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XXXV CICLO DEL DOTTORATO DI RICERCA IN

Biomedicina molecolare

The designed antimicrobial peptide B7-005: biocompatibility, mode of action and antimicrobial activity against ESKAPE pathogens

Settore scientifico-disciplinare: BIO/10 BIOCHIMICA

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Summary

Riassunto

L'Organizzazione Mondiale della Sanità ha elencato alcuni patogeni resistenti alla maggior parte degli antibiotici e responsabili della maggior parte delle infezioni nosocomiali. Questi patogeni sono indicati con l'acronimo ESKAPE: *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* ed *Enterobacter spp*. Sono urgentemente necessari nuovi farmaci che superino la resistenza agli antibiotici di questi patogeni. Grandi speranze sono rivolte ai peptidi antimicrobici ricchi di proline (PrAMP). I PrAMP presentano un'attività antimicrobica potente e specifica, penetrano in alcune specie batteriche, si legano ai ribosomi procariotici e inibiscono la sintesi proteica.

I PrAMP vengono attualmente ottimizzati per superare uno dei loro più grandi limiti, ovvero lo spettro d'azione limitato solo ad alcune specie di batteri, con il fine ultimo di sviluppare nuovi antibiotici. Nello screening di librerie di PrAMP corti di mammiferi, è stato selezionato il peptide B7-005 perché presenta attività antimicrobica efficace anche a basse concentrazioni, insensibilità alle proteasi del siero e dei dati preliminari hanno suggerito una bassa citotossicità nei confronti delle cellule eucariotiche.

I risultati indicano che B7-005 ha una minore propensione a selezionare la resistenza in *E. coli* rispetto ad altri antibiotici e PrAMP. B7-005 è risultato attivo su tutti i ceppi di riferimento ESKAPE gram-positivi e gram-negativi. La crescita dei preoccupanti patogeni nosocomiali *K. pneumoniae* e *A. baumannii* è stata inibita dal B7-005alle concentrazioni rispettivamente di 2µM e 4µM. Il B7-005 ha inibito la crescita anche di *P. aeruginosa*, *S. aureus* ed *E. faecium*, specie batteriche che spesso non vengono influenzate dai PrAMP nativi, poiché mancano del trasportatore batterico SbmA, sfruttato da questi peptidi per entrare nel citosol batterico. Il B7-005 non solo ha inibito la crescita della maggior parte delle ESKAPE, ma ha anche ucciso attivamente questi patogeni. Tra le specie sono state riscontrate grandi differenze in termini di efficacia, tempo di uccisione e soglia di concentrazione. Per verificare se la morte batterica fosse dovuta a un meccanismo di uccisione non litico, come ci si aspetterebbe per un PrAMP, l'integrità della membrana cellulare e la presenza del peptide intracellulare sono state monitorate mediante citometria a flusso e un derivato fluorescente del B7-005.

il B7-005 è stato osservato nel citoplasma delle cellule di *E. coli* e *K. pneumoniae*, lasciando allo stesso tempo intatta la membrana batterica. Mentre le cellule delle altre specie di batteri ESKAPE esposte al B7-005 hanno mostrato una destabilizzazione delle membrane, e una ridotta internalizzazione del peptide.

Per garantire che l'attività permeabilizzante di membrana di B7-005 osservata in alcune ESKAPE non fosse accompagnata da un aumento della citotossicità, sono stati eseguiti test di biocompatibilità su diverse cellule umane. Le normali cellule endoteliali primarie della vena ombelicale umana (HUVEC) sono risultate tolleranti al peptide anche ad alte concentrazioni e più tolleranti anche dei cheratinociti immortalizzati HaCaT, della linea tumorale polmonare A549 e delle cellule leucemiche MEC-1. I risultati complessivi sono coerenti con i dati della letteratura che indicano che gli AMP spesso colpiscono le cellule tumorali più di quelle non tumorali, principalmente a causa della loro diversa composizione di membrana. Nel peggiore dei casi, la citotossicità del B7-005 si è verificata a concentrazioni almeno 6 volte superiori a quelle richieste per l'attività antimicrobica, suggerendo che il peptide ha un'ampia finestra terapeutica.

Inoltre, sono stati eseguiti test di citotossicità *in vivo* per valutare la biocompatibilità di B7-005 nel sistema più complesso delle larve di Zebrafish (*Danio rerio*). B7-005 si è dimostrato sicuro per i pesci fino a concentrazioni millimolari elevate, cioè una dose molto distante sia dalle concentrazionie micromolari definite tossiche per le cellule eucariotiche in vitro, ma ancora di più da quelle battericide.

Lo studio dimostra che il peptide B7-005 è un composto antimicrobico con una bassa propensione a sviluppare resistenza batterica, ha un ampio spettro di attività contro le ESKAPE, è ben tollerato dalle cellule umane normali e non risulta tossico in un sistema in vivo come Zebrafish.

B7-005 supera così alcune delle limitazioni degli AMP come molecola terapeutica ed è quindi un buon candidato per l'uso come prima linea di difesa contro alcuni dei patogeni che destano preoccupazione nella salute umana.

Abstract

The World Health Organisation has listed a number of pathogens that are resistant to most antibiotics and are responsible for most nosocomial infections. These pathogens are referred to by the acronym ESKAPE: *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter spp*. New drugs that overcome the antibiotic resistance of these pathogens are urgently needed. Great hopes rest on antimicrobial proline-rich peptides (PrAMPs). PrAMPs have potent and specific antimicrobial activity, penetrate certain bacterial species, bind to prokaryotic ribosomes, and inhibit protein synthesis.

PrAMPs are currently being optimised to overcome one of their major limitations, namely their spectrum of activity limited to certain bacterial species, with the goal of developing new antibiotics. In screening libraries of short mammalian PrAMPs, peptide B7-005 was selected because it exhibits effective antimicrobial activity even at low concentrations, is insensitive to serum proteases, and preliminary data indicate low cytotoxicity to eukaryotic cells.

The results suggest that B7-005 has a lower propensity to develop resistance in *E. coli* than other antibiotics and PrAMP. B7-005 was active in all Gram-positive and Gram-negative ESKAPE reference strains. Growth of the nosocomial pathogens of concern, *K. pneumoniae* and *A. baumannii*, was inhibited by B7-005 at concentrations of 2 µM and 4 µM, respectively. B7-005 also inhibited *P. aeruginosa*, *S. aureus*, and *E. faecium*, bacterial species that are often unaffected by native PrAMPs because they lack the bacterial SbmA transporter used by these peptides to enter the bacterial cytosol. B7-005 not only inhibited the growth of most ESKAPEs, but also actively killed these pathogens. Large interspecies differences in efficacy, killing time, and concentration threshold were observed. To verify whether bacterial death was due to a non-lytic killing mechanism, as would be expected with a PrAMP, cell membrane integrity and the presence of the intracellular peptide were monitored by flow cytometry, and a fluorescent derivative of B7-005.

B7-005 was observed in the cytoplasm of *E. coli* and *K. pneumoniae* cells, whereas the bacterial membrane remained intact. In contrast, cells of other ESKAPE bacterial species exposed to B7-005 showed destabilisation of the membrane and decreased internalisation of the peptide. To ensure that the membrane-permeabilizing activity of B7-005 observed in some ESKAPE was not associated with increased cytotoxicity, biocompatibility tests were performed with various human cells. Normal primary human umbilical vein endothelial cells (HUVECs) were tolerant to the peptide even at high concentrations and more tolerant than even immortalised HaCaT keratinocytes, the A549 lung cancer line, and MEC-1 leukaemia cells. The overall results are consistent with literature data indicating that AMPs often affect tumour cells more than non-tumour cells, mainly due to their different membrane composition. In the worst case, cytotoxicity of B7-005 occurred at

concentrations at least 6 times higher than those required for antimicrobial activity, suggesting that the peptide has a broad therapeutic window.

In addition, in vivo cytotoxicity tests were performed to evaluate the biocompatibility of B7-005 in the more complex system of Zebrafish larvae (*Danio rerio*). B7-005 was found to be safe for fish up to very high millimolar concentrations, i.e., a dose far from both micromolar concentrations defined as toxic to eukaryotic cells in vitro, but even more from bactericidal concentrations.

The study shows that the peptide B7-005 is an antimicrobial compound with a low propensity to develop bacterial resistance, has a broad spectrum of activity against ESKAPEs, is well tolerated by normal human cells, and is not toxic in an in vivo system such as Zebrafish larvae. B7-005 thus overcomes some of the limitations of AMPs as a therapeutic molecule and is therefore a good candidate for use as a first line of defence against some of the pathogens of concern to human health.

1. Introduction

1.1 Antibiotic resistance and tolerance in bacteria

Antibiotics are defined as substances capable of causing the death of bacteria or preventing their growth, and are probably the most successful drugs in the history of medicine. The importance of these drugs in saving human lives and controlling infectious diseases certainly does not need to be reiterated.

The beginning of the antibiotic era is famously credited to the discoveries of Paul Ehrlich and Alexander Fleming. While Ehlrich described antibiotics as a "magic bullet" that selectively targets microbes rather than the host[1], Fleming had warned the scientific community as early as 1940[2] about the potential resistance of bacteria to the antibiotic penicillin if used too little or for too short a time[3]. At that time, however, the optimistic assumption was that communicable diseases would almost completely stop[1].

Because of these significant innovations, the period between the 1950s and 1970s has been called the golden age for the discovery of new classes of antibiotics [4]. In fact, most of the antibiotics in use today were discovered during this time. From this period on, these drugs were overused, and the demand for antibiotics came from areas other than medicine, such as agriculture and food production. However, the massive and often inappropriate use exerted relentless selection pressure on microbial populations, contributing to the emergence and selection of antimicrobial resistance mechanisms[5] (Fig. 1.1). In the past, the emergence of resistant strains was accompanied and counteracted by the production of new antibiotics, but today there is no such balance. For years, the biopharmaceutical industry has withdrawn many investments from research and development of new antimicrobial agents, considering them not profitable enough[6]. On the other hand, unfortunately, bacterial resistance was eventually found to almost all antibiotics developed[7]. In fact, since the turn of the 1990s, the development and commercialization of novel antibiotics have slowed, up to the very last years. Between 2017 and 2019, 11 new antimicrobial therapies were indeed approved by the U.S. Food and Drug Administration (U.S. FDA)[8].

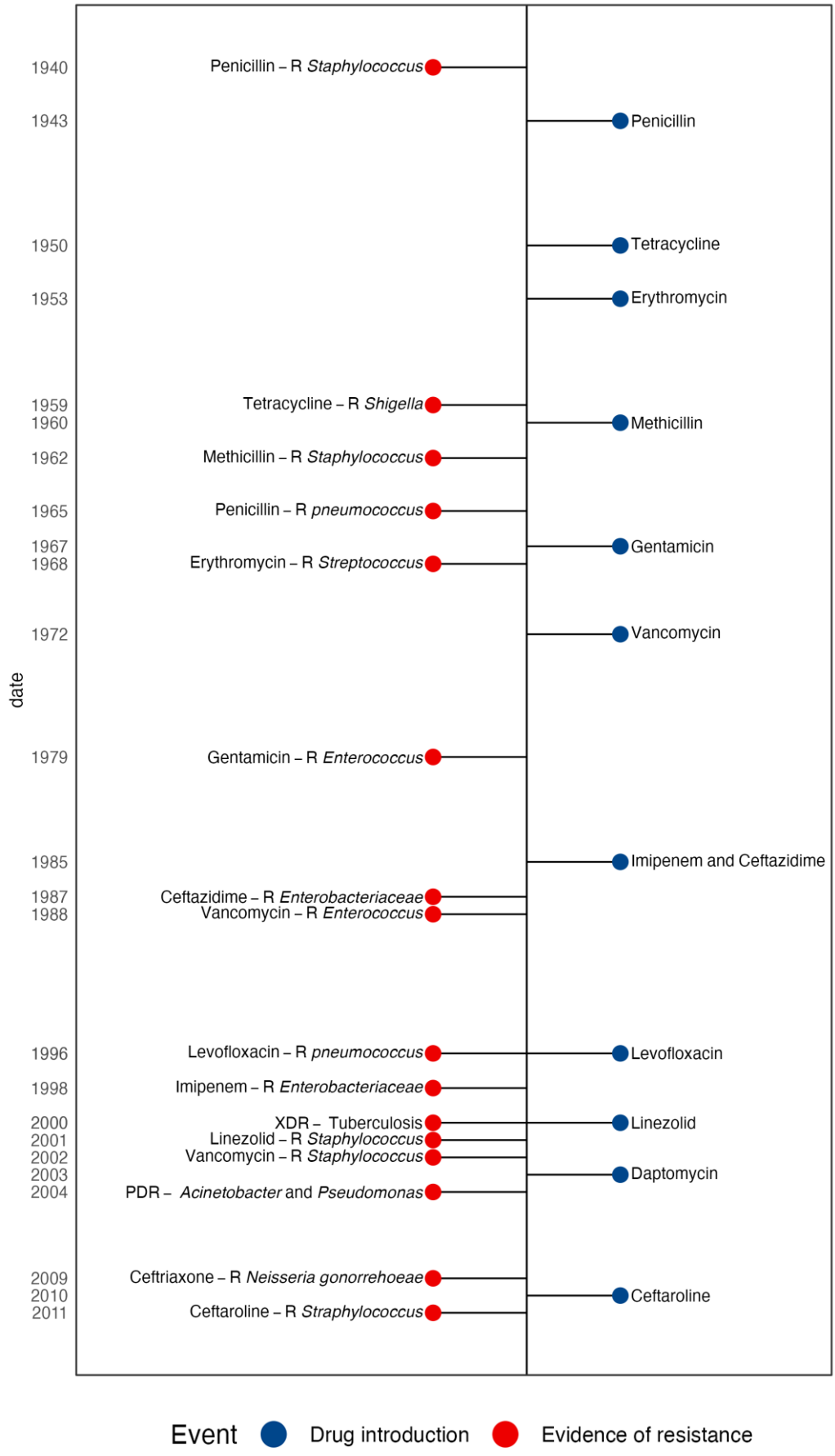

Fig. 1.1: **Graphical representation of the emergence of antibiotic resistance** versus the time of onset of discovery of a new antibiotic (image adapted from Ventola 2015)

However, the number and efficiency of these new drugs is far from covering all the existing needs, to fully combat highly adaptive microorganisms. Thus, there is a real need and urgency to develop novel antibiotic strategies and drugs to overcome the Antimicrobial resistance (AMR)[9]. AMR is defined as the ability of a micro-organism to resist the action of one or more antimicrobial agents, is one of the greatest threats to public health worldwide today, leading to rising health care costs, treatment failures, and deaths[5]. Resistance to antibiotics, especially to last-generation ones, undermines not only the effectiveness of life-saving medical interventions such as intensive care, cancer treatment, and organ transplantation, but also important and very diffused procedures in modern medicine (e.g., intestinal surgery, caesarean sections, hip replacement[10]). It is estimated that by 2050, 10 million lives and a total economic output of 100 trillion USD will be at risk each year from the increase in drug-resistant infections if proactive solutions are not found now to slow the increase of antibiotic resistance[10].

Reports from the European Centre for Disease Prevention and Control indicate that overall human antibiotic use in the European Union/European Economic Area (EU/EEA) decreased by 23% between 2011 and 2020, particularly during the 2019 coronavirus pandemic (COVID -19) (between 2019 and 2020, average total antibiotic use decreased by nearly 18%). However, the relative use of broad-spectrum antibiotics has increased (Fig. 1.2). Although recent trends are encouraging, resistance to commonly used antibiotics remains high (> 20% to 50%) or very high (> 50% to 70%) in foodborne pathogens in the EU/EEA region.

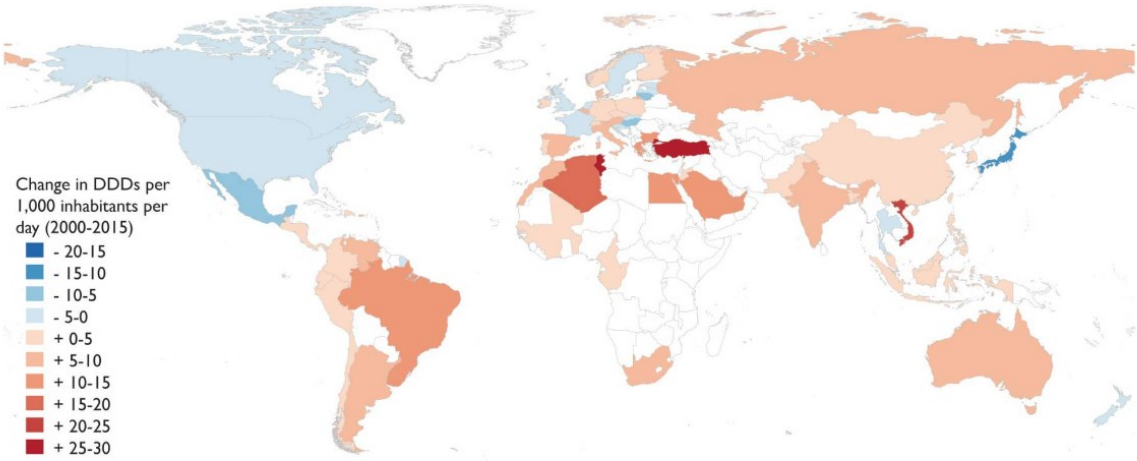

Fig. 1.2: Antibiotic consumption rates expressed as defined daily doses (DDDs) per 1,000 inhabitants per day over the period 2000-2015. The WHO definition of DDD is "the assumed average maintenance dose per day for a drug used for its main indication in adults." (Princeton Environmental Institute (PEI))

The emergence of 'multi-drug-resistant' (MDR), 'extensively drug-resistant' (XDR) and 'pan-drug-resistant' (PDR) strains has led experts to define this particular moment as the beginning of the post-antibiotic era, in which several infections are now intractable[11].

In this scenario, knowledge and understanding of the mechanisms underlying antibiotic resistance is crucial for the development of new antibiotics specifically tailored to circumvent these resistance strategies. The mechanisms of AMR can generally be classified as intrinsic, adaptive, or acquired resistance[12]. AMR, however, is often multifactorial, using multiple mechanisms simultaneously to evade the effects of specific antimicrobials.

- **Intrinsic resistance**: refers to properties of the microorganism that make it naturally insensitive to a particular antibiotic. An example of this is the outer membrane of Gram-negative bacteria, which acts as a permeability barrier and prevents antibiotics from reaching their target[13].

- **Acquired resistance**: in bacteria, it is caused by mutations in chromosomal genes or by the acquisition of exogenous resistance genes carried by mobile genetic elements that can spread horizontally between bacteria. Bacteria can acquire multiple resistance mechanisms and thus become resistant to several antimicrobial agents. This is particularly problematic because it can severely limit the available treatment alternatives for the infection.[11]

Such resistance can be further subdivided according to its effect.

Alteration of the antibiotic target: mutations of this type make the target protein less susceptible to the antimicrobial agent. One example is the *erm* gene. This gene is encoded by a plasmid and causes methylation of adenine 2058 of 50S rRNA, preventing the action of the antibiotic erythromycin[14].

Antibiotic modification/inactivation: this is a common resistance mechanism for natural antibiotics such as aminoglycosides (kanamycin, tobramycin, amikacin), which are inactivated by enzymatic phosphorylation, acetylation, or adenylation, as well as for β-lactams (penicillins, cephalosporins, carbapenems), which are inactivated by enzymatic hydrolysis by β –lactamases[15].

Increased antibiotic efflux and decreased permeabilization: drug efflux pumps are proteins that extrude antibiotics from the bacterial cell, reducing their cytosolic concentration to sublethal levels. They are the cell's first line of defence. When the organism is confronted with an antibiotic, there is a temporary upregulation of efflux pump expression[16]. Reduced permeability to molecules is another effective antibiotic resistance strategy. Hydrophilic antibiotics such as β –lactams and fluoroquinolones often pass through 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 synthesising porins with lower permeability[17].

Adaptive resistance: Bacteria have the ability to adapt and change their behaviour according to the environmental conditions in which they live. Clear examples of this are the development of

persistent cells and biofilms. Persistent cells are a subpopulation of cells that have altered their metabolism to grow slowly or stop growing as a result of environmental stress. This adaptation makes the bacteria less susceptible to the effects of antibiotics than the rest of the metabolically active colony, either because in this state the targets for antibiotics are less active or available, or because the uptake of antibiotics is significantly reduced.[18]

Biofilm is particularly problematic because it confers to micro-organisms resistance to conventional antibiotic therapies. The term 'biofilm' refers to a microbial community that is in close contact with the surface it colonises and is encased in an extracellular polymer matrix produced by the microorganisms themselves[19]. The transition from planktonic form to the sessile state of the biofilm is a response to various environmental stimuli. Environmental conditions such as desiccation, temperature shocks, pH changes, and the action of antimicrobial agents are a stress signal for the bacterial cells, which begin to adhere to the surface on which they are located[20]. As bacterial cell density increases, the biofilm begins to mature, assuming a complex spatial organisation. The mature biofilm allows most of the included microorganisms to come into direct contact with nutrients from the external environment, while the macro-community holds together thanks to the molecules that make up the extracellular matrix. The matrix is the most important part of the biofilm; it is composed of polysaccharides, proteins and extracellular DNA[21], and contains water-filled channels that allow better access to nutrients in the biofilm[20]. The formation of such an architecture is largely driven by the secretion of small signalling molecules that allow microorganisms to communicate with each other to organise in these structures and form a mature biofilm; this process is known as quorum sensing[22]. In this way, the bacteria activate a sophisticated mechanism of gene regulation by releasing molecules from the bacteria themselves. The mature biofilm also undergoes a detachment process, releasing groups of cells that can return to the planktonic state and eventually colonise a new surface (Fig. 1.3).

