4% and 0,4% (v/v). released hemoglobin (absorbance at 540 nm) is reported as percentages compared to the positive control, i.e. hRBCs treated with 0,1% Triton -X 100. Results with 4% hRBCs and 0,4% hRBCs are the average of three and two independent experiments (n=3 and n=2), respectively.

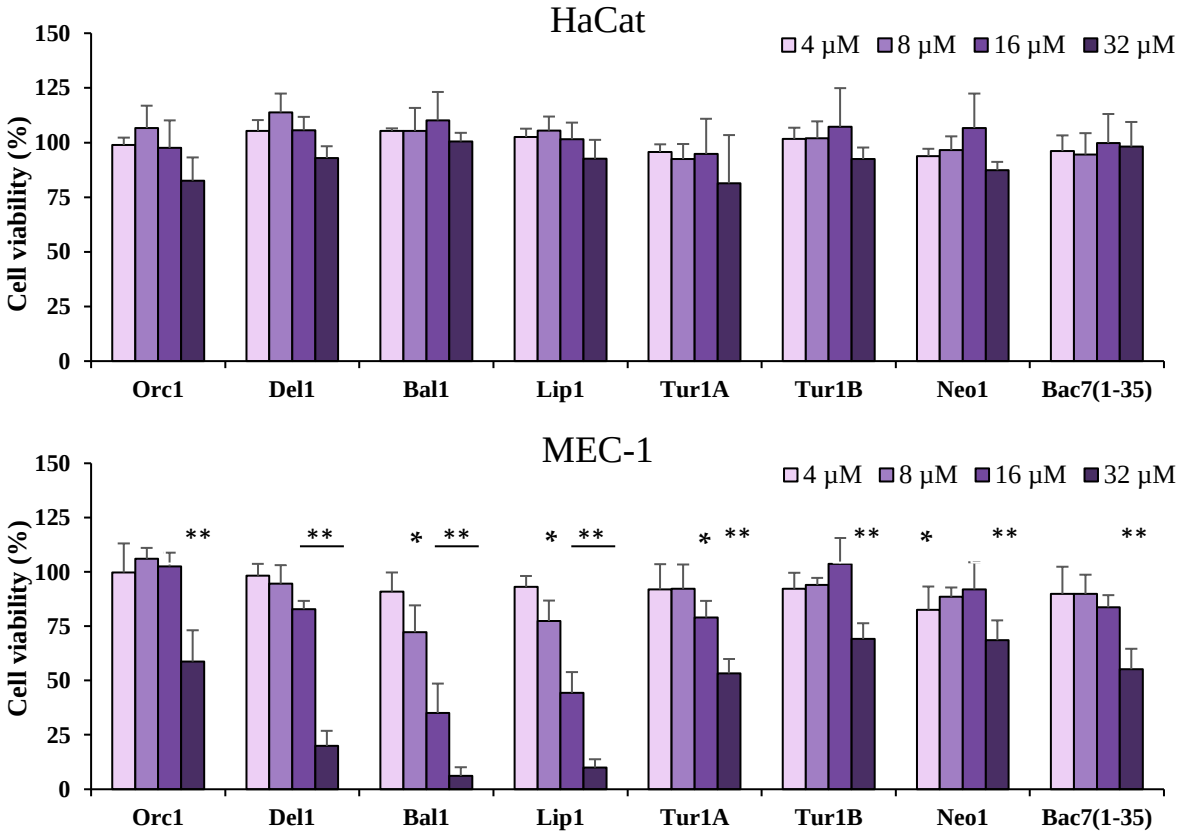

Figure 3.126. Effects of cePrAMPs and Bac7(1-35) on the viability of two human cell lines. MTT assays to assess the toxicity of the peptides against the human immortalized keratynocytes HaCat cells and the human lymphoid leukemic MEC-1 cells, exposed for 21 h and 24 h to the peptides, respectively. Average and standard deviations of three independent experiments (n = 3). * = $p > 0,05$; ** = $p < 0,01$ (T-test, comparing treated samples vs untreated controls).

Antimicrobial activity in presence of serum and high salt concentrations

Finally, we have assessed the antimicrobial activity of cePrAMPs and Bac7(1-35) Against *E.coli* ATCC 25922 in presence of 10% human serum or 2,7% NaCl, as was done for Bac selected derivatives (Tab 3.12). Bal1, Lip1, Tur1A and Bac7(1-35) were clearly not influenced by the presence of 10% HS, while the other four cePrAMPs Orc1, Bal1, Lip1 and Neo1 were more affected and significantly increased their MIC. Bal1, Lip1 and Tur1A were also the least affected by the high saline concentrations, changing their MIC of only two- or four-fold, unlike Bac7(1-35) and the other four cePrAMPs. In a few cases, results had poor replicability but still Bal1, Lip1 and Tur1A clearly showed to withstand high saline concentrations better than Bac7(1-35) and the other cePrAMPs.

Table 3.12. Variations of antimicrobial activity of cePraMPs and Bac7(1-35) against *E. coli* ATCC 25922 in presence of human serum or high saline concentration.

	MIC (μM)							
	Orc1	**Del1**	**Bal1**	**Lip1**	**Tur1A**	**Tur1B**	**Neo1**	**Bac7$_{(1-35)}$**
MHB	4	4	1	1	1	12	24	1
MHB +10%HS	>32	32	0,5	0,5	1	>32	>32	1
MHB + 2,7% NaCl	> 32	> 32	2	2	4	> 32	> 32	16

MIC (μM) of cePrAMPs and Bac7(1-35) after 18h of incubation in Mueller-Hinton broth alone (MH, on top), supplemented with 10% human serum (+10%HS), or with 2,7% (v/w) NaCl. Results are the median of three independent experiments (n=3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Design of optimized 'chimeric' PrAMPs

The characterization of Bac selected derivatives and cePrAMPs allowed us to identify some peptides which we deem are already promising on their own. Peptide B7_005, derived from the sequence optimization of Bac7(1-16), has a significantly wider spectrum of activity and shares the low toxicity of the original fragment against MEC-1 cells; it retains a good inhibitory action on bacterial translation and a low MIC against *E. coli* in presence of 10% human serum. Among cePrAMPs, we were mostly impressed by the properties of Bal1 and Lip1, and the nearly identical N-terminal 17-residues of these two peptides and Bac7(1-35). The N-terminal half of Bal1 and Lip1 granted a great inhibition of bacterial translation, like Bac7(1-35), whereas the differences in activity spectrum (and toxicity) of the two cePrAMPs with respect to the bovine PrAMP are most likely related to their C-terminal halves. Plus, Bal1 and Lip1, similarly to Tur1A and Bac7(1-35), did not change their MIC against *E. coli* in presence of 10% human serum and plus, unlike the bovine PrAMP, seemed to withstand quite well the high salt concentration.

We have designed novel 'chimeric' molecules by combining traits of B7_005 and cePrAMPs. Sequence and feature of these chimeric PrAMPs are reported in Tab 3.13. Given features of B7_005, we considered it as an optimized version of of Bac7(1-16), whose sequence is contained, nearly unchanged, in the N-terminal of Bal1 and Lip1. therefore, we tried to design novel chimeric PrAMPs by adding to the C-terminal of B7_005 tails of 7 or 11 residues derived from the C-terminal halves of Lip1. However, we were afraid that the Lip1-derived C-terminal tail, albeit incomplete, could give a toxic effect against MEC-1 to the chimeric peptide. Thus, other chimeric

peptides were produced, containing at their C-terminus fragments of the C-terminal halves of Tur1A and Bac7(1-35). This was done in the effort to boost the antimicrobial potency of B7_005 by elongating its sequence, choosing the residues from some of the most potent PrAMPs of this work. We named these chimeric peptides with 'B7_' plus the first letter of the peptide whose residues were used to design of the C-terminal tail (Tab 3.13).