The importance of biofilms in microbial infections is more than relevant: the annual cost of biofilm-associated infections is estimated at $94 billion and is responsible for over half a million deaths[23]. This picture is due to the increasing incidence of nosocomial infections contracted by patients in close contact with medical devices that are often infected with biofilms. Examples include intravenous, endotracheal, dialysis catheters, prosthetic heart valves, orthopaedic devices, tissue fillers, pacemakers and cerebrospinal fluid shunts. These materials can be very easily colonised by microorganisms, which thus gain access to the patient's internal organs and cause infections with often very serious consequences[19].

Crucially, biofilms are much less sensitive to antimicrobial treatment than the same bacteria in planktonic form. This is due to the presence of bacterial cells that have an inefficient programmed

cell death (PCD) that does not induce death even after cell damage, allowing them to survive even high doses of antibiotics[24].

These cells are normally localised in the innermost layers of the biofilm; if the biofilm is not completely removed, it can soon form again from these cells.

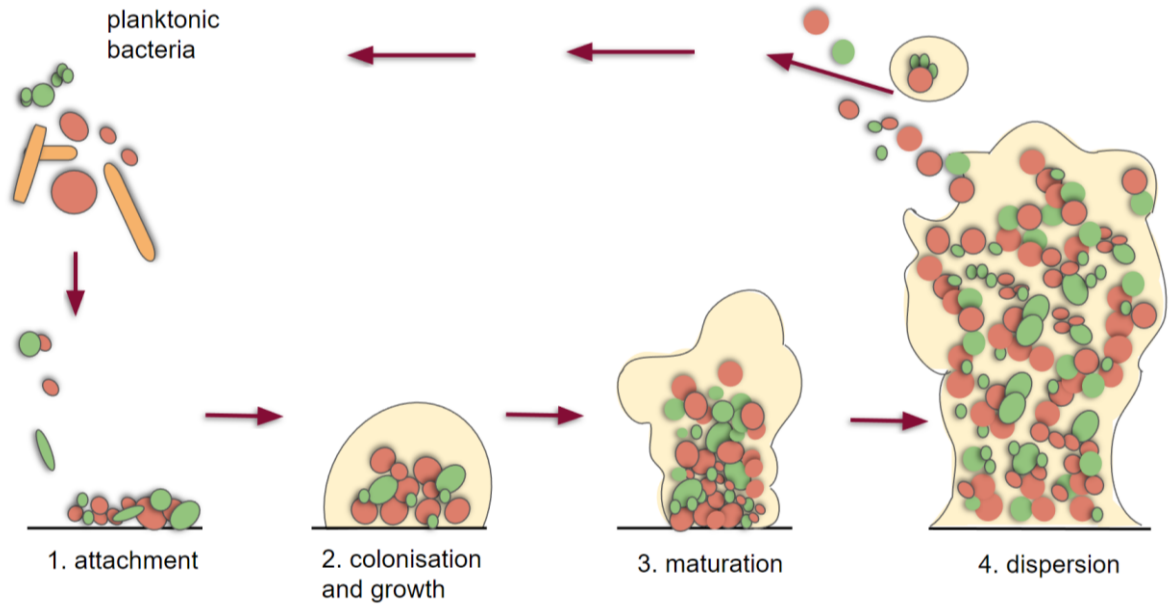

Fig. 1.3: Graphic representation of biofilm formation. (1) Reversible adhesion of bacteria to a surface, (2) Biofilm growth and irreversible adhesion, (3) Biofilm maturation, (4) Biofilm dispersion.

1.2 ESKAPE pathogens

In February 2017, the World Health Organization (WHO) published a list of pathogens that can "escape" the biocidal effects of antibiotics and for which the development of new antibiotics is urgently needed. These pathogens are grouped under the acronym ESKAPE (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa*, and ***E****nterobacter species*). These microorganisms pose a serious threat to human health, as the acquisition of antimicrobial resistance genes has reduced the therapeutic options for the treatment of serious infections and has increased the burden of disease and mortality due to treatment failure. As antibiotic development declines and resistant species increase, healthcare-associated infections are a constant threat, especially for high-risk patients requiring invasive devices, and ESKAPE pathogens are becoming increasingly common, particularly in hospitals. Indeed, these pathogens are the leading cause of nosocomial infections[25,26]. The WHO describes three categories of pathogens: critical pathogens, high-priority pathogens and medium-priority pathogens, based on the urgency of the need for new antibiotics. Carbapenemase-resistant *A. baumannii* and *P. aeruginosa*, as well as *K. pneumoniae* and *Enterobacter* spp. resistant to extended-spectrum β-lactamases (ESBLs) or carbapenems[27], are included in the critical priority

pathogen list, while vancomycin-resistant *E. faecium* (VRE)[28] and methicillin- and vancomycin-resistant *S. aureus* (MRSA and VRSA) [29] are included in the high-priority group.

E. *faecium* is a Gram-positive pathogen and VRE is a major cause of healthcare-associated infections[28]. Resistance to β-lactam antibiotics is almost guaranteed in this bacterial species, e.g. in the UK a prevalence of resistance to ampicillin of 98.8% is reported[30]. Studies have shown that VRE infections, especially in the case of sepsis, lead to a significant increase in mortality, prolonged hospitalization, and treatment costs. In fact, the treatment in this case includes the use of isolation rooms and specific sanitisation of the environment[31]. It has been shown that favouring the development of VRE strains by antibiotic treatment can lead to the dominance of this species in the gastrointestinal tract. This event appears to be associated with an increased risk for patients to develop VRE sepsis.[32].

S. *aureus* is a Gram-positive pathogen that commonly is part of the skin microbiota[33], so it is not surprising that it is an important wound pathogen that can cause both acute and chronic infections[34]. Clinical isolates of MRSA were first identified in the 1960s [35]; because these strains showed resistance to β-lactams, infections caused by MRSA were treated with newer antibiotics such as vancomycin[36], and it was not too long before clinical VRSA isolates were expected to appear[26]. This situation poses a major challenge for the development of new methods to treat MRSA and VRSA infections, especially since *S. aureus* also has other strategies to undermine the action of drugs, such as biofilms. Indeed, it is now recognised that *S. aureus* biofilms are a major cause of chronic infections following the insertion of implanted medical devices[37]. Numerous virulence factors, as well as the propensity to acquire interspecific or even intraspecific resistance mechanisms [38], make *S. aureus* a human pathogen difficult to treat.

K. *pneumoniae,* along with *Escherichia coli*, is a prominent member of the Gram-negative *Enterobacteriaceae* family. High mortality rates, often over 40%, have been reported in severe infections caused by carbapenemase-resistant Enterobacterales (CRE)[39]. Antibiotics of the cephalosporin and carbapenemase class have indeed been a mainstay in the treatment of severe infections caused by Enterobacterales, but their efficacy has been compromised by the spread of genes encoding enzymes such as ESBLs and carbapenemases that lead to resistance to these critical drugs[40]. For these reasons, effective antibiotics are often lacking today, and drugs with a toxicity risk (e.g. polymyxins) are used[41].

A*. baumannii* is a Gram-negative pathogen that typically causes infections in hospitalised patients[42]. Although infection rates with *A. baumannii* are relatively low compared to other ESKAPE pathogens, approximately 45% of all *A. baumannii* isolates worldwide are considered MDR [43]. This is due to its intrinsic resistance to antibiotics. *A. baumannii* exhibits some of the resistance mechanisms described in the paragraph above, such as the prominent membrane typical of gram-

negative bacteria, efflux pump systems, and low expression of outer membrane porins. The synergy between these intrinsic features significantly reduces antimicrobial permeability[44], which is exacerbated by its propensity to rapidly evolve resistance mechanisms acquired through gene mutations, such as resistance to carbapenems and β-lactams. From 2011 to 2016, the rate of identified *A. baumannii* isolates resistant to antibiotics of the carbapenem and lactam classes increased by more than 30% worldwide[45]. Of far greater concern, however, is the emergence of polymyxins-resistant isolates that render even these drugs ineffective[46].

***P**. aeruginosa* is an opportunistic Gram-negative pathogen associated with severe respiratory infections in immunocompromised patients; indeed, it is frequently found in patients with cystic fibrosis, where the presence of dense mucus creates a suitable environment for its survival [47]. Due to some intrinsic characteristics, *P. aeruginosa* it can easily evade antibiotic treatment: the gram-negative envelope, loss of porins, and presence of efflux pumps limit drug permeability, while biofilm production leads to antimicrobial persistence and resistance, resulting in chronic infections[13]. It is clear, therefore, that the spread of nosocomial isolates of *P. aeruginosa* resistant to polymyxin- and carbapenemase-class antibiotics as last resort is of great concern[48].

***E**nterobacter spp*. is a genus of a common Gram-negative bacteria and poses a significant risk to neonatal units and intensive care patients[49]. Prior to 2005, an estimated 99.9% of *Enterobacter* strains were sensitive to carbapenems[50], whereas resistance to these antibiotics is now reported in all health regions WHO[51]. In addition, *Enterobacter* clinical isolates resistant to colistin have also emerged[52], so as with the other ESKAPE pathogens, there are very few effective antimicrobials to address this growing health crisis.

Antimicrobial options are often not available for these bacterial species, are often lacking and drugs with a toxicity risk, such as polymyxins, are used to treat these infections. In recent decades, the emergence of polymyxin resistance of these pathogens has become increasingly widespread. Polymyxins remain the last line of treatment for these Gram-negative MDR bacterial infections, even though they are associated with nephrotoxicity and neurotoxicity, and are not active against Gram-positive bacteria or fungi. This raises the need to develop new antimicrobial agents that have a broad spectrum of activity against both Gram-positive and -negative bacteria, while being well tolerated and having a low propensity to develop resistance[53].

1.3 Antimicrobial peptides

1.3.1 Common Properties of Antimicrobial Peptides

In this scenario, the discovery of antimicrobial peptides (AMPs) has opened a new front in the fight toward against antibiotic resistance. The field of antimicrobial peptide research emerged in the early 1980s when Hans Boman reported that the humoral immune system of silk moths (*Hyalophora cecropia*) contains peptides (cecropins) with strong broad-spectrum antimicrobial activity[54]. Shortly thereafter, additional AMPs were identified and in the following years, the field of research rapidly expanded to characterise AMPs of various organisms, including humans. To date, more than 3000 AMPs are known[55], having been isolated from biologic samples or predicted based on gene sequence[56], and the scientific community is working to characterise their activity and function.

The AMPs are a complex class of molecule but they share some common aspects[57]. AMPs are small (generally between 12 and 100 amino acids in size) amphipathic, positively charged biomolecules (among +2 and +12) produced by many organisms, bacteria, plants and animals, both invertebrates and vertebrates, to defend against pathogens [58]. They show a broad-spectrum of activities against Gram- positive and – negative bacteria, fungi and viruses [59]. Their widespread occurrence in almost all domains of life indicates the important role that AMPs play in combating pathogens, representing an essential component of the innate immune system[60].

1.3.2 Charge and Amphipathicity

The charge and amphipathicity are strongly linked to their antimicrobial activity enabling the interaction with the bacterial membranes [61]. The net positive charge (due to the presence of lysine and arginine residues, both amino acids with a positively charged side chain) is essential for their action on the bacterial membrane and enables the initial electrostatic AMP -bacterial membrane interaction[63]. In fact, the most common are cation peptides. Anionic peptides also exist, but often require positive ions such as zinc as cofactors to carry out their antimicrobial activity directed towards Gram-positive and Gram-negative bacteria [64].

Among the characteristics of AMPs, there is the capability to structure themselves as amphipathic molecules, which results in the peptide's ability to interact with- and penetrate bacterial membranes. The result of such interactions is an increase in permeability and a loss of cell membrane function[65]. In the development of new AMPs, amphipathicity and hydrophobicity are properties that must be closely controlled.

Some studies hypothesised that increased levels of hydrophobicity could improve microbial activity, based on the principle that more hydrophobic AMPs could better permeabilise the bacterial

membrane. However, subsequent studies have shown that this also leads to increased toxicity, as these antimicrobial peptides show a lytic effect even against neutral membranes [57].

1.3.3 AMP's structure

AMPs do not have a conserved structure, but tend to fold, exposing hydrophobic and charged regions that are important for interaction and insertion into the bacterial membrane. AMPs can be divided into main structural groups based on their secondary structures. The α-helical peptides adopt an α-helical form in the presence of amphipathic environments such as membranes, whereas they have a disordered structure in aqueous environments[66], and exhibit selective activity against Gram-positive bacteria and fungi. For example, magainin and LL-37 belong to this group[67].

β-sheet AMPs contain highly conserved cysteine residues that allow disulfide bridges to form, making the structure stable and minimising degradation by proteases[68]. β-sheet peptides preferentially act against Gram-negative bacteria[67]. Mixed helix/sheet peptides and extended non-helix/sheet peptides [69] are usually classified by the presence of specific amino acids such as arginine, proline, or tryptophan[60] (Fig. 1.4).

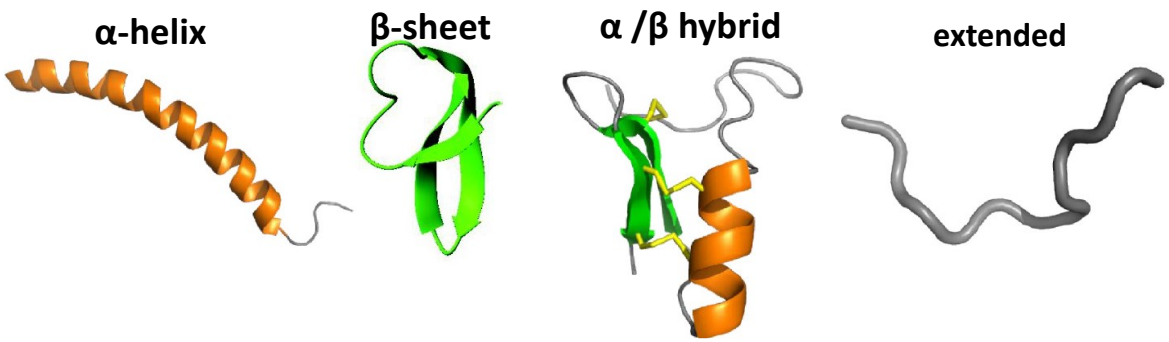

Fig. 1.4: Representative examples of the four "classical" structural classes, the α-helical, the β-sheet, the α/β- and the extended conformation, are exemplified by the peptides LL -37, human α-defensin, phormicin and indolicidin, respectively (J.Koehbach & D.J.Craik 2019).[70]

1.3.4 Mechanism of action of AMP

For simplicity, AMPs are divided into AMPs with membrane-lytic mechanism of action and AMPs with non-lytic mechanism of action according to the mechanism by which they exert their bactericidal action. This is a gross oversimplification because it is well known that some AMPs that exert antimicrobial action by membrane disruption can kill other bacteria via non-membrane-lytic pathways and vice versa.[71] Indeed, the mode of the antimicrobial action depends on many factors, such as AMP concentration, growth phase, and species of bacteria, that interact to provide efficient and rapid killing.[72]

The membrane-lytic mechanism of action of AMPs involves the rupture of the bacterial membrane and it is this that leads to lysis of bacterial cells[67]. This membrane lytic mechanism of action is mediated by electrostatic interactions between positively charged AMPs and microbial surfaces that are negatively charged. Teichoic acids in the cell wall of Gram-positive bacteria and LPS in the outer membrane of Gram-negative bacteria provide an electronegative charge to microbial surfaces, which enhances the interaction with AMPs[73]. The composition of the bacterial membrane is crucial for directing AMPs mainly to the bacterial membrane rather than to the host eukaryotic cell membrane. The latter has no negative charges on the outer face, and phosphatidylcholine and sphingomyelin have neutral charges at physiological pH, so the hydrophobic interactions that occur between AMP and the membrane are weak[74]. Once binding to the bacterial surface is established, AMPs cross the outer envelope by forming bonds with phosphate groups via electrostatic forces or by interacting with the lipid tails of LPS via their hydrophobic portions. In this way, the tight membrane bonds are further destabilised. Subsequently, AMPs diffuse inward into the periplasmic space and accumulate at the surface of the cytoplasmic membrane, where they exert their membrane lytic effect[73]. Three models have been proposed to explain the permeabilization of bacterial membranes by AMPs: the barrel model, the toroidal pore model, and the carpet model (Fig. 1.5). These models differ in the way AMPs penetrate the membrane and form pores that cause cell lysis. In the barrel model, AMPs penetrate perpendicularly, whereas in the toroid model, peptides interact with and deform membrane phospholipids[75]. AMPs can also arrange themselves parallel to the membrane, coating it and forming micelles with the lipid membrane, which has lost its integrity[76]. Because microbial membranes are the primary targets of AMPs, it is difficult for bacteria to develop resistance to AMPs as easily as to conventional antibiotics[77].

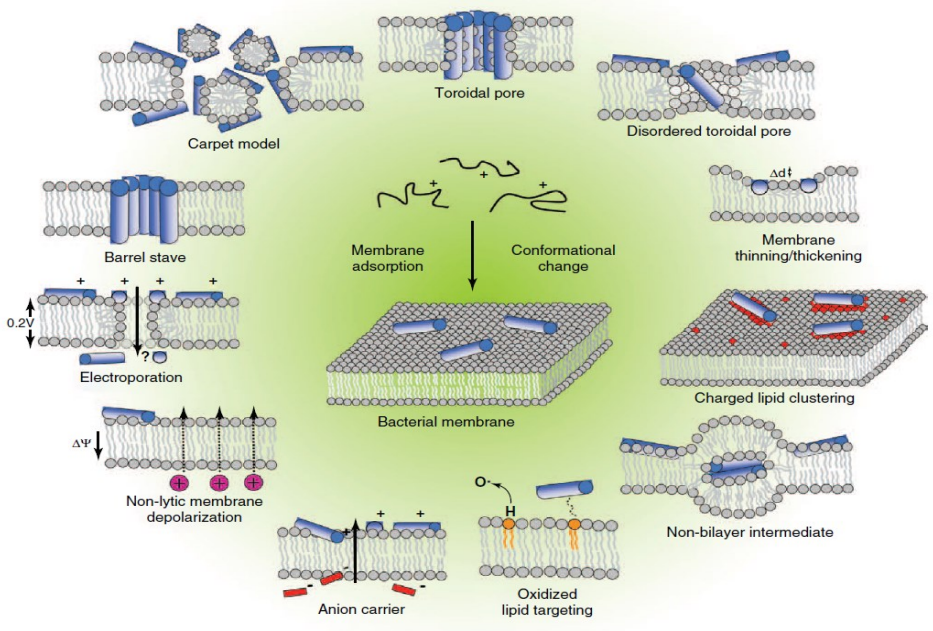

Fig. 1.5: Schematic representation of the mechanisms of action of AMPs targeting the bacterial membrane (L.T. Nguyen et al. 2011).

Although most AMPs kill bacteria by disrupting the bacterial membrane, there is growing evidence that some AMPs can exert antimicrobial activity by recognising intracellular components rather than triggering bacterial lysis[78]. These peptides must first cross the cytoplasmic membrane and have evolved unique mechanisms to be translocated[79,80], such as the use of specific transporters or 'self-promoted uptake' - e.g. by forming temporary pores in the membrane that are too short-lived to cause lethal damage[81]. Once in the cytoplasm, peptides can alter cell membrane septum formation, inhibit cell wall synthesis, inhibit nucleic acid synthesis, inhibit protein synthesis, or inhibit enzyme activity. For example, Lantibiotics, antimicrobial peptides from Gram-positive bacteria containing the amino acid thioether lanthionine, inhibits peptidoglycan biosynthesis[82]; Buforin II binds to the DNA and RNA of *E. coli* and interferes with their metabolism[83]. The proline-rich peptides Apidaecin[84] and the bovine peptide Bac7[85] have been shown to inhibit translation by binding to two different sites on ribosomes; and binding to DnaK, a 70 kDa heat shock protein, thereby decreasing activity and altering folding of proteins involving DnaK[86].

1.3.5 Advantages and disadvantages of AMPs as potential antibiotics

AMPs have been referred to as a "promising alternative to antibiotics"[87] or "potential to solve the growing problem of antibiotic resistance"[88], and great efforts have been made in recent years to fully understand how these molecules work. This is due to the great advantages that these molecules offer. AMPs have in fact multiple inhibitory effects, as they can act both non-selectively on bacterial membranes and selectively by recognising one or more intracellular targets, inducing less resistance in microbes than conventional antibiotics[89,90]. Indeed, it is very difficult for bacteria to develop resistance mechanisms towards such a non-selective effect like the membrane lysis. On the other hand, as non-lytic AMPs alter processes that are fundamental to cell life, the probability of developing resistance through genetic mutations would be very low, since this would require excessive metabolic effort[72]. Another distinctive feature of AMPs is their very broad spectrum of activity. AMPs are active on gram-positive gram-negative bacteria, fungi[91], and viruses[92], therefore representing a good option for the treatment of microbial co-infections. Unfortunately, despite these great advantages, most AMPs are currently limited to topical use, due to their main systemic toxicity, their susceptibility to proteases, and the rapid renal clearance of these peptides from the blood stream[67]. Indeed, oral administration would lead to proteolytic digestion by trypsin and pepsin in the digestive tract, whereas systemic administration would result in a short half-life of AMPs due to degradation by blood proteases and poor stability of peptides in serum [93]; factors to which must be added a non-negligible hemolytic activity[94]. In addition, the antimicrobial activity of AMPs has been shown to be influenced by the presence of salts or albumin in serum. The binding

of AMPs to serum albumin would greatly reduce the effective concentration of peptides available to fight bacterial infection, and consequently AMPs are less active.[95,96]

To limit the problems of toxicity and bioavailability and to improve the efficacy of AMPs, numerous strategies such as chemical modification[97], the use of transport vehicles[98], or peptidomimetic strategies[99] have been studied. Despite these studies on these new strategies, in recent years, researcher's attention has been focused on a subcategory of AMPs that naturally exhibits less cytotoxicity: proline-rich antimicrobial peptides, which will be discussed in more detail in the following sections.

1.4 Proline- rich AntiMicrobial Peptides (PrAMPs)

Among AMPs that prevalently act via a non-membrane lytic mechanism, we can identify a particular subgroup: the **P**roline-**r**ich **A**nti**M**icrobial **P**eptides (PrAMPs).

In general, PrAMPs have been isolated from various and phylogenetically distant animal sources, and share an unusually high content of proline and arginine residues in their sequence[100] (Fig. 1.6). These peptides have a spectrum of activity that is predominantly restricted to some Gram-negative bacterial species and possess a stereospecific mode of action mediated by their internalisation in the cytoplasm of the microorganism without damaging effects to its membrane[100].

The first peptides in this category to be discovered were the apidaecins, isolated from the lymphatic fluid of the bee *Apis mellifera* [101], bactenecins 5 and 7 (Bac5 and Bac7) identified in bovine neutrophils[102] and PR-39 isolated from the intestine of pigs [103]. Since then, PrAMPs have been identified in higher eukaryotes such as sheep [104], goats [104], and more recently in dolphins[105], but also in many arthropods such as crustaceans[106] and in numerous insects, including bees[101], flies[107], and beetles[108] , to BnPRP, the only PrAMP isolated from plants to date[109].

			[aa]	Organism
Arthropods	Apidaecin-1b	GNNRPVYIPQPRPPHPRL	18	*Apis mellifera*
	Drosocin	GKPRPYSPRPTSHPRPIRV	19	*Drosophil melagnog.*
	Oncocin	VDKPPYLPRPXPPRRIYNNR	20	*Oncopeltus fasciatus*
	PrAMP	XXVPYPRPFPRPPIGPRPLPFPGGGRP.	30	*Carcinus maenas*
Mammals	bt_Bactenecin-7	RRIRPRPPRLPRPRPRPLPFPRPGP..	60	*Bos taurus*
	PR-39	RRRPRPPYLPRPRPPPFFPPRLPPR.	39	*Sus scrofa*
	bt_Bactenecin-5	RFRPPIRRPPIRPPFYPPFRPPIRP..	43	*Bos taurus*

Fig. 1.6: Representation of sequence alignments of some PrAMPs derived from arthropods insects and crustaceans and mammals. Similar and identical residues are shown in blue and red respectively. (Modified image from Graf et al.2017.)

1.4.1 Biosynthesis of PrAMPs in animals

PrAMPs are produced by the immune system as a host response to bacterial infection and thus play a defensive role. Interestingly, their processing and activation differs between species. Mammalian PrAMPs belongs to the cathelicidin family which are synthesised as pre-pro sequences and directed into the large granules, where they undergo proteolytic editing of the addressing sequence and are then processed and stored as inactive pro-peptides. Bacterial infection results in the fusion of the large granules containing the pro-PrAMPs and the azurophil granules with the phagosome that phagocytosed the microorganism. In the latter, the elastases of the azurophil granules allow the activation of the mature PrAMPs by removing the pro- sequence. Alternatively, the large and azurophil granules may fuse with the plasma membrane, instead of the phagosome and exert their effects outside the eukaryotic cell[110] (Fig. 1.7 A).

This storage system provides significant advantages for the cell, which is protected from the potential action of the peptides by storing them as pro-forms, and in addition protein synthesis time is saved, allowing a faster response time to infection[111].

Most insect antibacterial peptides, like apidaecin, are synthesised as precursors containing repeated copies of mature peptides. The multi-unit precursor is preceded by a pre-pro -N-terminal sequence, which must be processed to release the mature peptide. The processing takes place in several steps. Upon activation, amino-, endo- and carboxypeptidases process the precursor and release multiple copies of the mature apidaecin[110] (Fig. 1.7 B).

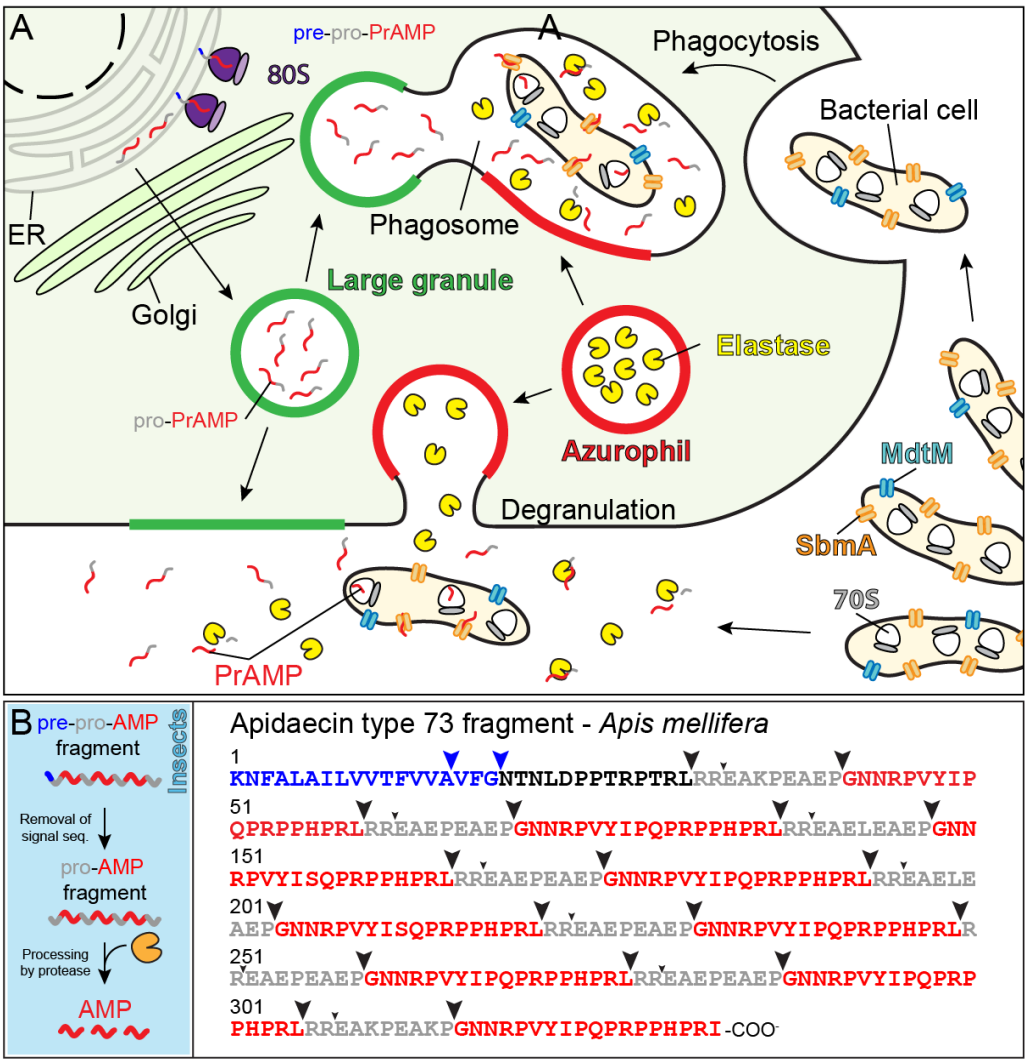

Fig. 1.7: **A) Schematic representation of the synthesis of PrAMPs in mammals** from pre-pro sequences that are directed to the large granules. The peptides are activated during bacterial infection by fusion of the large granules containing the pre-pro peptide with the azurophilic granules containing elastase. Elastase activates the mature PrAMP by removing the pro-sequence. Subsequently, the activated peptide is transported into the bacterial cell via SbmA.
B) Diagram illustrating the activation of insect PrAMP synthesised as multiple copies within an open reading frame. The peptide is processed by amino, carboxy and endoproteases. On the right is an example of a pre-pro sequence of apideacin type 73. The arrows indicate possible cut points. (Graf et al. 2017)

1.4.2 General features of PrAMPs

Although they originate from a wide variety of sources, these peptides share common characteristics that allow them to be grouped together in the PrAMP family.

- **Positive electrostatic charge**: they are often called cationic because they have positive charges up to +11. This property is essential for electrostatic interaction with the negatively charged bacterial membrane[110]. Generally, the positive charge is mainly conferred by arginine residues:
- **The presence of proline residues in the sequence**. In literature over the last few years, the percentage of proline residues that a peptide should reach to be defined proline-rich, has

been controversal, ranging from "> 25% [112]" to "about 30% [113]", to a general "high" proline content[111]. Analysis of PrAMP sequences finds that proline content varies from 14 to 49%, with an average of 34%[114], and proline residues are often arranged in conserved patterns along with arginine residues to form the Pro-Arg-Pro (PRP) motif [113]. The first insights into which residues are critical for the bactericidal activity of PrAMPs, and thus the first evidence for a sequence-activity relationship, were obtained by a comparative analysis of natural apidaecin-type peptides from a variety of insects. Comparison of these peptides revealed a conserved core with the sequence R/KPxxxPxxPRPPHPRI/L. Deviations from the C-terminal consensus greatly reduced antimicrobial activity [110]. This aspect was also verified for Bac5[115] and Bac7 [116], where modifications of certain key residues (but mainly on the N-terminus) significantly impaired the antimicrobial activity of these peptides. More recently, the introduction of proline residues has been used to improve the therapeutic index of PrAMPs[117]. Therefore, understanding the primary structural features of naturally occurring PrAMPs is of great importance for future therapeutic development.

- **<u>They act mainly on intracellular targets</u>**. In 2000, Otvos and colleagues using immunoprecipitation experiments identified an intracellular target of PrAMPs pyrrocoricin, drosocin and apidaecin, i.e. the DnaK chaperone [118,119]. However, later studies cast doubt on the identification of this target for PrAMPS, as DnaK-deleted *E. coli* strains showed no different susceptibility than wild-type *E. coli* strains [86,120]. Over the years, the search for alternative targets has not ceased and has led to the identification of the ribosome as a main target of PrAMPs[121] , proposing inhibition of bacterial translation as a mechanism of action for the antimicrobial activity of PrAMPs[121]. Although the intracellular mechanism of action is the predominant one, the identification of PrAMPs with a membranolytic mechanism, such as certain proline-rich peptides from cetaceans[122] and myticalins from bivalves[123], should not be neglected.

Since their discovery, several sequence- activity-based strategies have been used to develop improved analogues of PrAMPs. For example, consensus sequence analysis led to the creation of an A3- APO (All Peptide Optimised) peptide *de novo* [124], while the creation of a library of analogues led to bactenecin 5 derivatives with an improved activity spectrum of activity [115]. The recent use of deep mutational scanning methods to study how changes in the sequence of PrAMPs affect their antimicrobial activity opens up new opportunities to select a large number of amino acid variants for a single peptide, study its antimicrobial activity, and rapidly identify optimised derivatives[125].
Proline-rich peptides have several advantages: they have very low cytotoxicity, they have specific targets, and they offer the possibility of being used as a new class of peptides that can enter bacterial cells.

Because of these properties, PrAMPs represent a potential new class of peptides that can be used *per se* or as vehicles for the internalisation of non-membrane permeable molecules into cells and for the development of new compound anti-infective.

1.4.3 Uptake of PrAMPs in target bacteria

Once PrAMPs have been proteolytically cleaved, they can exert their effects on the pathogens that they are designed to combat. Since PrAMPs act in the bacterial cytosol, they must be internalised by bacterial cells. Most PrAMPs are actively transported into the cells of some species of Gram-negative bacteria by a mechanism involving the membrane transporters SbmA[79] or YgdD[126]. A secondary transport mechanism for PrAMPs is represented by MdtM an inner membrane protein that plays an important role in the elimination of antibiotics from the bacterial cytosol[80] (Fig. 1.8). SbmA is a 406- residue protein that has been identified in *Escherichia coli* but that is also present in several *Enterobacteriaceae* and in some *Pseudomonadales*, such as *Acinetobacter baumannii*, whose exact physiological function is still unknown [110,127]. SbmA transports PrAMPs into the bacterial cytosol by exploiting the electrochemical gradient at the inner membrane[127]. As evidence, bacterial strains with a mutated or deleted form of this protein show reduced sensitivity to treatment with mammalian and insect-derived PrAMPs, such as Bac5, Bac7, PR -39, and apidecin, but not to lytic peptides[79]. The presence of the membrane transporter in different species of microorganisms correlates with the antimicrobial activity of PrAMPs. For example, *Pseudomonas aeruginosa* lacks SbmA, making it less sensitive to PrAMPs because they are not internalised and cannot use their main bacterial killing mechanism [128]. Although uptake of PrAMPs is necessary for antimicrobial activity, several studies show that PrAMPs can act without internalisation, albeit in a different manner. Indeed, at high concentrations, PrAMPs are also able to act directly on the membrane rather than from within the bacteria[129]. The concentration value at which PrAMPs show this switch in their effect varies from peptide to peptide and also depends on the bacterial strain on which they act [129].

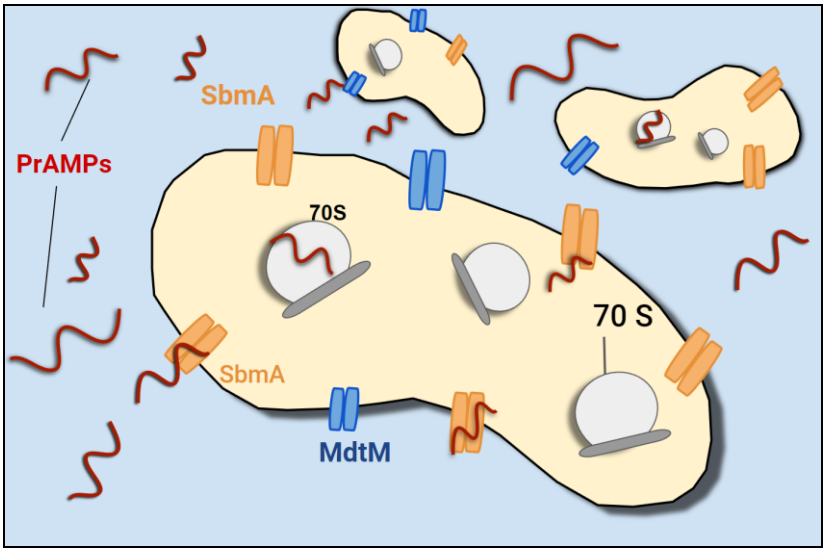

Fig. 1.8: Schematic representation of the uptake of PrAMPs in target bacteria via the transporters SbmA and MdtM.

1.4.4 Mechanism of action of PrAMPs on bacterial ribosomes

As mentioned earlier, several binding assays and in vitro translation experiments have shown that protein synthesis is the primary process inhibited by PrAMPs[120,121]. Following this discovery, many studies have focused on identifying the specific binding site of PrAMPs to the bacterial 70S ribosome, including whether or not all PrAMPs bind to the same ribosomal site and whether or not they all share the same mechanism. Based on the results of these studies, it is possible to distinguish PrAMPs into two different binding types and mechanisms of action: Type I PrAMPs, including Bac7, Onc, Pyr, Met, and Tur1A, all act as inhibitors of the first elongation step, while type II PrAMPs, such as apidaecin 1b and Api137, act predominantly as inhibitors of translation termination[85,105,110,130,131].

PrAMPs interact with the major subunit (50S) of the bacterial ribosome. The binding site of PrAMPs within the ribosome is very specific. It is located within the exit tunnel of the nascent protein, adjacent to the binding site of the nascent peptide to tRNA. Type I PrAMPs bind in the ribosome in an orientation opposite to that of the nascent polypeptide [110], and this PrAMPs-ribosome interaction is facilitated by a multitude of hydrogen bonds and weak interactions between the peptide backbone and the 23S rRNA nucleotides (Fig. 1.9) [85]. At the binding site, PrAMPs adopt a conformation consisting predominantly of unstructured regions interspersed with stretches in which the numerous proline residues give the peptide the appearance of a poly-proline helix of the II type[110]. Once bound in the ribosomal tunnel, PrAMPs protrude to the A-site of the ribosome (Fig. 1.9). Notably, it is the N-terminal sequence of PrAMPs that places steric stress on the binding region of the tRNA[85], resulting in the inability to accommodate the tRNA in the A-site, which is therefore unable to load amino acids into the neo-forming peptide chain (Fig. 1.9). The presence of

the N-terminal end is indeed crucial for the activity of PrAMPs, which deletion of this portion results in greatly reduced antimicrobial activity[132]. Importantly, PrAMPs enable the formation of the 70S initiation complex but prevent subsequent rounds of translation because other tRNAs cannot be accommodated in the A-site[85]. In addition, a very important observation is the sequence similarity of class I PrAMPs: there is a particularly conserved core in which the motif PRP is located. In all ribosome complexes with class I PrAMPs solved so far, the PRP motif of each PrAMP was in exactly the same position and conformation [85,115,130]

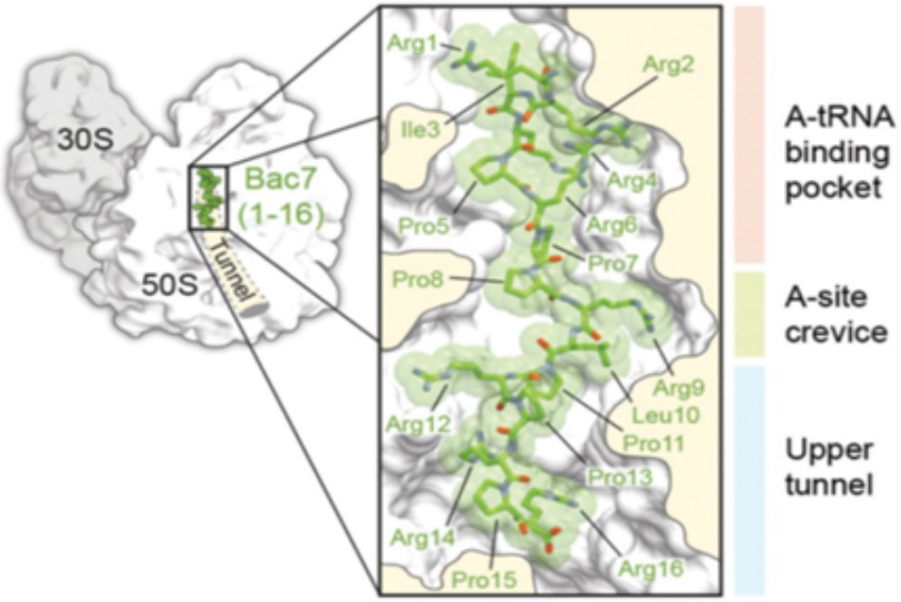

Fig. 1.9: Structural details of the binding site of PrAMP. Structure of Bac7(1-16) in the ribosomal exit tunnel. (Seefeldt et al., 2015.)

1.5 The mammalian PrAMP peptide Bac7 and its fragments

In the laboratory where this thesis project was conducted, PrAMPs have been studied for many years, with a focus on those isolated from mammals. In particular, two PrAMPs named Bactenecins 5 and 7 were isolated in our laboratory in 1989 from *Bos taurus* neutrophils[102], belonging to the group of cathelicidins[133] that includes PR-39 isolated from pigs[103], as well as several homologs from sheep and goats[104,134]. The present work is particularly focused on the Bac7, named according to its molecular weight of 7 kDa. Since its discovery, this peptide has been intensively studied and is now very well characterised. Bac7 is a linear peptide composed of 60 amino acid residues (Fig. 1.10) and has a peculiar primary structure that includes an arginine-rich cationic N-terminal region followed by 14 hydrophobic residues repeated three times in tandem [129]. The high arginine content gives the peptide a net positive charge of +17, while the numerous proline residues force the

molecule to maintain a linear structure, or at least impair its structuration in α-helix and β-sheet. The arginine and proline residues are the most abundant residues and the PRPX motif is repeated 11 times. These characteristics meet the above criteria to classify this peptide in the subgroup of PrAMPs[114].

RRIRPRPPRLPRPR $(PRPLPFPRPGPRPI)_3$ PRPL

Fig. 1.10: Sequence of Bac7

Bac7 is synthesised as previously described for mammalian PrAMPs (section 1.4.1). Processing of the 23.5 kDa pre-proform, produced by bovine bone marrow cells, results in an inactive 20 kDa preform that is stored in the large granules of neutrophils. Upon infection, cleavage by elastase allows release of the mature 7 kDa Bac7.[133].

The N-terminal region of Bac7 is essential and sufficient for the antibacterial activity of the peptide[132]. The N-terminal fragment of Bac7(1-35) has antibacterial activity comparable to that of the native peptide consisting of 60 amino acids: the minimum inhibitory concentration (MIC) of microbial growth against *E. coli*, *Salmonella typhimurium*, and *K. pneumoniae* is ≤ 1 μM, and the antimicrobial activity against certain multidrug-resistant clinical isolates of *A. baumannii* and *P. aeruginosa* is also excellent (MIC ≤ 8 μM). The minimum length required to Bac7 fragments for antimicrobial activity is 16 residues. In fact, significant antimicrobial activity against *E. coli, S. typhimurium* and *K. pneumoniae* was maintained even by the shortest Bac7(1-16). In contrast, when the four N-terminal RRIR residues of peptide 1-35 are removed to yield Bac7(5-35) and Bac7(5-16) fragments, the antimicrobial activity decreases dramatically, highlighting the critical importance of these four N-terminal residues[132].