Therefore, at the end of this thesis work, we made a preliminarily assessment of the antimicrobial activity of these chimeric PrAMPs, by determining their MIC against *E. coli* ATCC 25922 (Tab 3.13). Peptide B7_B6 exhibited a MIC of 0,5 µM, lower and equal to the MIC of B7_005 and Bac7(1-35), respectively (the peptides from which this chimera derives), and was the most active peptides of these preliminary assays. Generally, antimicrobial activity against *E. coli* of the novel peptides was quite similar to that of the PrAMPs they derive from (Tab 3.11 and 3.13), with one peptide having even higher MIC (peptide B7_T6, MIC 4 µM). Moreover, the peptides B7_L6 and B7_L11 clearly formed precipitates in MHB, as was observed for other hydrophobic cePrAMPs. Nevertheless, further analysis to clarify the spectrum of activity and toxicity of these novel molecules will be performed after this thesis work.

Table 3.13. Sequence, properties and MIC (µM) of the novel chimeric PrAMPs against *E. coli* ATCC 25922.

Name	Sequence	Origin of the C-term	q	H(GI)	MIC (µM)
B7_005	**W**R**R**R**R**W**R**L**R**R**P**R**W**R**		+ 8	-2,20	2
B7_B6	WRIRRRWPRLPRPRWRPLPFP**R**	Bac7(1-35)	+ 9	-1,72	0,5
B7_T6	WRIRRRWPRLPRPRWRP**R**FPPP	Tur1A	+ 9	-1,97	4
B7_L6	WRIRRRWPRLPRPRWRP**W**FPP**R**	Lip	+ 9	-1,94	1
B7_B11	WRIRRRWPRLPRPRWRPLPFP**R**PGP**R**P	Bac7(1-35)	+ 10	-1,76	2
B7_T11	WRIRRRWPRLPRPRWRP**R**FPPPFPIP**R**	Tur1A	+ 10	-1,62	2
B7_L11	WRIRRRWPRLPRPRWRP**W**FPP**R**FPIP**R**	Lip	+ 10	-1,59	2

The sequence of peptide B7_005, identical in the N-terminus of all the novel peptides is indicated on top, highlighting arginines in black and tryptophans in violet. The c-terminal tails added to B7_005 are underlined, highlighting arginines and tryptophans. 'q' = charge; H(GI) = average hydrophobicity score (GRAVY index). MIC is reported as the median of three independent experiments (n = 3); in the single experiments, the MIC differed from the median value of a factor 2, at most.

Discussion

Antimicrobial peptides have drawn a great interest in the research of new antibiotic molecules because of their antimicrobial, immune-modulatory and sometimes anti-LPS activities, but many AMPs have failed clinical trials due to problems of cytotoxicity, chemical stability and rapid clearance. Great research efforts to optimize AMPs were most often directed to address these issues.

In this work we have focused on peptides belonging to the class of PrAMPs, which share features that should (at least partially) solve one of the main problems in the development of peptide antibiotics i.e. toxicity towards host cells. Successful attempts of optimization of PrAMPs have already been reported in literature [99,102,202,207,235,236], managing to improve the stability against proteases, reduce the clearance as well as improving the antimicrobial activity, while keeping a low toxicity profile. Some of these strategies, however, were based on chemical modifications such as C-terminal amidation or the introduction of non-natural amino acids. In this work, we have temporarily avoided these modifications, relying instead on natural amino acids, thus finding promising peptide sequences that in a future could be translated from chemical synthesis to recombinant production.

By introducing substitutions with natural amino acids in the sequences of Bac7(1-16) and Bac5(1-17), we have discovered some peptides with significant improvements in those directions, with a broader spectrum of activity. Firstly, the Ala-scans and the other libraries of mono-substituted peptides helped us identifying 'core' motifs with the most crucial residues for the peptide's activity. These libraries revealed that residues 9-11 in Bac7(1-16) and 8-15 in Bac5(1-17) were particularly important for the action of the two PrAMPs (Fig. 3.4), because substitutions in those regions most often caused a drop in the antimicrobial activity and *in vitro* translation inhibition. Crystallography data obtained by collaborators with Bac7(1-16), B7_001 and B7_002 [230] revealed that the -R_9LP_{11}- residues were almost perfectly superimposable in all the crystal structures, confirming the importance of the interactions of these core residues with the ribosome. Structural data of the binding mode of Bac5(1-17) to the ribosome are currently missing and could shed light on the role of its crucial residues 8-15.