Like many PrAMPs, Bac7 (1-35) has a non-membrane- lytic mechanism of action that depends on the presence of the SbmA transporter or its homologues, in the bacterial membrane[135]. The transporter is then exploited for internalisation, allowing subsequent compounds to reach the specific intracellular target represented by the 70S ribosome. The *sbmA* gene is not essential for pathogens, which therefore may mutate or lose it and become less sensitive to PrAMPs [136]. As a consequence Due to the emergence of resistant microorganisms, the use of PrAMPs as antibiotics would be limited.

However, Bac7(1-35) has been shown to permeabilize the bacterial membrane of *P. aeruginosa* - which does not express the SbmA transporter- suggesting a possible dual mechanism of action.[127]Bac7(1-35) is a well-characterised peptide. Displayed anti-biofilm activity at sub-MIC concentrations against clinical isolates of *A. baumannii*[137]; it also showed promise in vivo, significantly reducing mortality in mouse models infected with *S. typhimurium*, albeit with

disadvantages such as rapid excretion from the mouse organism and deterioration of antimicrobial activity due to the presence of serum proteases[138].

1.6 Optimisation of Bac7(1-16)

In recent years, several studies have focused on the optimization of PrAMPs to develop new peptides with significant antimicrobial activity, low toxicity to eukaryotic cells, good stability in biological fluids, and last but not least, to reduce their production costs. With this aim, PrAMPs such as Apidaecin[136,], Oncocin[139,140] and Bac5[115] were subjected to systematic sequence and length modifications. The libraries synthesised in these studies led to the identification of variants with better antimicrobial activity than the original peptides. In light of these data, Mardirossian and colleagues in 2020 in our laboratory attempted a series of optimizations starting from the Bac7(1-16) fragment to design new peptides. The first important data from this work was the identification of Bac7(1-16) residues essential for antimicrobial activity.

To this purpose, an alanine-scan was set up. A library of 16 peptides was prepared, in which each residue was individually substituted with alanine and then the antimicrobial activity of all these peptides was evaluated. The residues 9-12 in Bac7(1-16) were evidenced as particularly important for Bac7(1-16) activity, as substitutions in this region lead to a decrease in antimicrobial activity and translation inhibition in vitro. These data then served as the basis for the development of new derivatives with more advantageous antimicrobial properties. Subsequent substitutions were performed with other amino acids, however only substitutions with Arg and Trp improved peptide activity when placed in specific regions. In the study, one of the selected derivatives proved to be particularly promising: B7-005[116] (Fig. 1.11).

B7-code	Sequence
wt	RRIRPRPPRLPRPRPR
001	WRIRPRPPRLPRPRPR
002	RRIRPRPPRLPRPRWR
003	WRIRPRPPRLPRWRPR
004	WRIRRRPPRLPRPRWR
005	WRIRRRWPRLPRPRWR

Fig. 1.11: The derivatives of Bac7(1-16) and their aminoacidic substitutions compared to the original sequence of Bac7(1-16) peptide. (Mardirossian et al. 2020)

B7-005 is a peptide with four substitutions compared to Bac7(1-16), namely an arginine and two proline residues at positions 1, 7, and 15, respectively, of Bac7(1-16), which have been replaced in B7-005 by three tryptophan residues, and an arginine substituted for the proline of Bac7(1-16) at position 5. B7-005 still retains 8 positive charges at neutral pH like Bac7(1-16) after these substitutions, while the tryptophan residues slightly increase the hydrophobicity of B7-005 compared to Bac7(1-16). B7-005 showed excellent activity against *E. coli*, which was less dependent on the presence of the SbmA transporter than native Bac7(1-16). It also showed antimicrobial activity against *P. aeruginosa* and *S. aureus*, which are not sensitive to the action of Bac7(1-16), giving B7-005 a broader spectrum of activity than the native peptide. In addition, in vitro transcription/translation assays confirmed that B7-005 has an inhibitory effect on bacterial translation, so the mechanism of action of B7-005 does not appear to differ from that of the original peptide. B7-005 also showed good tolerance to serum proteases, and an exploratory assay showed low toxicity in a blood cell line.[116]

With these properties, B7-005 overcomes some of the limitations of PrAMPs and could therefore be considered as a good broad-spectrum antimicrobial compound for use in the fight against antibiotic resistance. Therefore, in this thesis, an in-depth characterization of this promising peptide was performed, which holds out the prospect of its future clinical application as a first line of defence against most ESKAPE pathogens.

2. Aim of the thesis

The emergence of bacterial strains resistant to one or more antibiotics is now considered a serious problem worldwide, as many common infections become untreatable. Of particular concern is a group of pathogens against which the World Health Organisation has prioritised the development of new effective antibiotics. These pathogens are referred to by the acronym ESKAPE: *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter spp*.

Antimicrobial proline-rich peptides (PrAMPs) have attracted considerable interest scientific community as potential therapeutics. PrAMPs exhibit potent and specific antimicrobial activity, penetrate some bacterial species, bind to prokaryotic ribosomes, and inhibit protein synthesis. PrAMPs are currently being optimised to develop new antibiotics.

In screening libraries of short mammalian PrAMPs, peptide B7-005 was selected because it exhibits antimicrobial activity, is insensitive to serum proteases, and appears to have low toxicity based on preliminary data.

With this in mind, the project of this work had the following objectives

1) To characterise the antimicrobial potential and anti-biofilm activity B7-005 on ESKAPE pathogens;

2) To test its propensity to select de novo resistance in bacteria;

3) To investigate the mode of action of B7-005 on ESKAPE;

4) To evaluate its biocompatibility towards human cells;

5) To assess its potential in vivo toxicity by using a Zebrafish larvae model.

The ultimate goal was to contribute to the development of new drugs for the treatment of antibiotic-resistant bacteria by characterising B7-005 characterising it to estimate a therapeutic window and to collect data on which to base future studies of specific types of infections.

3. Materials and Methods

3.1 Peptides

All peptides were synthesised by the company NovoPro Bioscience (Shanghai,China) by solid-phase synthesis with F-moc chemistry, purified by RP-HPLC to a purity of >95%, and then their molecular weight was checked by mass spectrometry. The peptides were shipped lyophilised. Upon arrival, the peptides were resuspended in 500 µl of 10 mM HCl and lyophilised to remove the TFA replacing it with the chloride counterion (Cl-), which is less toxic. This process was repeated three times. Then, the peptides were resuspended in sterile milli-Q water and quantified by spectrophotometer (Ultraspec 2100 pro, Amersham Bioscience). The concentration of the peptides was calculated according to a work of Kuipers and Gruppen[141] for the calculation of molar extinction coefficients, and by measuring their absorbance at 214 nm and 280 nm according to Lambert-Beer's law. The peptides were stored at -20°C.

3.2 Antibiotics

The used antibiotics were chloramphenicol (CHL), solved in ethanol (95% v/v) and subsequently diluted in H_2O, colistin (COL) and rifampicin (RIF), both solved in sterile milli-Q-H_2O. All antibiotics were purchased from SIGMA-ALDRICH (USA).

3.3 Bacterial cultures

The reference bacterial strains used were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA), and were: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Acinetobacter baumannii* ATCC 19606, *Enterobacter cloacae* ATCC 13047, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecium* ATCC 19434. All strains were stored at −80 °C as glycerol stocks until used. The bacterial cultures were grown for all the experiments in sterile Müller-Hinton broth (MHB) (Difco Inc). For each experiment, the bacterial cultures were grown overnight (o/n) at 37°C. The following day, overnight cultures of each strain were diluted 1:30 in new MHB and incubated at 37°C with agitation (140 rpm) for approximately 2 hours (mid-log phase) until an optical density (OD) of about 0.3 at 600 nm was reached. Then, bacteria were diluted in new MHB to the desired concentration and used for the experiments.

3.4 Evaluation of the antimicrobial activity against ESKAPE pathogens

The antimicrobial activity was determined by the Minimum Inhibitory Concentration (MIC) assay, according to the guidelines of CLSI (Clinical and Laboratory Standards Institute). Briefly, the peptide was dispensed in the first wells of a round bottom 96-well microtiter plate (Sarstedt, Milan, Italy) and serially diluted twofold in MHB into the subsequent wells in a final volume of 50 µl. Then, a mid-log bacterial culture (see above) 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 MHB sterility control wells), halving the final concentration of bacteria and peptides. The plate was incubated at 37°C for 18h. The MIC value was calculated as the lowest concentration of compound resulting in the complete inhibition of visible bacterial growth. Every MIC was determined through at least three independent experiments ($n \geq 3$).

The Minimal Bactericidal Concentration (MBC) was evaluated by plating on MH-agar (MHA) (Difco Inc.) plates 25µL from the clear wells of the MIC assay, and incubating agar plates at 37°C overnight. The MBC value was taken as the lowest concentration of compound reducing the viability of the initial bacterial inoculum by ≥99.9%. Results derive from at least three independent experiments ($n \geq 3$).

3.5 Resistance selection by serial passages in *E. coli* ATCC 25922

A modified serial passage protocol was developed based on previously described method[142]. As reference antibiotics recognising intracellular bacterial targets and for which the development of resistance mechanisms in bacteria is well known, rifampicin and chloramphenicol were used. Colistin, a well-known lytic-acting antibiotic was used as reference for membrane permeabilizing compounds and for which the development of resistance in bacteria is a rare event. Bac7(1-35), Bac7(1-16) and B7-005 were used as representative for PrAMPs. Briefly, MIC values for the selected compounds were first determined for *E. coli* ATCC 25922 and recorded. The day after, the first serial passaging was started by collecting all bacterial cells growing at ½ MIC values and inoculating into fresh MHB, added with the same ½ MIC concentration of compound to maintain the evolutive pressure. This inoculum was incubated o/n and subjected to another MIC assay. After 18h incubation, cells growing at ½ MIC from the previous passage were once again harvested and used for another MIC. The process was repeated for 14 passages. Concentrations of antimicrobial compounds were adjusted during the process to compensate the increase of MIC values.

3.6 Time-Kill Assay

The rate at which B7-005 kills bacteria was determined using a mid-log phase bacterial suspension of the bacterial species reported above (see point 3.3 of Materials and Methods) diluted to $2{,}5x10^5$ CFU/mL in fresh MHB and incubated at 37°C with each peptide used at concentrations corresponding to their MIC or 4xMIC values. After 1h, 2h and 4h of incubation, samples were diluted in new MHB, plated on MHA in duplicates, and incubated o/n to determine viable colonies. An untreated control containing only the bacterial suspension, without peptide, was also considered for each time-point and underwent the same procedure as the other samples. The bacterial suspension was plated at time zero to verify the starting bacterial load. Data represent the average ± SD of three independent experiments (n=6).

3.7 Biofilm inhibition formation and biofilm eradication assays

The biofilm inhibition assay was performed against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. The assay was performed using plates from the MBEC Assay® Kit (Innovotech, Canada), consisting of a plastic lid with 96 pegs and a corresponding 96-wells base. Serial dilutions of B7-005 in 75 µL of MH broth were prepared in the plate base at twice the final concentration. Each well was inoculated with 75 µL of *E. coli* ATCC 25922 or *S. aureus* ATCC 25923 at a concentration of $5x10^5$ CFU/mL, halving therefore the concentration of bacteria and compound.

After 18 hours of incubation at 37°C, the lid with the pegs was transferred to a 96-well round-bottom plate (Sarstedt) containing 175 µL of PBS for 10 seconds to wash the pegs. After washing, the lid was transferred to a flat-bottomed black 96-well plate containing 175 µL of resazurin (TOX - 8 kit, Sigma), diluted 1:20 in MHB. The plate was incubated in the dark at 37 °C for at least 4 hours. The viability of the biofilm was determined hourly by measuring the fluorescence at FLUOstar Omega-BMG Labtech spectrofluorometer (λ_{ex}= 544nm, λ_{em}= 590nm). The time required for the biofilm to metabolise resazurin varied depending on the bacterial species. Therefore, the time when the untreated control reached its maximum fluorescence was chosen as the time point for measurements.

Biofilm eradication activity was tested against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. 125 µL of bacterial cells diluted in MHB were added to the MBEC Assay® Kit plate (Innovotech, Canada) at a concentration of 10^6 CFU/mL. The plate was incubated at 37 °C for 24 hours. During incubation, biofilms settled on the pegs, and these biofilms, once formed, could then be transferred to a new 96-well plate for the antimicrobial efficacy test. The next day, the lid with the pegs was transferred to a 96-well round- bottom plate (Sarstedt) containing 150 µL of PBS for 10 seconds to wash the pegs. The lid was then transferred to a second 96-well round bottom plate in which

different concentrations of B7-005 in 150 µL MHB were prepared. After incubation at 37°C for 18 hours, the pegs were transferred to a flat-bottomed black 96-well plate in which 175µL resazurin (TOX -8 kit, Sigma), diluted 1:20 in MHB, was prepared. The plate was incubated at 37°C, and the protocol was performed as described above for biofilm quantification.

3.8 Analysis of bacterial membrane integrity

The flow cytometric assays were performed with Attune NxT®, (ThermoFisher). The integrity of the bacterial cell membrane was assessed by measuring the uptake of propidium iodide (PI), whereby membrane permeabilisation was calculated as % PI-positive cells, and the extent of membrane damage was quantified by observing the mean fluorescence intensity (MFI) of PI. Briefly, bacterial cultures of the species considered in the study (see point 3.3 of Materials and Methods) were diluted to a final concentration of $2,5x10^5$ CFU/mL in MHB previously filtered at 0,2 µm, then incubated at 37°C for 1h with concentrations of B7-005 corresponding to corresponding ¼ MIC and MIC values for each bacterial species. PI was added to all samples at a final concentration of 10 µg/ml for 5 minutes before measurements. At the end of the incubation, the bacterial cells were analyzed by flow cytometry. As positive permeabilization control samples, bacteria were treated with the membrane-disrupting antibiotic Colistin at ¼ of the MIC and the MIC.While as a negative control for membrane damage, water was added to the cell suspension instead of peptides. Data analysis was performed with the Attune NxT Software. Data are expressed as means ± SEM of three independent experiments ($n \geq 3$).

3.9 Peptide internalization into bacterial cells

The uptake of peptide B7-005 in bacteria was studied by flow cytometry, using a fluorescently labelled derivative of B7-005 and Bac7(1-16) purchased from JPT (Berlin, Germany), obtained adding a further C-terminal cysteine that was reacted with BODIPY™ FL Maleimide. Bac7(1-16) was used as an internalised peptide reference.

The uptake of the B7-005 peptide in the different species above (see section 3.3 of Materials and Methods) was assessed in the flow cytometry (Attune NxT®, ThermoFisher) using fluorophore-labelled B7-005 (B7-005-BY) and a quencher (Trypan Blue, TB, Sigma-Aldrich) to get rid of all the fluorescent of all the compound that was not internalized in bacteria. Mid-log phase bacterial cultures were diluted in filtered MHB to a final concentration of $2,5x10^5$ CFU/mL. Bacteria cultures were then incubated with B7-005-BY at the desired concentrations for 0,5h at 37°C in a thermostat water bath. To have more information regarding the uptake of the peptide, also a sub-lethal concentration was used, i.e. ¼ MIC other than MIC, specific to each bacterial species. The samples were divided into two parts: one aliquot was analysed in flow cytometry by observing the MFI of

the bacteria cells. The other aliquot was incubated with TB (1 mg/mL diluted in MHB) for 10 minutes at room temperature. Tt the end of the incubation, both the aliquots were analysed by flow cytometry. MFI values obtained in the absence and presence of TB of each sample were compared. Data analysis was performed with the Attune NxT Software and results are expressed as means ± SEM after three different experiments ($n \geq 3$).

3.10 Cell lines and growth condition

Four different types of human cell lines were used: B lymphocyte precursors (MEC-1), human lung carcinoma epithelial cells (A549) and the human keratinocyte cell line (HaCaT) and Human umbilical vein endothelial cells (HUVECs). All cell lines used were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA).

Human MEC-1 cells were cultured in suspension culture flasks (Sarsted) using Complete RPMI (EuroClone), supplemented with 100 U/mL penicillin (Sigma), 100 µg/mL streptomycin (Sigma), L-Glutamine (2mM) and 10% v/v foetal bovine serum (FBS, EuroClone).

Human A549 and HaCaT cells were grown in adhesion cell culture flasks (EuroClone) with complete Dulbecco's MEM with high glucose content (DMEM, EuroClone) supplemented with 100 U/mL penicillin (Sigma), 100 mg/mL streptomycin (Sigma), L-Glutamine (2 mM) and 10% v/v of FBS.

HUVECs were cultured with Human Endothelial Serum-Free Medium (HESFM, Life Technologies, Carlsbad, CA, USA) supplemented with 20 ng/mL of epidermal growth factor (EGF), 10 ng/mL of basic fibroblast growth factor (bFGF) (Immunological Sciences), 1% penicillin – streptomycin (Sigma-Aldrich) and 10% FBS (Life Technologies).

Both cell lines were incubated at 37°C with 5% CO_2.

3.11 *In vitro cy*totoxicity against human cell lines

The cytotoxicity of MEC-1, A549, and HaCaT cell lines was evaluated as cell's vitality by the MTT assay. After reaching confluence, A549 and HaCaT cells were removed from the flask and counted in a Burker- Türk chamber to dilute them in complete DMEM to a concentration of $2x10^5$ cells/mL. 100 µL of these cell suspensions were seeded in 96-well flat-bottom microtiter plates (EuroClone). The plate was incubated o/n 37°C and 5% CO_2. The following day, the exhausted medium was removed from each well and replaced with 100 µL of B7-005 prepared at the desired concentration in DMEM medium. After 20 hours of incubation at 37°C, 5% CO_2, 25 µL of 1mg/mL of MTT (Sigma) in PBS was added to each well, and the plate was incubated again in the dark for 4h at 37°C and 5% CO_2. After incubation, the solution was carefully removed from each well to prevent loss of MTT crystals, and then each well was washed with 100 µL sterile PBS. Then, 100 µL of IGEPAL (Sigma) (10% w/v in 10 mM HCl) was added to each well to solubilise the MTT

crystals and incubated o/n at 37°C and 5% CO_2. The next day, absorbance was measured at 570 nm in a Nanoquant Infinite-M200Pro plate reader.

For MEC-1 cells, the MTT assay was performed in a 96-well flat-bottomed plate (EuroClone) with 50 µL of peptides in RPMI concentrated to twice the desired final concentration added to the designated wells. Cells were counted in a Burker-Türk chamber and diluted to a concentration of 2×10^6 cells/mL. Finally, 50 µL of the cell suspension was aliquoted into the wells of a microtiter plate, and the peptide concentrations and cell density were halved. Subsequently, the protocol was performed as described above.

To investigate the cytotoxicity of the peptide to HUVEC cells, 7500 cells/well were seeded in HESFM + 10% FBS in a 96-well flat-bottomed microtiter plate (euroClone). The plate was incubated o/n at 37°C in a humidified atmosphere in an incubator containing 5% CO_2. The following day, the cells were treated with peptide B7-005 at the desired concentrations and incubated for 24 hours. At the end of the treatment period, the medium containing the peptide was removed and 200 ul/well of a solution of MTS (Promega) diluted 1:5 in PBS + glucose (4.5 g/L) + 0.7 mM Ca^{2+} + 0.7 mM Mg^{2+} was added. The plate was then incubated at 37°C for 4 h in a humidified 5% CO2 atmosphere, and the absorbance at 490nm, measured with a Nanoquant infinite M200pro (Tecan) plate reader spectrophotometer, was recorded after 4h. The peptide cytotoxicity was calculated by comparing the OD of treated samples with that of untreated control. The results are average ± SEM of three independent experiments.

3.12 Effects of B7-005 on Eukaryotic Cells Membrane Integrity

Membrane integrity of MEC-1, A549, HaCaT, and HUVEC cell lines was measured by flow cytometric assays after 24-hour treatment with peptide B7-005.

For the A549, HaCaT, and HUVEC cell lines, 120000 cells were seeded in 600 µL DMEM per well in a 24-well flat-bottom microtiter plate (EuroClone), and the plate was incubated o/n at 37°C and 5% CO_2. The following day, the medium was removed from each well and replaced with an equal volume of B7-005 diluted in DMEM to the concentration selected for the treatment. The plate was further incubated for 24 hours.

The following day, to remove adherent cells from the plate, the cells were treated with 2mL of trypsin 1x/EDTA (EuroClone) and then incubated for 5 minutes at 37°C, CO2 5%. At the end of the incubation, the cells were harvested and transferred into a sterile tube. Then, PI was added to reach a final concentration of 10 µg/mL. PI uptake was assessed using the Attune NxT flow cytometer (Thermo Fisher Scientific), which is equipped with a single 488nm laser to measure the fluorescence of PI.

Results are expressed as % PI -positive cells compared with untreated control from three independent experiments in which 10000 events per sample were collected. Cells of the MEC-1 cell line were seeded in a 24-well flat-bottom microtiter plate (EuroClone) at a concentration of 500,000 cells in a final volume of 250 µL per well, twice the final concentration. A serial double dilution of the peptide was then prepared and 250 µL was aliquoted into each well, resulting in a halved concentration compared with the initial concentration. The protocol was then performed as described above.

3.13 Short-time cytotoxicity

The membrane state, morphology and mitochondrial viability of cells from MEC-1, A549 and HaCaT lines were assessed by flow cytometry after being treated with the peptide for a short time. For each cell line, $2x10^6$ cells were harvested and incubated with 24 µL of DiOC6 (3-3'-dihexyloxacarbocyanine iodide) probe (FluoProbes, Interchin, Montlucon Cedex, France) 10 µM in PBS (i.e. 10uL per 10^6cell/0.5mL) at 37°C, 5% CO_2 for 15 minutes. At the end of incubation, the cell sample was washed appropriately with PBS to remove the excess of probe and resuspended in new culture medium. Then PI was added at a final concentration of 10 µg/mL. Finally, the peptide was added to the prepared samples at the required concentrations and incubated for 15, 30 and 60 minutes. Then each sample was analysed by flow cytometer. To check for loss of mitochondrial function, a cell sample was also prepared to which CCCP (carbonyl cyanide-3-chlorophenylhydrazone) was added at a final concentration of 625 µM. Data on the percentage change in mean fluorescence intensity (MFI), morphology, and percentage of PI -positive cells were analysed after 15, 30, and 60 minutes compared with the control.

3.14 Cytotoxicity test in Zebrafish larvae

Zebrafish eggs were placed in E3 1X medium supplemented with methylene blue 0.5%, incubated at 28°C, and 24 hours after fertilisation (hpf) the eggs were manually decorated. Subsequently, the embryos were placed in E3 1X medium. The assays were performed on healthy embryos anaesthetised with tricaine at a final concentration of 0.02%. Toxicity studies of B7-005 were performed using different concentrations of the peptide, 16 mg/kg, 24 mg/kg, 32 mg/kg, 48 mg/kg and 64 mg/kg. PBS was used as an untreated control. For each concentration of B7-005, 4.6nL/embryo was injected into the duct of Cuvier of the embryos, using capillary glasses and a Nanoject II Auto- Nanoliter Injector (Drummond Scientific Co., Broomall, PA, USA). The entire process was conducted using a SteREO Discovery.V8 microscope (Zeiss,Oberkochen, Germany, EU). Embryo survival was observed and recorded after 24, 48 and 72 hours post injection (hpi) at 28°C.

3.15 Model of Infection in Zebrafish larvae

In order to set up a model of bacterial infection in zebrafish larvae, bacterial suspensions of *E. coli* ATCC 25922 were prepared re-inoculating 1 mL of an overnight culture in 40 mL of MH broth at 37°C under agitation. When the bacterial suspensions reached an OD of approximately 0.3, bacteria were centrifuged for 10 minutes at 1000 g (ALC centrifuge PK 120) and the supernatant (MHB) was discarded. The bacterial pellet was then resuspended and washed in 10 mL of sterile PBS and centrifuged as before. The washing procedure step was then repeated, in order to remove as much MHB as possible, to prevent any toxic effect on fish. At the end of the last centrifugation, the pellet was resuspended in 1.5 ml of sterile PBS. Subsequently, the OD_{600} of the concentrated bacterial suspension was measured (upon *ad hoc* dilution 1:10). The number of CFU/mL was then calculated, and the bacterial suspension was diluted to the desired concentrations, i.e. 2×10^6, 2×10^7, 2×10^8, 4×10^8 and 8×10^8 CFU/mL to provide the injection of 10, 100, 1000, 2000 or 4000 CFU of *E. coli* in each fish, considering a micro-injection volume of approximately 5 nL.

Bacteria were injected into the Cuvier's duct of the zebrafish embryos, dechorionated 24 h prior to injection, using a Nanoject II Auto- Nanoliter Injector (Drummond Scientific Co., Broomall, PA, USA) and a pulled glass capillary. The entire procedure was conducted using a SteREO Discovery.V8 microscope (Zeiss, Oberkochen, Germany, EU).

In order to check the consistency of the bacteria inoculum, the bacterial viability, as well as to control if bacteria sedimented during their permanence into the capillary during the procedure, single injections of bacteria were performed into drops of PBS, subsequently diluted in MHB and plated on MH-agar. Plates were incubated overnight at 37°C and, the day after, colonies were counted.

3.16 Statistical analysis

For statistical analysis, data from at least three independent experiments were used, which were internal triplicate experiments. Differences between groups were evaluated using the unpaired Student t test, or the ANOVA test using SigmaPlot (Systat Software Inc,). Values of $p < 0.05$ were considered statistically significant.

4. Results

B7-005 displays four substitution relative to the natural peptide Bac7(1-16). Three tryptophan residues at position 1, 7 and 15 replaces one arginine and two proline residues respectively, and one arginine residue at position five replaces a proline. Both B7-005 and Bac7(1-16) have eight positive charges at neutral pH and B7-005 is a slightly more hydrophobic peptide than the Bac7(1-16) and halves the proline number in its sequence (from 6 to 3) (Table 4.1).

Table 4.1: Amino acid sequence and physical properties of PrAMPs B7-005 and Bac7(1-16)

Peptide	Sequence	MW*	Charge *	GRAVY*
Bac7(1-16)	RRIRPRPPRLPRPRPR	2076.52	+8	-2.331
B7-005	WRIRRRWPRLPRPRWR	2343.81	+8	-2.200

*Molecular weight, charge and grand average of hydropathicity were calculate by using ProtParam tool of Expasy (https://web.expasy.org/cgi-bin/protparam/protparam)

4.1 Rate of resistance selection in *E. coli* grown in presence of B7-005

Given the alarming spread of antibiotic-resistant pathogens that render antibiotics ineffective in a short time, it was initially investigated whether and how quickly the peptide B7-005 could enable the selection of de novo resistance in bacteria. We set up an assay, in which the appearance of resistant mutants was indicated by the increase in the relative minimum inhibiting concentration (MIC) (ratio between the MIC values obtained every single day and the MIC value of the first day) after consecutive exposure to sub-MIC concentrations of the drug. The wild-type (*wt*) counterparts of B7-005, Bac7(1-35) and Bac7(1-16) peptides were used for comparison. The antibiotics rifampicin and chloramphenicol were chosen as reference antibiotics that bind intracellular bacterial targets[143,144] and for which the development of resistance mechanisms in bacteria is well known, whereas colistin was selected as a reference molecule for which the development of resistance in bacteria is a rare event due to its membranolytic mechanism of action[145].

The relative MICs of B7-005, those of the other PrAMPs, and the other antibiotics after serial passages of the reference strain *E. coli* ATCC 25922 are shown in Fig. 4.1

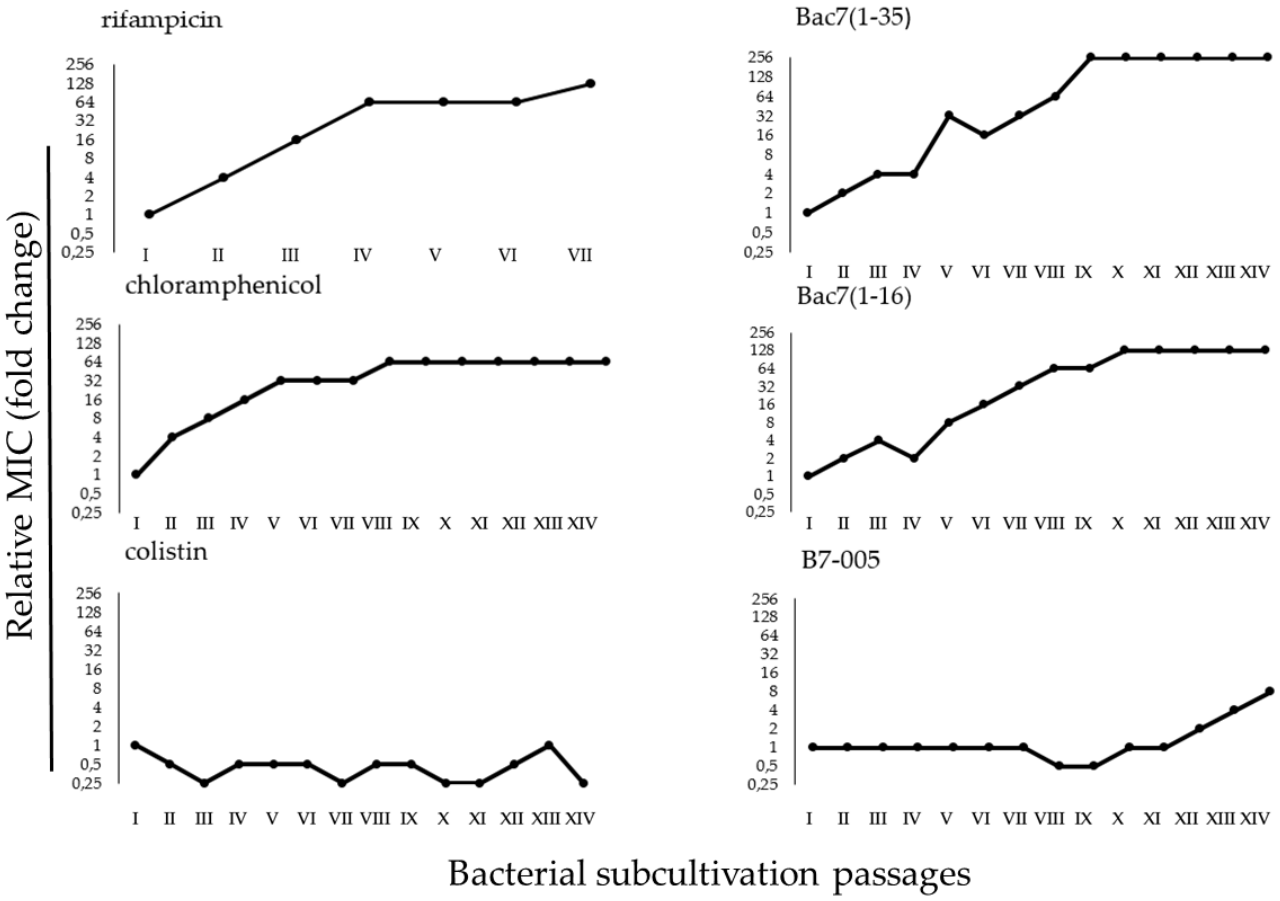

Fig. 4.1: **Relative susceptibility of *E. coli* ATCC 25922 along time to different peptides and antibiotics**. Bacterial cells grown with the highest concentration of antimicrobial agent (1/2xMIC) were sub-cultured and used for the next MIC assay. The procedure was repeated for 14 passages, except for rifampicin, for which seven steps were performed because the molecule's solubility limit was reached. Plots are representative result of assays repeated at least three times with similar results.

Table 4.2: **MIC values for each passage of** rifampicin (RIF), chloramphenicol (CHL), colistin (COL), Bac7(1-35), Bac7(1-16) and B7-005 against ***E. coli* ATCC 25922.**

	MIC values (µM)					
Number of passage	**RIF**	**CHL**	**COLIST**	**Bac7(1-35)**	**Bac7(1-16)**	**B7-005**
I	8	16	0,5	0,5	1	1
II	32	64	0,25	1	2	1
III	128	128	0,125	2	4	1
IV	512	256	0,25	2	2	1
V	512	512	0,25	16	8	1
VI	512	512	0,25	8	16	1
VII	1024	512	0,125	16	32	1
VIII		1024	0,25	32	64	0,5
IX		1024	0,25	128	64	0,5
X		1024	0,125	128	128	1
XI		1024	0,125	128	128	1
XII		1024	0,25	128	128	2
XIII		1024	0,25	128	128	4
XIV		1024	0,125	128	128	8

Values expressed in µM for each individual MIC passage in which *E. coli* ATCC 25922 was exposed to increasing drug concentrations for 14 steps.

B7-005 showed increase its MIC from the 12th passage, whereas the other PrAMPs increased their MIC after only 5 passages. Moreover, the MIC value of B7-005 (MIC= 8 µM) at the 14th final passage was significantly lower than those of Bac7(1-35) and Bac7(1-16), both of which have a MIC of 128 µM (Table 4.2).

As expected, the antibiotics rifampicin and chloramphenicol increased the MIC against *E. coli* ATCC 25922 128- and 64-fold, respectively, after 7 and 14 passages. This increase occurred rapidly, already after the first passage. On the contrary, the MIC values of colistin did not increase during serial passages (apart from the inherent variability of the experiment) (Table 4.2).

Overall, results suggest that bacteria decreased susceptibility to B7-005 more slowly than to the *wt* peptides Bac7 (1-35) and Bac7(1-16), other than to reference antibiotics. However, the appearance of *de novo* resistance emerges in shorter time than that of the lytic antibiotic colistin.

4.2 Antimicrobial activity of B7-005 against ESKAPE pathogens

Given the encouraging result of B7-005 regarding its low propensity to select *de novo* resistance activity of B7-005 was tested against a panel of reference strains representing all the ESKAPE pathogen group (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *E. cloacae*).

The antimicrobial activity of B7-005 was compared with that of *wt* peptide Bac7(1-16). B7-005 was found to be active against all ESKAPE strains tested, with MICs ranging from 2 µM to 32 µM (Table 4.3). B7-005 showed the same MIC as Bac7(1-16) against *E. coli* ATCC 25922 (Table 4.3). However, the sequence changes increased B7-005 activity against Gram-negative *K. pneumoniae* ATCC 700603 and *A. baumannii* ATCC 19606 strains (MIC 2 µM and 4 µM, respectively), and also against *P. aeruginosa* ATCC 27853 (MIC=16 µM), which was not inhibited by the native peptide. Interestingly, B7-005 was also active against Gram-positive bacteria not sensitive to other PrAMPs, showing MIC values of 16 µM and 32 µM against *S. aureus* ATCC 25923 and *E. faecium* ATCC 19434 respectively.

Table 4.3: **Antimicrobial activity of B7-005 and Bac7(1-16) wt against a panel of reference strains of ESKAPE bacterial pathogens.**

Bacteria strain		B7-005		Bac7(1-16) wt	
		MIC (µM)	MBC (µM)	MIC (µM)	MBC (µM)
E. coli	ATCC 25922	1	2	1	2
E. *cloacae*	ATCC 13047	8	16	n.a.	n.a.
S. *aureus*	ATCC 25923	16	32	>64	>64
K. *pneumoniae*	ATCC 700603	2	4	4	64
A. *baumannii*	ATCC 19606	4	4	32	64
P. *aeruginosa*	ATCC 27853	16	16	>64	>64
E. *faecium*	ATCC 19434	32	>64	n.a.	n.a.

Results are the mode of at least three independent experiments (n = 3). MIC and MBC were recorded after 18h of incubation at 37°C. n.a.= not available data.

The MBC test was performed following the MIC test (Table 4.3). The results showed a similar trend to the MIC test, with some exceptions (*E. faecium*, MBC > 64 µM).

Comparison of the MIC and MBC data showed that these concentrations were very close, if not identical, indicating that B7-005 display bactericidal activity. Moreover, these data indicate that the activity of the B7-005 peptide is enhanced compared to the *wt* peptide and that the spectrum of activity has been significantly broadened to include bacterial species lacking the SbmA transporter[79], such as *P. aeruginosa* and Gram-positive bacteria.

4.3 Time-kill assay using B7-005

The rate at which B7-005 kills bacteria was determined against *E. coli* ATCC 25922 and against ESKAPE strains using the viable colony count method. The bacterial cells were treated at MIC and 4xMIC and the number of viable colonies was observed after 1h, 2h and 4h of treatment with the peptide. The number of viable colonies without peptide treatment was also observed as a growth control. Exposure of *E. coli* cells to B7-005 at MIC concentrations for 1 hour resulted in decrease in the number of viable cells by a 1-log (Fig. 4.2). A greater decrease in viable cells, up to 2,6 log, was observed when the peptide concentrations was increased to 4xMIC (Fig. 4.2) suggesting that the concentration-dependent killing action of the peptide was quite rapid.

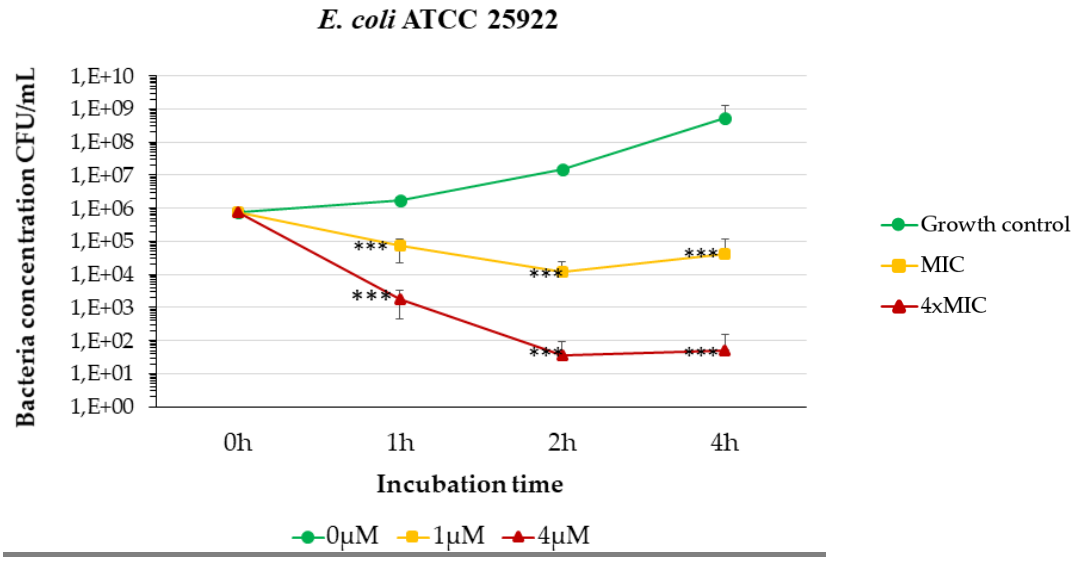

Fig. 4.2: Evaluation of the bactericidal activity of B7-005 against *E. coli* ATCC 25922 cells. Time-kill was determined using a mid-logarithmic phase bacterial suspension, diluted in fresh MHB to a final concentration of 2,5x10^5 CFU/mL, and incubated for 1h, 2h and 4h at 37°C in the presence of different concentrations of B7-005. Samples were then diluted in MHB, plated on MH agar, and incubated overnight to allow the colony counts. Data are the average± SD of at least three independent experiments in internal duplicate (n=6). Green line= growth control; yellow line=_bacteria exposed to MIC of B7-005; red line= bacteria exposed to 4xMIC of B7-005. On the right is the general key to the graph, below the graph is the key with the used concentrations of B7-005 in µM.*= $p < 0.05$, **= $p \leq 0.01$ and ***= $p \leq 0.005$ versus the starting inoculum (Test t-student).

The time–kill assay was then repeated with all the ESKAPE pathogens (Fig. 4.3).

B7-005 reduced the number of viable cells of *A. baumannii*, *E. cloacae*, and *S. aureus* by at least 3 log and by 6 log for *P. aeruginosa* cells within 1 hour at 4xMIC (Fig 4.3 B-E), demonstrating that the bactericidal activity was faster than for *E. coli*. When the treatment with B7-005 at 4xMIC was prolonged for 4 hours, there are no more viable cells of *A. baumannii*, and *P. aeruginosa*, while the bacterial concentrations of *E. cloacae* and *S. aureus* are further reduced by 1 and 2 log, respectively. On the other hand, 4-hour treatment was required to observe 1-log reduction in viable cells of *K. pnumoniae* and *E. faecium* (Fig. 4.3 A and F), underscoring that the bactericidal effect of B7-005 on these species is much less rapid. Overall, these data indicate that B7-005 has bactericidal activity against all ESKAPE, even if the efficacy and speed of this effect varies among the species.