Nevertheless, Trp- and Arg-scans revealed that some substitutions with these residues could improve the peptide's activity, in some rare cases even when they fell into the 'core motifs'. Hopefully, other high-resolution structural data could clarify the role of such advantageous substitutions in the future.

These results are in agreement with other studies on insect PrAMPs optimization. Antimicrobial activity of apidaecin 1b and oncocin was also successfully improved by substitutions with tryptophan and arginine [235,236], suggesting that these two residues can be used for improving many PrAMPs. This is consistent with a proposed general model of PrAMP binding to the ribosome, where the side chains of arginine and aromatic residues can perform π stacking or other interactions with the nitrogenous bases of rRNA, besides the charge interactions of arginine with polar/charged residues (perhaps such putative general model could not apply for apidaecin, whose target is different from other PrAMPs) [142,200,230].

The introduction of multiple arginine and tryptophan substituents generated derivatives of Bac7(1-16) and Bac5(1-17) with more desirable properties than the original fragments for three reasons. Firstly, the spectrum of activity of the optimized fragments was significantly broader than their original counterparts, and some derivatives of Bac5(1-17) showed MICs eight-to-sixteen fold lower than the unsubstituted peptide. The most potent derivatives, i.e. B7_005 and B5_291, did not rely much on the SbmA transporter for their action, and they both permeabilize bacterial membranes and inhibit *in vitro* protein translation. Importantly, B7_005 shares with Bac7(1-35) and other cePrAMPs a sequence motif that has been proposed as consensus motif related to protein synthesis inhibition (see below). The lesser dependence of these peptides on the SbmA transporter means that the downregulation of SbmA expression would no longer be effective in evading the peptides' action, and that the double mode of action can be detrimental to bacteria, which are less likely to develop resistance against both mechanisms. One can speculate that this different behavior compared to the unsubstituted fragments may be ascribable to the tryptophan residues. Such hypothesis would be in accordance with the behavior of cePrAMPs, which also caused membrane permeabilization in a way that seemed related to the number of tryptophan residues in their sequence (see below).

Secondly, Bac7(1-17) derivatives and B5_291 showed no significant toxicity on the MEC-1 cells at concentrations many fold higher their MICs. The permeabilizing activity of these derivatives is clearly detectable but it is not as marked as for colistin; such weaker membrane permeabilization could partly explain their very low cytotoxicity, despite the acquisition of a certain 'membranolytic' behavior.

Thirdly, peptide B7_005 maintained a MIC $\leq$ 4 μM against nearly every Gram-negative strain tested in presence of both normal and heat-inactivated sera. The efficacy of B7_005 in 10% human serum and its low cytoxicity against the MEC-1 cells raises the hope to employ I future B7_005 (or other variants) in human blood, i.e. for the treatment of bloodstream infections, although further assays (i.e. hemolysis assays) will be needed. The retention of activity in presence of serum has

been observed in the cePrAMPs Bal1, Lip1 and Tur1A. This feature prompted us to use their sequences to design the Bac-cePrAMP chimeric peptides.

Serum proteases generally represent a problem for antimicrobial peptides, mining their bioavailability; however, in our case the loss of activity of our PrAMPs did not seem caused by a proteolytic cleavage: in fact, the PrAMPs' activity decreased equally, or even more, when they were incubated with a serum in which proteins have been inactivated by pre-heating at 60°C. Thus, we speculated that such loss of activity can be due to the binding with certain serum components, although the different influence of serum on Bac7(1-16) and Bac5(1-17) derivatives remains hard to explain.