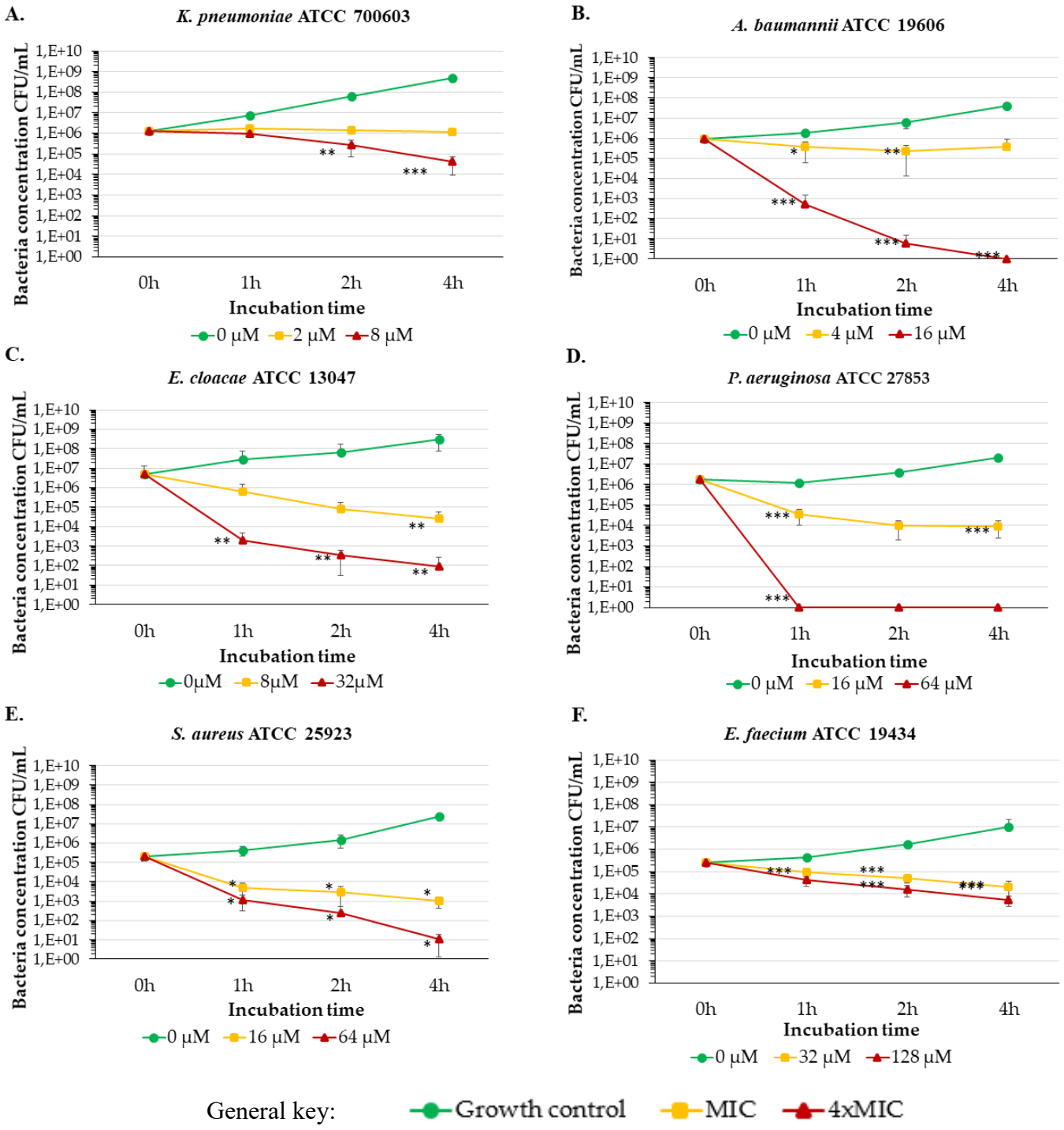

Fig. 4.3: **Evaluation of the bactericidal activity of B7-005** against *K. pneumoniae* ATCC 700603 (A), *A. baumannii* ATCC 19606 (B), *E. cloacae* ATCC 13047 (C), *P. aeruginosa* ATCC 27853 (D), *S. aureus* ATCC 25923 (E), *E. faecium* ATCC 19434 (F). The time-kill was determined using a mid-logarithmic phase bacterial suspension, diluted in fresh MHB to a final concentration of $2{,}5x10^5$ CFU/mL, and incubated for 1h, 2h and 4h at 37°C in the presence of B7-005. Samples were then diluted in MHB, plated on MH agar, and incubated overnight to allow the colony counts. Below each graph, the legend shows the peptide concentrations in µM used for each bacterial species. Below the panel is the generic key. Green line= growth control; yellow line= bacteria exposed to MIC of B7-005; red line= bacteria exposed to 4xMIC of B7-005. Data are the average± SD of at least three independent experiments in internal duplicate (n=6). *= $p < 0.05$, **=$p \leq 0.01$ and *** = $p \leq 0.001$ versus the starting inoculum (Test t-student).

4.4 Anti-biofilm activity of B7-005

Exploratory assays were performed to investigate the activity of B7-005 against the deposition of new biofilm and against the pre-formed biofilms of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, which were used as representatives of Gram-negative and Gram-positive bacteria.

At sub-inhibitory concentrations, B7-005 did not inhibit biofilm formation of *E. coli* ATCC 25922 (Fig. 4.4 A), a phenomenon that was only evident at MIC. On the contrary, the peptide inhibited biofilm deposition of *S. aureus* ATCC 25923 at a concentration of 1/4xMIC. At this concentration less than 50% neosynthesized biofilm was present compared to the untreated control (Fig. 4.4 B).

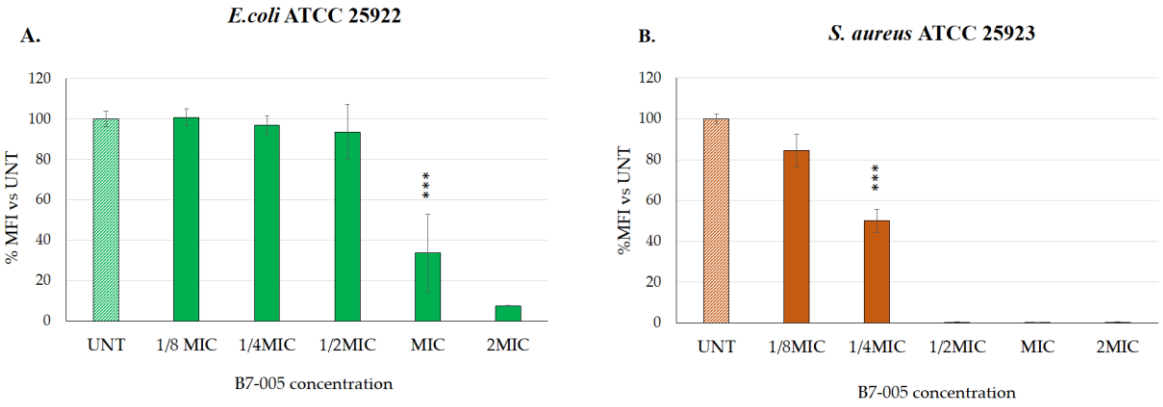

Fig 4.4: Inhibition of *E. coli* ATCC 25922 (A) and *S. aureus* ATCC 25923 (B) biofilm formation. Inhibition of biofilm formation was calculated after 18 hours of incubation with different B7-005 concentrations. The data are expressed as percentages of mean fluorescence intensity (MFI) compared to the untreated growth control (UNT). Given the same initial inoculum, resazurin conversion times differ between bacterial species, so the conversion of the compound inside the biofilm of UNT was monitored over time, and the time required to produce the maximum fluorescence signal was chosen as the time point of the experiment. (2h for *E. coli* and 4h for *S. aureus*). Results are the average of three independent experiments in internal triplicate (n=9) ± SEM. ***=p< 0.001 (Test t-student).

The anti-biofilm activity of B7-005 was also evaluated in the treatment of pre-formed biofilms of the same two reference strains. 60% of the pre-formed *E. coli*, biofilm was eradicated at 16-fold MIC (Fig. 4.5-A), and 96% of the pre-formed *S. aureus* biofilm was eradicated at the MIC compared to the untreated control (Fig. 4.5 6-B).

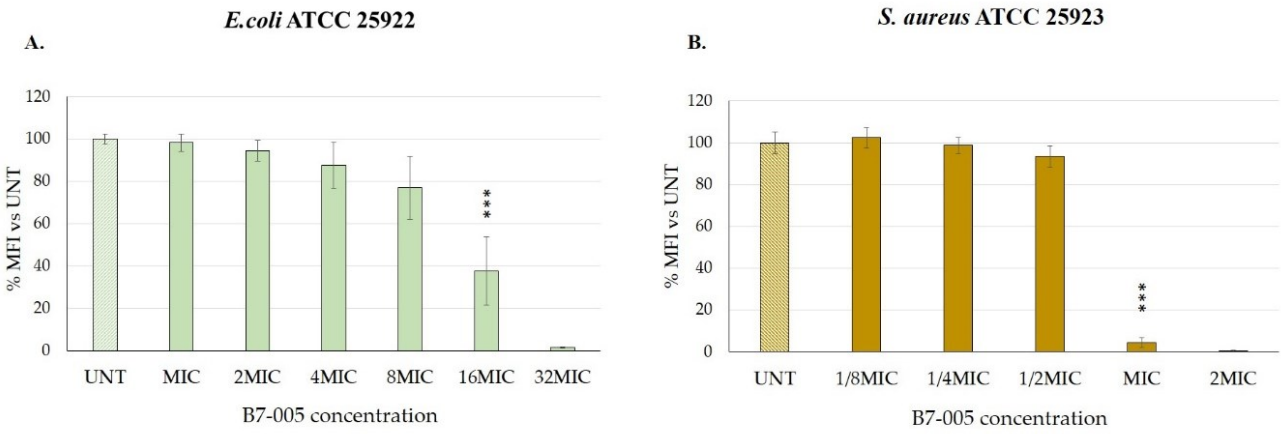

Fig 4.5: Biofilm eradication of *E. coli* ATCC 25922 (A) and *S. aureus* ATCC 25923 (B) after 24h-treatment with different concentrations of B7-005. Eradication activity has been reported as percentages of MFI of metabolic active biofilm in comparison with the untreated control of growth (UNT). The results are the average of three independent experiments in internal triplicate (n=9) ± SEM. ***=$p<0.001$ (Test t-student).

These preliminary data indicate that B7-005 has anti-biofilm activity, both in inhibiting biofilm formation and both in eradicating preformed biofilm, especially against *S. aureus*. It is worth extending the same tests to all ESKAPE strains in future.

4.5 Assessment of the Integrity of the Bacterial Cell Membrane

To determine whether the rapid and bactericidal activity of B7-005 was due to a membrane-perturbing effects, the membrane integrity of *E. coli* ATCC 25922 and of ESKAPE treated with the peptide was examined using the propidium iodide (PI)-uptake assay. The assay is based on the use of the cell-impermeant dye PI, which reaches its DNA target and emits fluorescence only when the bacterial membrane is damaged. Therefore, cell fluorescence indicates membrane permeabilization. Bacteria were treated with B7-005 at 1/4xMIC and at MIC for 1 hour and the degree of permeabilization was compared with that of the lytic peptide antibiotic colistin also used at 1/4xMIC and at MIC (Fig. 4.6). The bacterial assays involving the use of the flow cytometry were carried out in collaboration with Dr. Sara Capolla at the University of Trieste.

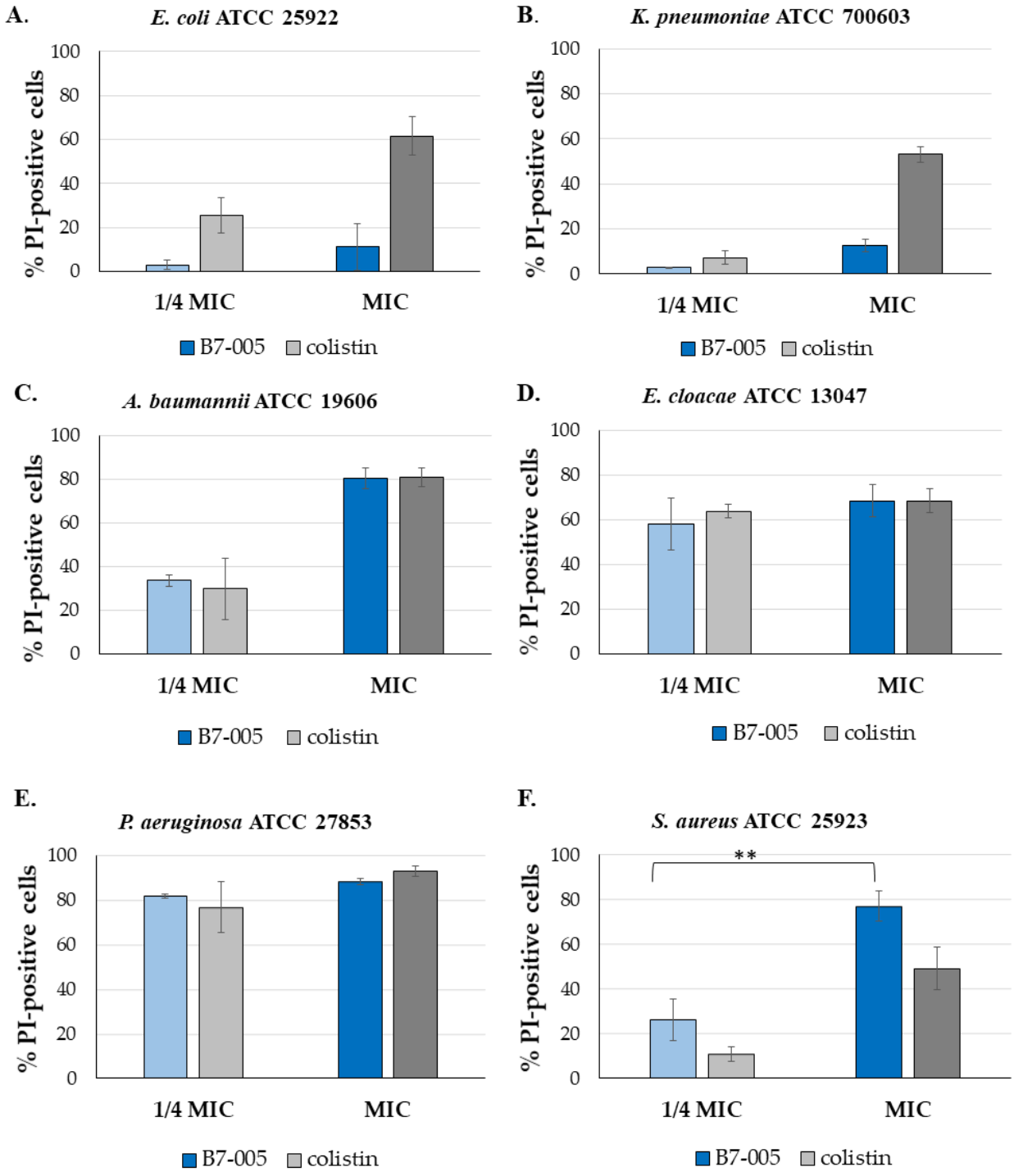

Fig. 4.6: Membrane-damaging activity of B7-005 on *E. coli* ATCC 25922 (A) *K. pneumoniae* ATCC 700603 (B), *A. baumannii* ATCC 19606 (C), *E. cloacae* ATCC 13047 (D), *P. aeruginosa* ATCC 27853 (E), *S. aureus* ATCC 25923 (F). The permeabilization assay has been performed in MHB. 2,5 x10^5 CFU/mL cells were treated with B7-005 or colistin at 1/4xMIC and MIC of peptide or colistin (0,5-8 µM). To provide the same experimental conditions for all bacterial species, colistin was also used as a control for *S. aureus* (MIC=128 µM), although its scarce antimicrobial activity against Gram-positive bacteria. The assays were not performed with the *E. faecium* strain because its too high MIC. The background level of permeabilized cells of untreated samples was always below 2% and was subtracted to the corresponding peptide-treated sample. Data are the average ± SEM of three independent experiments (n=3). **=p≤0.01 (ANOVA with Bonferroni's test).

B7-005 activity resulted in less than 20% PI -positive cells in *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603; even at MIC and with values much lower than colistin (Fig. 4.6). In the other bacterial species, the percentage of PI -positive cells was much higher and comparable to that of colistin. Membrane damage by B7-005 at MIC observed in *P. aeruginosa* ATCC 27853*, E. cloacae* ATCC 13047, and *A. baumannii* ATCC 19606, was considerably high (about 80% of cells). In addition, a high degree of permeabilization was also observed in *P. aeruginosa* and *E. cloacae*, even when sub-inhibitory concentrations of the peptide were used. *S. aureus* ATCC 25923, treated with 1/4xMIC, showed a low degree of permeabilization (26% of PI-positive cells) but increased at MIC at the same level of other bacteria. From these data, it appears that B7-005 causes varying degrees of membrane damage depending on the bacterial species.

4.6 Internalization of B7-005-BY into Bacterial Cells

To determine whether B7-005 retains the ability to enter target bacterial cells as Bac7(1-16), internalisation assays were performed using *E. coli* ATCC 25922 and ESKAPE. Specifically, flow cytometry studies were performed using a fluorescently-labelled derivative of B7-005 peptide (B7-005-BY, BY=Bodipy) and a quencher, trypan blue (TB). After treating the cells with B7-005-BY, their mean fluorescence intensity (MFI) was analysed with or without the presence of TB to assess the amount of B7-005-BY accessible at the extracellular level by fluorescence quenching.The assay was first performed on *E. coli* ATCC 25922 using comparatively B7-005-BY and Bac7(1-16)-BY, the latter because it is effectively internalized in *E. coli* cells [146]. Both bodipylated peptides, that displayed the same MIC as the non-fluorescent labelled derivatives against *E. coli* (data not shown), were incubated with the cells at 1/4xMIC and MIC for 30 min. Bacterial cells were analysed with or without TB (Fig. 4.7 A-H).

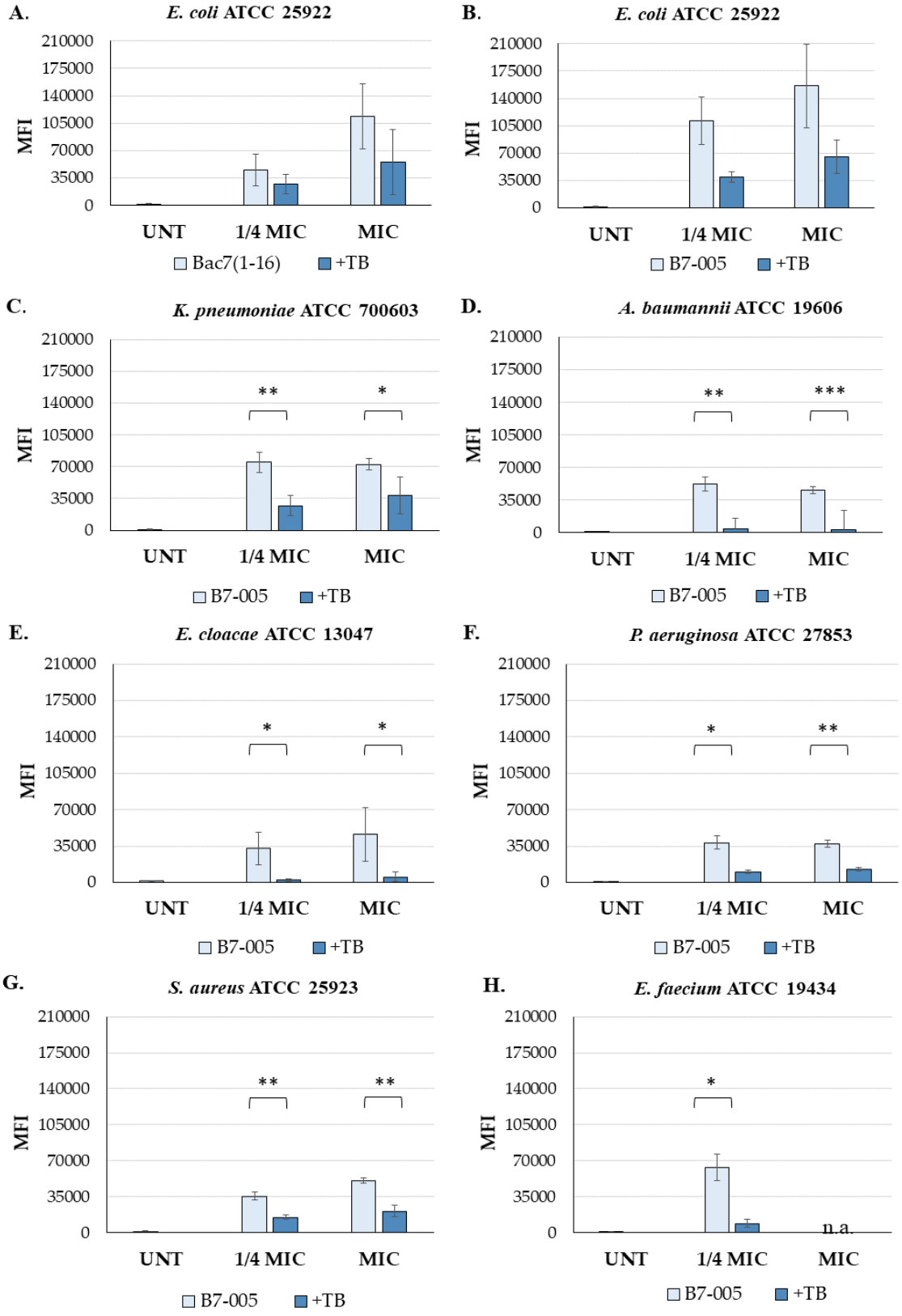

Fig. 4.7: Evaluation of peptide internalization into bacterial cells.
Mean fluorescence intensity (MFI) of *E. coli* ATCC 25922 treated with B7(1-16)-BY (A) and B7-005 (B), and of of *K. pneumoniae* ATCC 700603 (C), *A. baumannii* ATCC 19606 (D), *E. cloacae* ATCC 13047 (E), *P. aeruginosa* ATCC 27853 (F), *S. aureus* ATCC 25923 (G) and *E. faecium* ATCC 19434 (H) cells treated with B7-005. Bacterial cells ($2{,}5 \times 10^5$ CFU/ml) were incubated with 1/4xMIC or MIC of B7-005 for 30 min, washed, and analysed by flow cytometry after or without incubation with 1 mg/ml TB for 10 min at 37°C. UNT= untreated control. n.a= not available data. Data are expressed as the average MFI with SEM from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.005$ (Test t-student).