Arguably, when screening the libraries aiming to identify the most promising peptides, we should have carefully evaluated stability and toxicity as well; assessing these parameters in all the peptides of the libraries could have led us to choose different peptides than the ones we selected. However, extending such investigations to all the peptides would be really costly, considering that toxicity and stability can seriously vary, according to cell lines and biological fluids [237,238] . Therefore, we limited the screening to antimicrobial activity and toxicity towards at most one cell line – as was done by other groups working with peptide libraries [233,236,239,240] – and decided to solve the issues of toxicity and stability by relying on other chemical 'fine-tuning' approaches, such as C-terminal amidation [202,207] cyclization or d-isomerization[169] .

We chose Bac7(1-16) and Bac5(1-17) as starting peptides, not only to optimize the shortest PrAMPs possible – so as to reduce the cost of a possible large-scale synthesis –, but also because we wanted to produce libraries of mutants with the SPOT synthesis. The error rate of the SPOT technique discouraged the synthesis of peptides longer than 17 residues, because they would be too likely to have unwanted amino acids in their sequence. Arguably, the low purity (< 60%) of the SPOT synthesis could maybe generate some peptides whose scarce activity is due to the low purity of the preparation. Even considering this chance, however, the large screening allowed by the SPOT technique would provide us with a great number of experimental data, avoiding *in silico* predictions, and this would balance the risk of losing few promising hits in the screen.

Nevertheless, optimization of longer fragments should be considered in the future, as we saw that both Bac7 [204] and Bac5 longer fragments generally have a better antimicrobial activity. Admittedly, other scans of Bac7(1-16) and Bac5(1-17), especially with other cationic residues such as lysine and histidine, could reveal even better peptides than the ones we found. Some Arg → Lys substitutions, for example, have recently been found to improve the antimicrobial activity of Bac7(1-16) and pyrrhocoricin[102] - which shares the binding site with Bac7(1-16)[143,200] - . In addition, some of the selected Bac7 derivatives were designed without a screening of multiple mutants but just by putting

together a number of advantageous single substitutions; with such approach, we may have missed excellent peptides with multiple substitutions. Peptide 282 is another example of promising multi-substituted peptide which was left behind; this peptide will surely deserve further analyses in the future.

Besides the work on optimized PrAMPs, our work on the research and characterization of cetacean PrAMPs has also allowed interesting results. Firstly, all the cePrAMPs appeared to be homologous to Bac7, given the significant degree of sequence similarity with Bac7(1-35) (for instance, 15 out of 35 residues of Bac7(1-35) are present in more than half of cePrAMPs). However, these peptides seemed less dependent on the membrane-transporter SbmA, as was already demonstrated for Tur1A and Tur1B[189]. Secondly, all cePrAMPs, except Neo1, showed to have an appreciable spectrum of activity. However, the most interesting results in terms of MIC assays came from Bal1 and Lip1. In fact, these two (nearly identical) peptides showed an impressive spectrum of action, with MIC $\leq$ 2 μM against eight Gram-negative species and a fungus, and even MIC $\leq$ 8 μM against two Gram-positive pathogens. However, what was most surprising was how their properties differed from Bac7(1-35) despite the resemblance of their N-terminal halves. Bac7(1-35), Bal1 and Lip1 have a similar charge distribution and GRAVY index, and their first 17 N-terminal residues differ only in the fifth amino acid; however, they displayed impressive differences in their action against Gram-positives and in highly salty environments, and in their toxicity towards MEC-1 cells (plus slightly different interactions with bacterial membrane). Such dissimilarity is, most likely, entirely given by the residues of their C-terminal half. More studies and comparisons of the properties of Bal1/Lip1 and Bac7(1-35) may provide useful hints on the functional role of short sequence motifs on the properties of PrAMPs. Thirdly, despite their degree of similarity, the sequences and experimental results of cePrAMPs could quite clearly distinguish two subgroups of peptides. While Bal1, Lip1 and Tur1A shared with Bac7(1-35) a strong non-permeabilizing, translation-inhibiting mode of action, the other four peptides displayed a membrane-damaging behavior, which is rather unusual for PrAMPs.

Most interestingly, the sequences of Bal1, Lip1 and Tur1A, along with Bac7(1-35) and the optimized Bac7(1-16) derivatives, share the sequence motif: **+XX(R/Y)LPRPRX**. Such sequence motif is also present in the insect PrAMPs oncocin, pyrrhocoricin and metalnikowin-1 and was already proposed by Mardirossian[230] as a consensus for the inhibition of bacterial protein synthesis. These findings reinforce the validity of the consensus proposed by Mardirossian and might be very useful in the design of peptide-based protein-synthesis inhibitors.

Also surprisingly, despite being membrane-permeabilizing, Orc1, Del1, Tur1B and Neo1 showed a narrower and less potent antimicrobial action compared to the other non-permeabilizing cePrAMPs. Arguably, this may also be related to their scarce solubility in both 100% MH and 20% MH.

Among the four permeabilizing cePrAMPs, Orc1 was the only one not showing structural rearrangements in presence of a membrane-mimicking environment. Another interesting feature of Orc1 is its significant inhibition of transcription/translation at 10 μM, which makes it tempting to speculate a double mechanism of action for this peptide, including a slight reduction of bacterial protein synthesis. These data should be further investigated.

Despite all these four cePrAMPs significantly permeabilized *E. coli* membranes, Del1 was the only one showing an evident hemolytic effect. We speculated that the lack of hemolytic effects of Neo1 could derive from its shorter size but we found it harder to explain it for Orc1 and mostly for Tur1B. Del1 is actually the most hydrophobic of the 'long' cePrAMPs, but hydrophobicity alone may not be enough to explain. Again, the careful comparison of Del1 and Tur1B sequence can be another source of hints on the effects of short sequence motifs.

Bal1 and Lip1 were the most promising cePrAMPS. Their excellent antimicrobial, translation-inhibiting activity, was combined to a non-hemolytic effect, scarce dependence on SbmA and a weak but measurable destabilizing effect on the membranes of *E. coli*. Their two modes of action would render them less susceptible to antimicrobial resistance, for the same reasons indicated for B7_005. Bal1 and Lip1 maintained their high effectiveness against *E. coli* also in presence of 10% human serum and 2,7% NaCl. Although these are just few preliminary data about the peptides stability, they make us optimistic about the possible administration routes for peptides Bal1 and Lip1. Unluckily, their detrimental effect on the lymphoid leukemic cells MEC-1 may discourages their use in blood, but the features of these cePrAMPs remain remarkable, even before any chemical optimization. Plus, tumoral cell lines such as MEC-1 have generally a higher negative charge on their membrane which can contribute to their susceptibility to AMPs [241] , while non-tumoral lymphocytes may be less damaged by Bal1 and Lip1.

Given their activity in MH with 2,7% NaCl, we hope Bal1 and Lip1 can be employed in aerosol formulations with salty water to treat cystic fibrosis[234]; we will start verifying this possibility by assessing their spectrum of action in presence of 2,7% NaCl and assessing their toxicity towards lung cell lines.

In our work with bovine optimized and cetacean PrAMPs, we did not carefully evaluate the action of our PrAMPs against biofilms. At the moment, data about the action of these peptides against

biofilms are limited to some preliminary experiments (not shown), which revealed some inhibition of biofilm formation by Lip1 and B7_005 at ½ MIC against two reference strains of *A. baumannii* (ATCC 17978 and 19609) and a (non-MDR) clinical strain of *K-pneumoniae*. More experiments will be soon performed to clarify the minimal biofilm-inhibiting concentration and, hopefully, the biofilm-eradicating concentration of these peptides.

Another important parameter to evaluate the rate would be the induction of antimicrobial resistance. These assays could confirm whether PrAMPs with a putative double mechanism like B7_005, Orc1 or Lip1 are equally or less prone to induce AMR than 'classical', non-membranolytic PrAMPs.