Internalisation of B7-005 in *E. coli* cells showed a comparable trend to internalized peptide Bac7(1-16) which showed a non-significant decrease in MFI after addition of the quencher (Fig. 4.7 A and B). The analysis has been repeated with all the ESKAPEs and all became fluorescent after peptide treatment and in all cases the addition of TB significantly reduced fluorescence (Fig. 4.7 C-H). The MFI values of *K. pneumoniae* cells treated with B7-005-BY are lower than those of *E. coli*, so B7-005 appears to recognise the membrane of *K. pneumoniae* less effectively (Fig.4.7 C). Moreover, the interaction between the peptide and the bacterial membrane appear to be non-concentration dependent, as the fluorescence values obtained at 1/4xMIC and MIC are almost identical. The presence of the quencher resulted in 63% decrease MFI at 1/4x MIC and to 41% at MIC, indicating that some of the peptide is nevertheless internalised. In the Gram-negative *A. baumannii* and *E. cloacae* (Fig. 4.7 D and E), MFI values obtained were similar (around 35000) at both 1/4xMIC and MIC, indicating that there is no concentration dependence in bacterial membrane recognition. Moreover, at both concentrations tested, approx 90% fluorescence is quenched by the TB. Similar results have been obtained with *P. aeruginosa* cells, despite a slightly higher proportion of peptide appeared to be internalised (27% at 1/4xMIC) (Fig. 4.7 F). Interestingly, incubation with the quencher reduces the MFI of *S. aureus* cells by 57% at 1/4xMIC and 59% at MIC (Fig. 4.7 G), suggesting that some of the peptide is internalised. The *E. faecium* strain was tested only at 1/4x MIC, due to its too high MIC values, , and showed a similar trend to *A. baumannii* and *E. cloacae.*
B7-005 appears to be internalised only by *E. coli* and *K. pneumoniae* cells, albeit with different efficiency, whereas the ESKAPE strains show comparable levels of fluorescence and quenching in the presence of TB. The internalisation of B7-005 thus seems to be dependent on the bacterial species, confirming what has already been observed through permeabilisation experiments. B7-005 thus preserved the non-lytic membrane mechanism of action of the original peptide against only 2 out 7 bacterial species examined.

4.7 Cytotoxicity of B7-005 towards human cells

PrAMPs such as Bac7(1-16) are not toxic to eukaryotic cells even at concentrations well above than the antimicrobial doses, an important aspect in the development of a potential antibiotic. Therefore, the biocompatibility of B7-005 and its mechanism of interaction with the eukaryotic cells were evaluated on four different human cell lines. Based on the *in vitro* data, an *in vivo* study was conducted to test toxicity in a Zebrafish model. To test the antimicrobial efficacy of B7-005 in a more complex system that more closely resembles a real clinical situation, a zebrafish infection model was also developed.

The assays analysing the effect of B7-005 on eukaryotic cells were carried out with the collaboration of Prof. Sabrina Pacor (University of Trieste). The use of the zebrafish model organism was possible thanks to the collaboration with Dr. Sara Bozzer (University of Trieste).

Cytotoxicity tests were carried out by testing the viability of four different human cell lines after 24-h incubation with different peptide concentrations. Cell viability was determined using MTS assays on human umbilical vein endothelial cell line (HUVECs) and MTT assays on human epidermal keratinocytes (HaCaT), the adenocarcinomic human alveolar basal epithelial cells (A549) and against the human line of lymphocyte precursors (MEC-1). B7-005 did not cause significant decrease in the viability of HUVEC cells up to 128 µM (Fig. 4.8 D), and its IC_{50} values was 256µM.

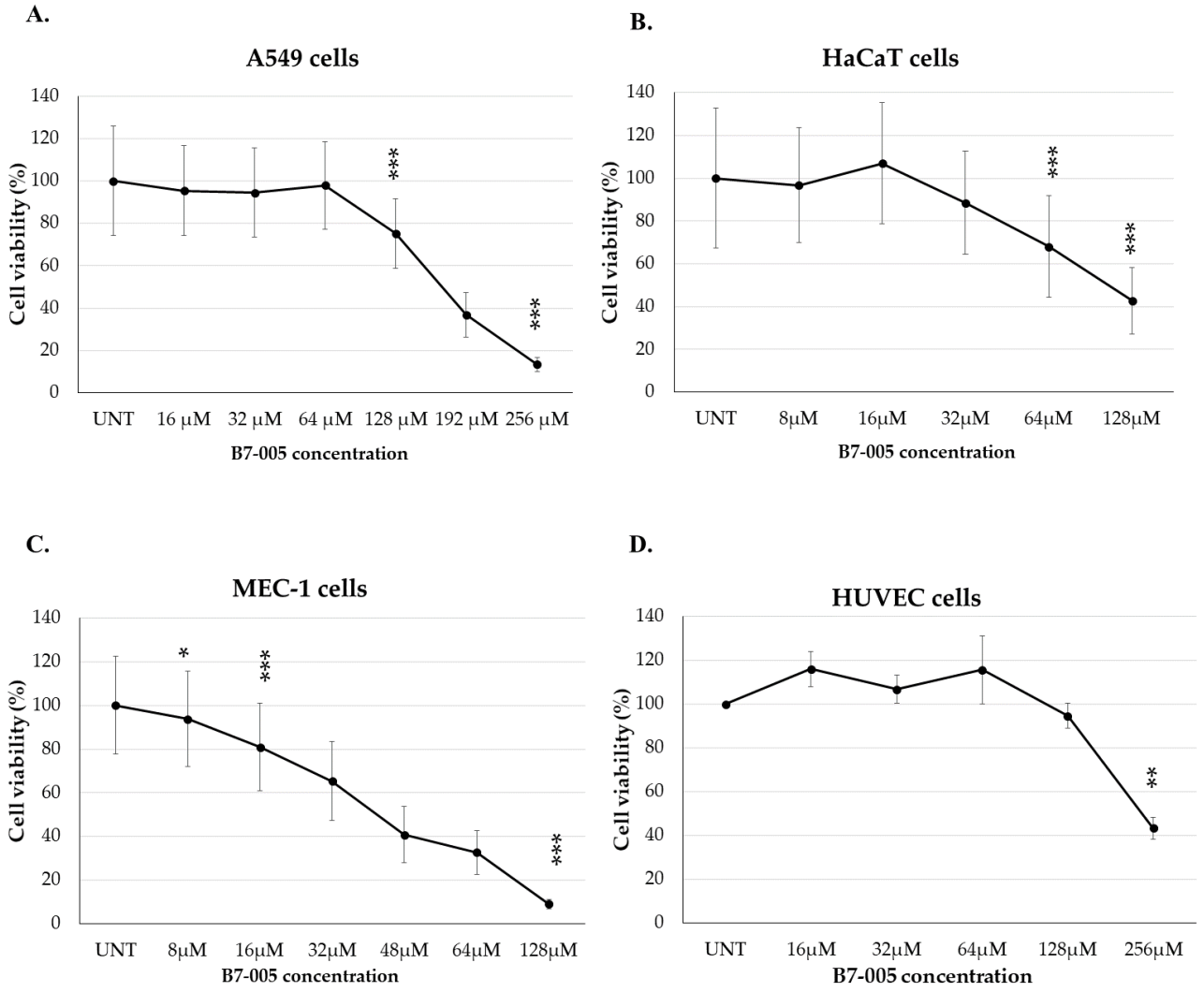

Fig. 4.8: Effects of B7-005 on A549 (A), HaCaT (B) MEC-1 (C) and HUVEC (D) cell lines viability. MTT assay on A549, HaCat and MEC-1 cells and MTS assay on HUVEC cells after treatment with different concentrations of B7-005. The cell viability was measured (as absorbance at 570 nm for MTT and 490 nm for MTS) after 24 h from the exposure to the peptide. Results are reported as percentages of viable cells with respect to the untreated control cells (UNT) (set as 100% of viability). Data are the average ± SEM of at least three independent experiments in internal triplicate (n=9). *p< 0,05, ** p < 0,01, *** p < 0,005 versus the untreated control (Test t-student).

B7-005 also proved to be non-toxic to the A549 and HaCaT cell lines at bactericidal concentrations (Fig. 4.8 A and B)., showing IC_{50} values of 170 μM and 109,4 μM, respectively. At last, B7-005 caused a 50% loss of viability of the MEC-1 cells with a calculated IC_{50} value of 42,3 μM, proved to be the most sensitive cell line (Fig. 4.8 C). These results indicate that the normal HUVEC cells, appear to be less susceptible to the peptide than A549 and MEC-1 tumor cells, and also than the immortalized epithelial HaCaT cells. However, the viability of all cells examined decreased significantly only at concentrations far above than the antimicrobial concentrations (1-4 μM in 4 out 7 bacterial species).

Cytotoxicity in the same cell types was also investigated by assessing membrane permeabilization. For this purpose, cells were treated with various concentrations of B7-005 for 24 hours and then examined by measuring the uptake of PI. Results indicated that PI-positive HUVECs cells increased with raising peptide concentration n (Fig. 4.9 D) and that less than 50% of the cells were damaged at the IC_{50} determine by MTS assay.

A peptide concentration-dependent increase in PI-positive (permeabilized) cells was also observed in A549, HaCaT, and MEC-1 cells (Fig. 4.9). A significant increase in percentage permeabilized cells was observed at 64 μM B7-005 with HaCaT and A549 cells and at 48 μM with MEC-1 cells highlighting a good correlation between the two methods of analysis and confirming the threshold concentration raising cytotoxicity observed with the MTT viability test.

Taken together, these data indicate that B7-005 is not toxic to primary normal HUVEC cells even at very high concentrations, whereas some cytotoxic effects are detectable in A549 tumour cells and immortalised HaCaT cells at 64 μM a concentration well above the bactericidal concentrations. Evidence of toxicity in leukaemic MEC-1 cells at lower concentrations, indicate that these cells are most sensitive to the peptide.

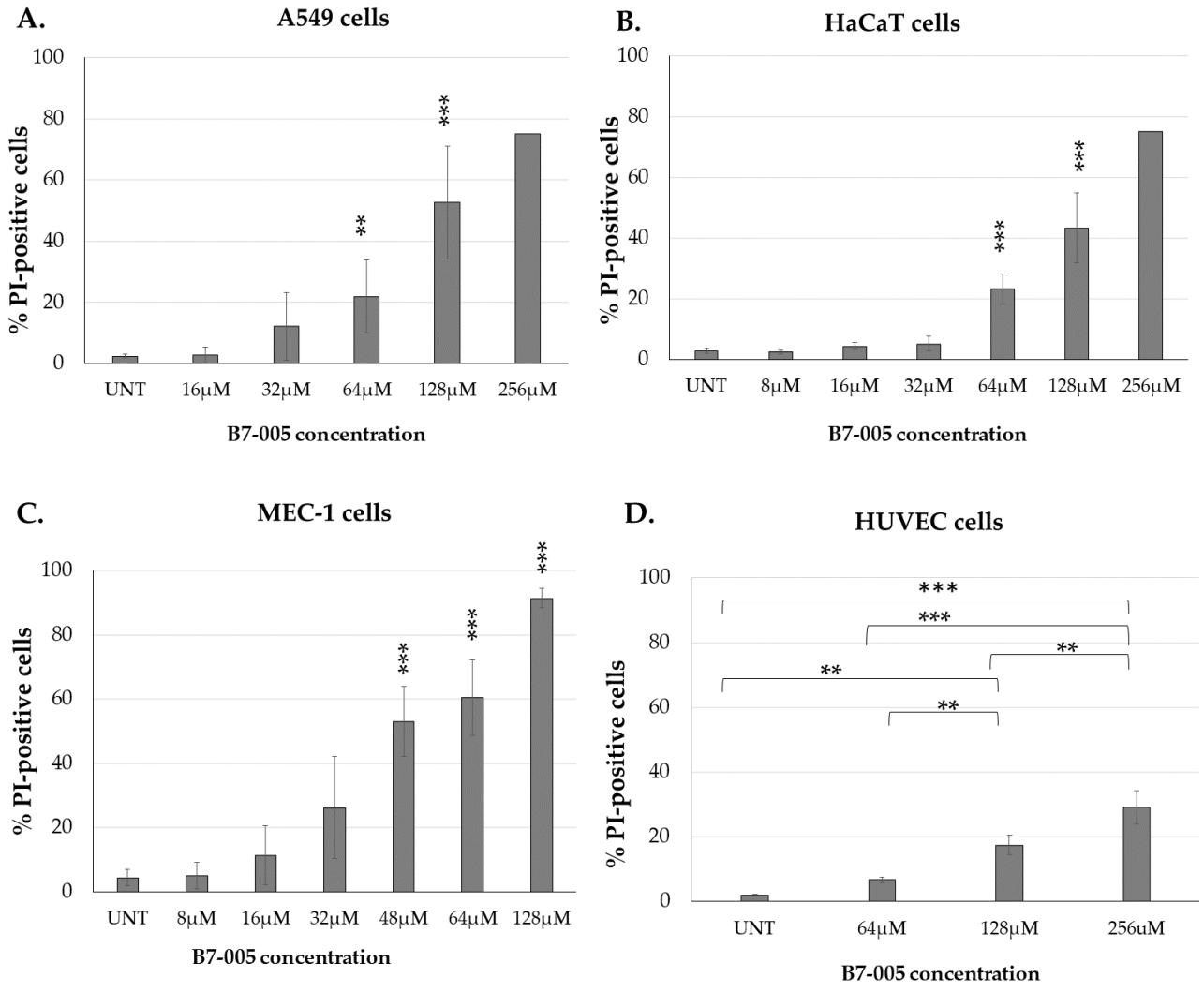

Fig. 4.9: Assessment of A549 (A), HaCaT (B) MEC-1 (C) and HUVEC (D) cell lines membrane permeabilization by flow cytometry. The uptake of PI was checked after incubating cells for 24 hours in the presence of increasing B7-005 concentrations evaluating the percentage of PI-positive cells. UNT= untreated control. Error bars are the standard deviations calculated on the average of three independent experiments performed in duplicate (n=6). *p< 0,05, ** p < 0,01, *** p < 0,005 versus the untreated control (Test t-student).

To investigate the mechanism of cytotoxicity the susceptible cell types were analysed multiparametrically by flow cytometry after one-hour incubation with the peptide. The analysis included: morphological observation (measurement of the forward scatter signal to obtain information about the size of the cells and of the side scatter signal related to cell surface properties) (Fig. 4.10) assessment of membrane permeabilization (PI positive cells) and depolarization of the mitochondrial membrane measured using the fluorescence probe DiOC6(3).

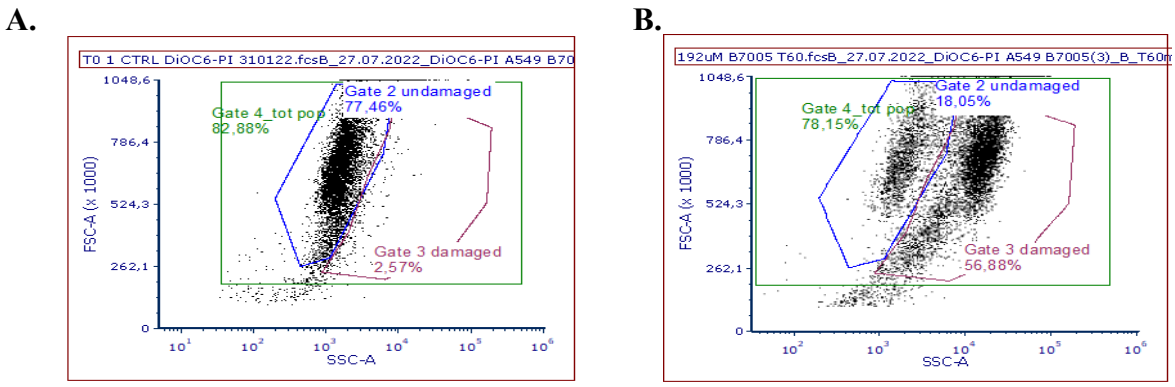

Fig. 4.10: **example of dot-plot of untreated cells (A) and cells after treatment with B7-005(B).** the cell population were subdivided into subpopulations identified as morphologically normal or with altered morphology (damaged).

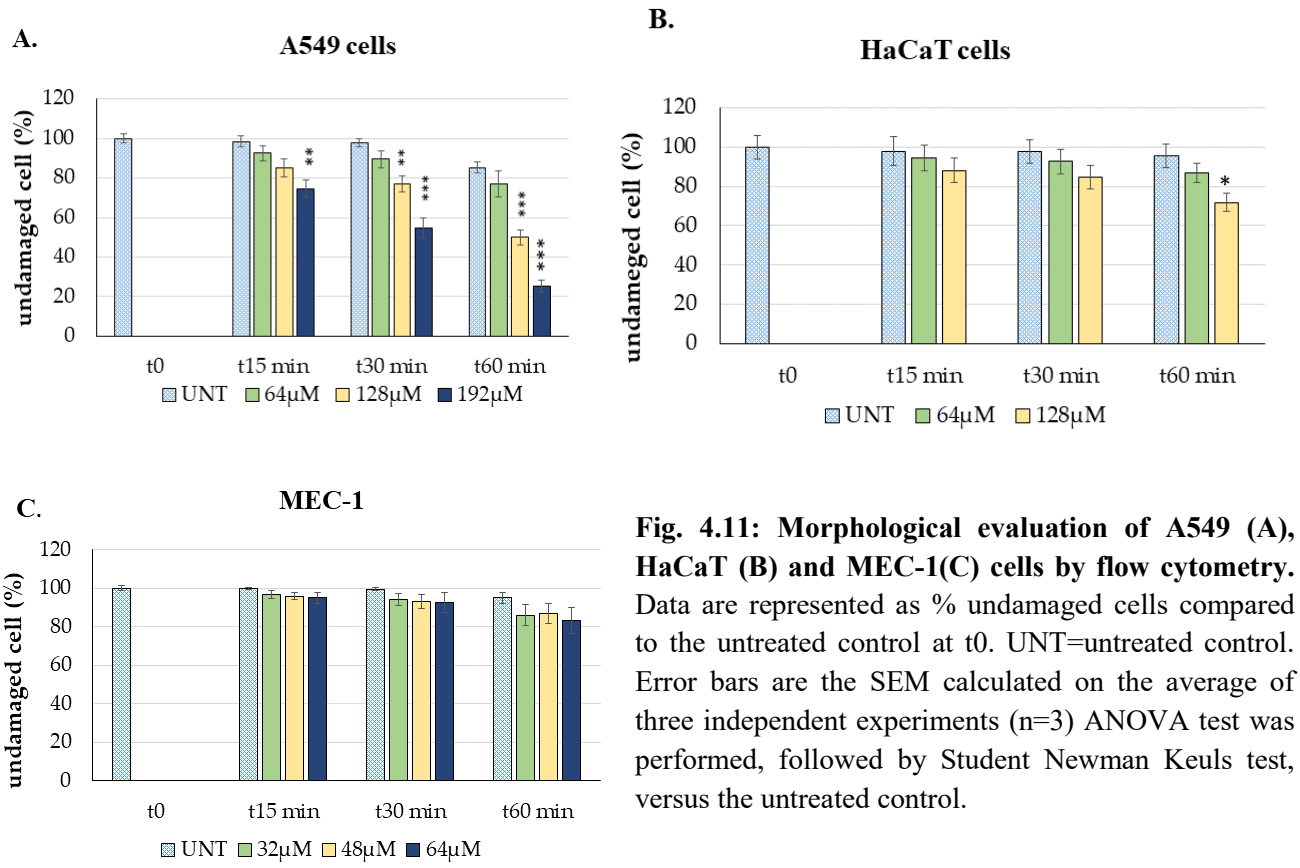

Fig. 4.11: Morphological evaluation of A549 (A), HaCaT (B) and MEC-1(C) cells by flow cytometry. Data are represented as % undamaged cells compared to the untreated control at t0. UNT=untreated control. Error bars are the SEM calculated on the average of three independent experiments (n=3) ANOVA test was performed, followed by Student Newman Keuls test, versus the untreated control.

Cells were examined for morphological changes after incubation with the peptide at concentrations close to the IC_{50} calculated by the 24-hour MTT test. Cells remained intact after treatment suggesting that B7-005 did not cause significant morphological changes in MEC-1 (Fig .4.11 C) and HaCaT cells, except at 128 µM (Fig. 4.11 B). At contrast, treatment of A549 cells with B7-005 for one hour caused significant concentration-dependent morphological changes after 1 hour of treatment, suggesting that most cells are likely damaged (Fig. 4.11 A).