When we designed the chimeric PrAMPs, we tried to 'gather' the best properties of Bac7_005 and cePrAMPs by combining their sequence motifs. We decided not to add all the 16 C-terminal residues of Lip1 to peptide B7_005; although this may deprive the chimeric peptides of all the properties of Lip1, we tried a shorter C-terminal moiety in order to keep a shorter peptide sequence (max 27 residues). Moreover, trying to avoid the toxicity on MEC-1 cells, we designed four other chimeric peptides with the sequence of B7_005 followed by 6 or 11 residues from the C-terminal halves of Bac7(1-35) and Tur1A. In the preliminary experiments we made, our chimeric peptides did not show an improved antimicrobial activity against *E.coli* ATCC 25922. This may be partly because some of these peptides are hardly soluble in MH, like it was for Del1 or Tur1B. This scarce solubility was also quite unexpected, given their high cationicity and low hydrophobicity. Studies may be required to explain this behavior.

Besides joining sequence motifs into chimeric PrAMPs, another way to join the forces of our PrAMPs would be by using those PrAMPs in combination. Synergistic activity was reported for combinations of AMPs with conventional antibiotics[88] and with other AMPs[178]. Some preliminary experiments were made, looking for synergistic activity between Lip1 and B7_005 against *K. pneumoniae* and *S. aureus*, but results seemed to show an additive effect rather than a synergy (data not shown). Instead, preliminary checkerboard assays revealed synergy of Orc1+Lip1 against *S. typhimurium* and Orc1+B7_005 against *K. pneumoniae* (data not shown). This promising data prompt us to extend the investigation to more bacterial strains and even more drug combinations; for instance, including known AMPs active against biofilms such as LL-37[242,243] or CRAMP[147] .

Finally, but not less importantly, none of these peptides was tested for its action on the immune response. However, these analyses will be performed after the aforementioned experiments, once that fully-optimized peptides, stable in biological fluids and with a low toxicity profile, will be generated.

The *in vivo* therapeutic potential of PrAMPs is not a novelty[101,208,212,213]. Oncocin derivatives were shown to rescue murine models of systemic *E.coli* infections , with a relatively low $T_{1/2}$ but a strong post-antibiotic effect [213] ; apidaecin derivatives rescued 50 to 100% mice infected intraperitoneally with *E.coli* albeit with a shorter half-life in serum than oncocin derivatives [101]; Bac7(1-35) rescued the 36% of typhoid fever murine models infected with *S. typhimurium*, [212]. These peptides showed efficacy combined with relatively low toxicity in the murine models they were tested in, although suffering of a rapid clearance. These are encouraging findings which make us hope that some of our PrAMPs can also have good results used *in vivo*.

Conclusions

In conclusion, both the sequence optimization of Bac7(1-16) and Bac5(1-17) and the characterization of cePrAMPs provided us with some interesting novel peptides, which we believe promising enough and worth of future analyses and optimization. Some of the PrAMPs we found have excellent antimicrobial activity and a satisfactory level of stability in serum. Some of our best PrAMPs showed some cytoxicity, but limited (at the moment) to one cell line.

At present, chimeric peptides combining sequence motifs of some of our best PrAMPs are just entering the first characterization; some promising data came from combinations of PrAMPs in preliminary synergy assays with a cePrAMP and one optimized derivative of Bac7(1-16).

We believe that the characterization of our best PrAMPs should be extended; aims for the near future could be to extend the cytotoxicity analyses to more cell lines, assess the action of these PrAMPs against biofilms and test them in combination with other molecules looking for synergistic effects. Next, further sequence optimization could help to overcomes some issues such as toxicity and stability. Finally, after this characterization, chemical modifications can be tried to further improve the peptide's activity, stability biocompatibility, before starting to test the performance of the resulting molecules into animal models.

The *in vivo* application of the PrAMPs of this work may still be far away, but these molecules represent a good starting point to generate PrAMP-based drugs to deploy in animal models of infections.

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