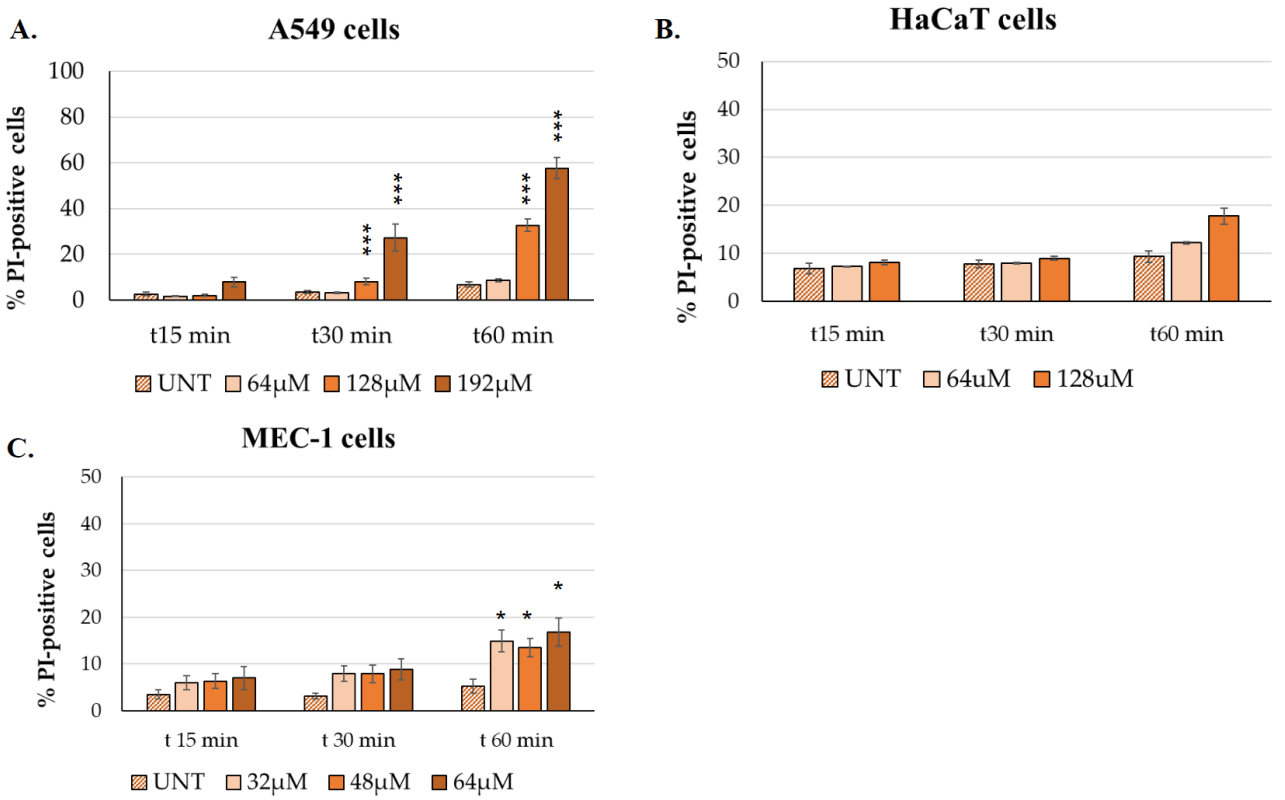

Fig. 4.12: Percentage of damaged cells, evaluated by flow cytometry on A549 (A), HaCaT (B) and MEC-1 (C) cell lines after 1h of treatment. Percentage of PI-positive cells respect to the untreated control (UNT) at time 0. The % of PI-positive has been evaluated after 15, 30 and 60 minutes of incubation at different concentrations of B7-005. Error bars are the SEM calculated on the average of three independent experiments (n=3). ANOVA test was performed, followed by Student Newman Keuls test. * $p < 0,05$, *** $p < 0,005$. versus the untreated control

Cytometric methods using double staining with propidium iodide and DiOC6(3) probes were then used to assess cell membrane permeabilization and disruption of mitochondrial membrane potential. Cells were treated with various concentrations of B7-005 in the presence of PI and DiOC6(3) for 15, 30 and 60 minutes and then analysed. The highest peptide concentration was different for each cell type and corresponded to IC_{50} values calculated by the 24-hour MTT test.

The results were quite different for the three cell lines. A549 cells appeared permeabilized (PI – positive) after only 30 minutes at 128 µM and after 60 minutes more than 50% of the cells were damaged (Fig. 4.12 A). MEC-1 cells were significantly damaged within 1 hour of incubation with 32 µM peptides (Fig. 4.12 C), a concentration lower than the IC_{50} values calculated by the 24-hour MTT test. In contrast, the percentage of permeabilised HaCaT cells was not significant after 60 minutes incubation with 128 µM B7-005 (Fig. 4.12 B), a higher concentration than the IC_{50} previously identified.

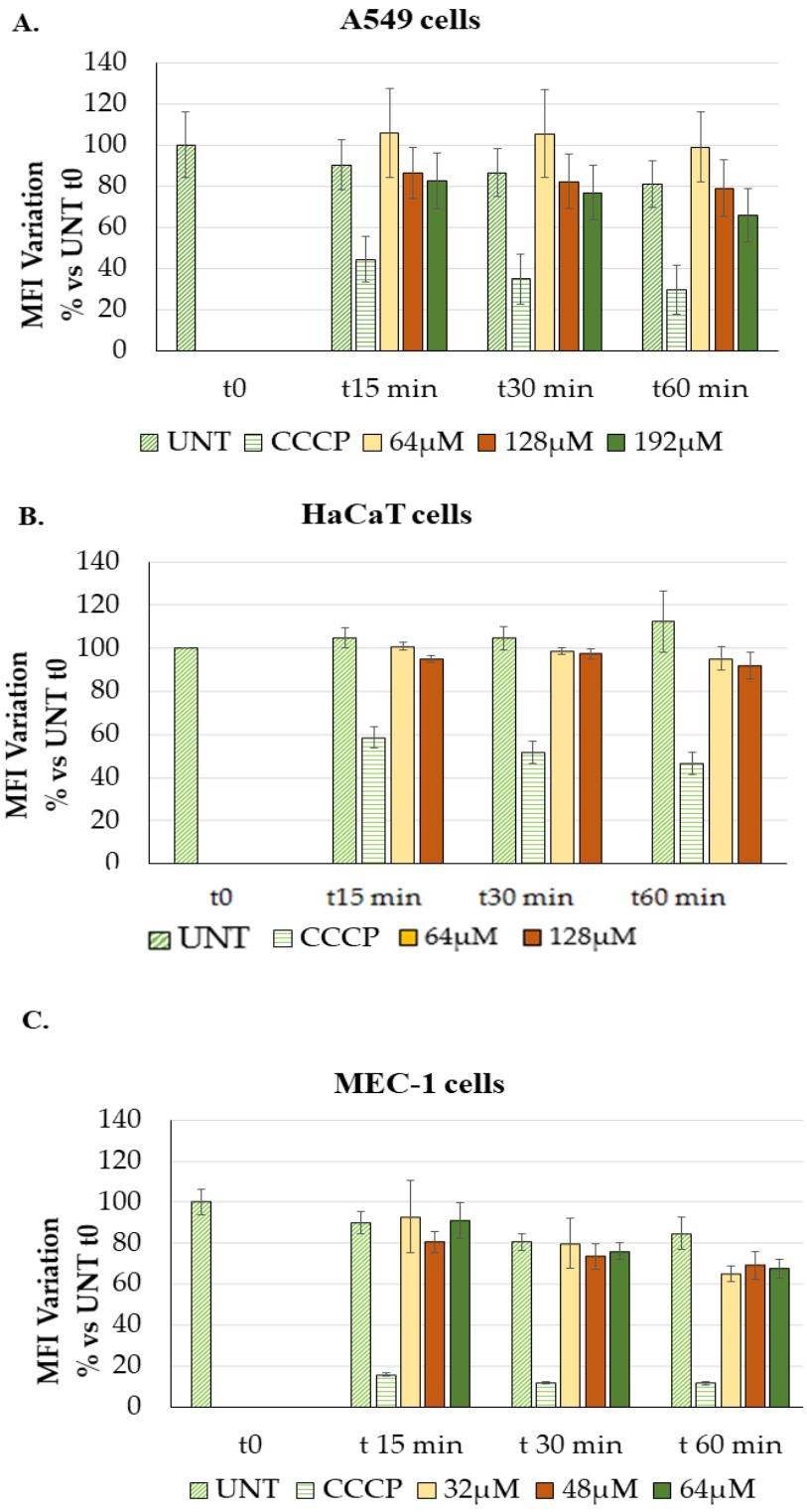

Fig. 4.13: Evaluation of the mitochondrial membrane potential by flow cytometry on A549 (A), HaCaT (B) and MEC-1 (C) cell lines. Variation of MFI expressed in percentage respect to the control (UNT). The MFI has been evaluated at time 0 for the UNT only and after 15, 30 and 60 minutes for all the samples. Cells were treated with DiOC6 and PI as control.CCCP = Carbonyl cyanide m-chlorophenylhydrazone is an uncoupler used as positive control of disruption of membrane potential. Error bars are the SEM of the mean calculated on the average of three independent experiments (n=3). ANOVA test was performed, followed by Student Newman Keuls test.

To observe whether B7-005 damages the mitochondrial membrane in addition to the plasmatic membrane, the mitochondrial membrane potential of PI -positive cells was examined. B7-005 did not cause mitochondrial membrane depolarization as measured by the DiOC6(3) probe in any of the cell lines during 1-hour treatment (Fig. 4.13). This indicates that although membrane damage occurs, it is not accompanied by a loss of membrane potential in the mitochondrial membrane.

Overall these results suggest that B7-005 affects A549 and MEC-1 cell lines within 1 hour and causes membrane permeabilization but only at concentrations several times higher than the microbicidal concentration. This result is supported by the observed changes in cell morphology, particularly in A549, and the increase in PI-positive cells. In contrast, the effect of the peptide on HaCaT cells appears to be delayed and any damage is detected within 1 hour of incubation. On the other hand, as A549, this cell type was injured after 24 hours, raising the hypothesis of an initial mechanism of damage distinct from membrane lysis. In both HaCaT and the other cells, the membrane potential was intact ruling out detrimental effects due to depolarization of the mitochondrial membrane.

4.8 Cytotoxicity test in zebrafish larvae and setting up of an infection model

In vivo citotoxicity tests were also performed to evaluate the biocompatibility of B7-005 in the more complex system of zebrafish larvae (*Danio rerio*). Prior to hatching, the zebrafish embryo is surrounded by an envelope, the chorion, which was manually removed 24 hours before peptide injection. Five different concentrations of B7-005 were injected into the duct of Cuvier, a venous structure that leads blood directly to the heart, thus allowing B7-005 be injected directly into the bloodstream. 30 larvae per each concentration were used. Survival of the Zebrafish was observed at 24, 48 and 72 hours after injection (hpi).

Most larvae survived to 32 mg/kg B7-005 after 72 hours, although data varied widely, so exact numbers could not be determined. A significant decrease in viability was observed when fish were treated with 48 and 64 mg/kg peptide concentrations and after 72 hours of incubation (Fig.4.14). The lethal dose 50 (LD_{50}) of B7-005 after 72 hours could be tentatively estimated between 48 mg/kg and 64 mg/kg.

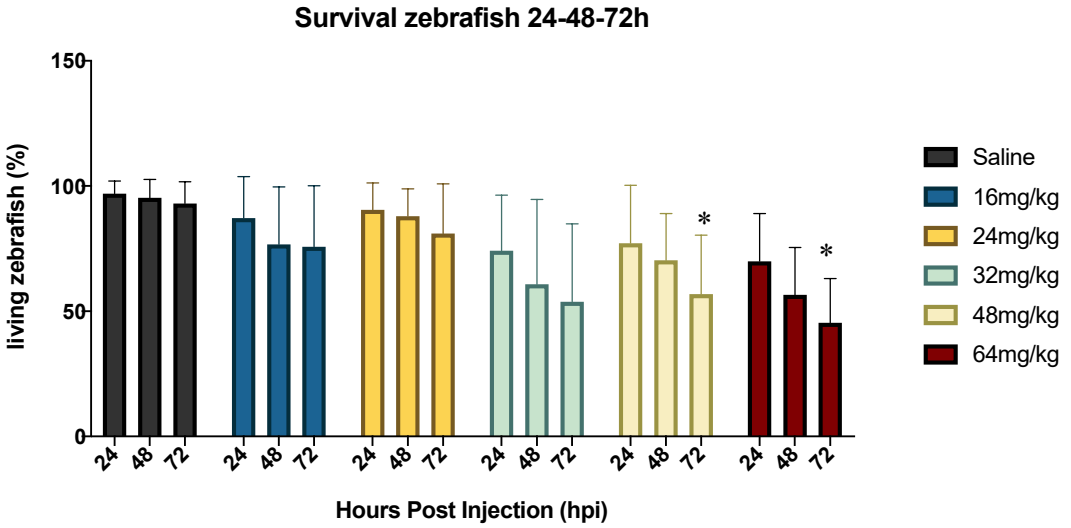

Fig. 4.14: Toxicity of B7-005 in zebrafish animal model. The zebrafish were used for a total of 5 days. The reference point was set at the time of injection (0 h post-injection, hpi), which was performed 48 h after fertilisation. At 24 hours before injection (-24hpi), embryos were manually decorticated. PBS for the untreated control and different concentrations of B7-005 were injected into the duct of Cuvier and analysed at 24, 48 and 72 hpi. (Control: n=30, Treatment: n=30) The assay was performed at least three times for each concentration tested (n=3). The percentage of zebrafish surviving was calculated in each group. Data were expressed as mean ± SD. Control vs. treated: * $p < 0.05$.

This relatively high concentration encouraged us to test the efficacy of B7-005 in fish. In view to future study of the efficacy of B7-005 and its bactericidal activity in a living organism that has a complete immune system, an infection model in Zebrafish was developed using *E. coli* ATCC 25922 as infecting bacterium.

Zebrafish embryos were deprived of the chorion 24 hours before injection. The following day, anaesthetised fish embryos were infected with different concentrations of *E. coli* ATCC 25922 by injecting them into the duct of Cuvier. By measuring the percentage of surviving larvae, a number of 2000 CFU/animal was selected as the most appropriate for the model, because this number of CFU resulted in a low number of survivors within 72 hours post infection (Fig. 4.15). These data are essential for near future testing of the efficacy of B7-005, as the peptide treatment is expected to increase the percentage of surviving larvae to determine if it is capable of producing an effect in live fish.

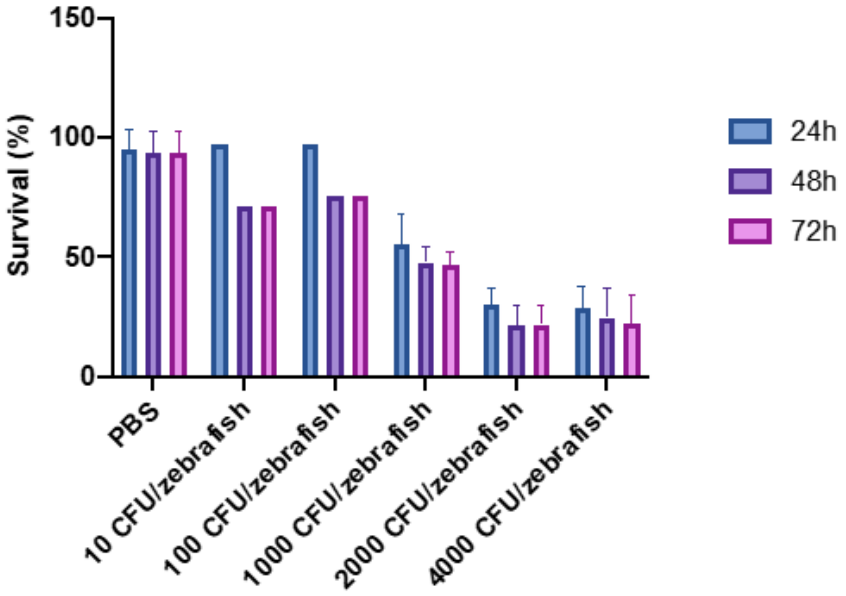

Fig. 4.15: Zebrafish as a model of infection. Different concentrations of *E. coli* ATCC 25922 were microinjected into zebrafish. In the untreated control, PBS was injected instead of bacteria. (Control: n=30, Treated: n=30) the assay was performed at least 3 times for each bacterial concentration tested (n=3). Data were expressed as mean ± SD.

5. Discussion

Antimicrobial peptides (AMPs) are a class of molecules produced by the innate immune system that have a remarkable ability to kill bacteria. For this reason AMPs could be potentially an alternative to or flank the current antibiotics. Unfortunately most of them have important weaknesses as antibacterials such as a narrow spectrum of activity, cytotoxicity and susceptibility to proteases. Generally, the group of PrAMPs has low cytotoxicy to host cells, but are only active mainly to *Enterobacteriaceae* e few other Gram-negative bacteria.

In this study, we focused on the properties of B7-005, a small PrAMPs designed from Bac7(1-16), a peptide that served as a template because it offers some interesting advantages such as tolerance to serum proteases and low cytotoxicity.

First we demonstrated that B7-005 has a broadened spectrum of activity than the original Bac7(1-16). Indeed, B7-005 is active on all strains of ESKAPE species tested with a MIC range from 2 µM to 32 µM. Notably, B7-005 is active against Gram-negative species on which the *wt* peptide did not act, such as *P. aeruginosa*, and unlike other PrAMPs, B7-005 is also active against Gram-positive species, such as *S. aureus* and *E. faecium*. We have also shown that B7-005 not only inhibits the growth of the species tested, but also has a bactericidal effect. Taken together, these data suggest that B7-005 has a reduced dependence on the SbmA transporter compared to Bac7(1-16), as it is also active on species lacking the membrane transporter such as *P.aeruginosa* and Gram-positive bacteria.

Another property of B7-005 is its rapid action. We have observed that B7-005 had significant bactericidal activity toward four out six ESKAPE pathogens within one hour. The only exceptions to this general trend were *K. pneumoniae* and *E. faecium*, towards which B7-005 still significantly reduced viability, albeit within 4 hours of treatment. This is an aspect to be taken into account for an antibacterial drug, especially when treating infections in clinical cases where there is a risk of complications requiring more rapid recovery, such as in immunocompromised patients. In this case, B7-005 proves to be a molecule with excellent property.

However, the broad spectrum of activity and rapid bactericidal activity of B7-005 are aspects rarely reported for PrAMPs, which are not membranolytic AMPs. Therefore, we wondered what such rapid bactericidal activity of B7-005 was due to. The antimicrobial activity in several bacterial species could be related to the ability of B7-005 to act from within the bacterial cytosol independently of the presence of the bacterial SbmA transporter, either through the use of an alternative transporter or by self-promoted uptake. However, another possibility is that B7-005 could act on bacterial membranes by a lytic mechanism rather than by internalisation and block of the protein synthesis, as Bac7(1-16) does.

Based on permeabilization and internalisation experiments with ESKAPE pathogens, we found that B7-005 acquires a permeabilization capacity for the membrane of certain bacterial species, and in these, its internalisation appeared to be reduced. From these data, we were able to deduce that the mechanism of action of B7-005 has partially changed compared to the original molecule, and interestingly, it is specie-specific. Indeed, B7-005 still manages to be internalised by *E. coli* and *K. pneumoniae* cells, although in the latter with less efficiency, whereas on the other species we observed a marked destabilisation of the membrane. Furthermore, internalisation in *K. pneumoniae* cells would also explain why B7-005 takes longer to kill bacteria of this species than the others.

Thus, B7-005 displays more than one mechanism of action, depending on the species with which it interacts. Interestingly, B7-005 appears to have a non-lytic mechanism towards *E. coli* and *K. pneumoniae* that express the membrane transporter SbmA, but it also appear to act mainly with a lytic mechanism towards other species carrying a *sbma*-like gene, such as *E. cloacae* and *A. baumannii*. Thus we can conclude that the presence or absence of a *sbma* homolog is not sufficient to predict its mechanism of action of B7-005, as may have been the case for Bac7(1-16).

Another very interesting finding is that B7-005 did not allow the selection of resistant mutants in *E. coli* cultures, and only partially after prolonged exposure, a desirable property for a new antibiotic that could thus be used in the clinical setting for longer than conventional antibiotics.

The tendency of B7-005 not to select resistant mutants seems, at least initially, to be more similar to what has been observed for the lytic peptide colistin, which is known to be very unlikely to induce resistance in bacteria, than to what has been observed for the PrAMPs Bac7(1-35) and Bac7(1-16). Bac7(1-35) and Bac7(1-16), which use the SbmA transporter that could easily mutated, rapidly increased MIC values during the experiment. For colistin, on the other hand, we did not observe any increase in MIC against *E. coli*. B7-005 in this respect does not seem to behave as distinctly as the reference molecules tested, because it does not induce the development of resistant mutants at least until 12th day, but subsequently this occurs, and its behaviour again seems more similar to the original peptide than to colistin.

Another interesting aspect is the ability of B7-005 to inhibit the formation of a new biofilm of the gram-positive *S. aureus* at subinhibitory concentrations. At anti-biofilm concentrations B7-005 did not show significant membranolytic activity against *S. aureus*. Therefore, we cannot entirely attribute the inhibition of biofilm to an exclusively membranolytic mechanism. B7-005 could possibly also act on biofilm formation and/or eradication via other mechanisms. What is certain, however, is that this finding would certainly deserve further study, especially since a molecule with good, well-characterised anti-biofilm activity would provide considerable benefits in the clinic, where biofilm is a serious problem for relapses and recurrent infections.

We also observed that the biofilm eradication activity of B7-005 towards *E. coli* is much weaker than towards *S. aureus* biofilm, in fact we only observed it at high concentrations. However, this did not surprise us much, because the bacteria within the biofilm are in a very different metabolic state than they are in the planktonic form on which we tested the antimicrobial activity of B7-005.

At present, we cannot know whether a mechanism of action based on inhibition of protein synthesis would be a successful strategy for a peptide when it is acting against bacterial cells that are in a 'slowed down' metabolic state such as the cells in biofilm. In this regard, it would also be necessary to investigate whether or not SbmA is expressed within the biofilm of *E. coli* cells.

Therefore, it is clear that the mechanism of action of B7-005 is not easy to determine, especially as it appears to be a quite different mechanism, i.e. non-membranolytic towards some species and membranolytic towards others. It should also be noted that all the tests were only conducted on a representative strain of each bacterial species of ESKAPE pathogens, so it cannot be excluded that B7-005 may act differently on other strains of the same species.

Nevertheless, since a strong interaction with the membrane is usually accompanied by reduced selectivity for the target and consequently increased cytotoxicity[147], we wondered whether the lytic mechanism of action of B7-005 toward certain bacterial species also has implications for its biocompatibility with eukaryotic cells. We therefore investigated whether B7-005 causes destabilisation of eukaryotic cell membranes. In our study, we showed that the cytotoxicity of B7-005 also appears to be cell-type dependent and detectable only at high peptide concentrations.

We tested cytotoxicity in several cell lines and found that B7-005 was not toxic to primary cells such as HUVECs. In these cells, the first toxic effects of the peptide were observed only at concentrations above 128 μM, a concentration about 30 times higher than the antibacterial effective dose. These are excellent data as they provide a first assessment of the therapeutic window of B7-005, which in this case is very broad and suggests that B7-005 may be a safe drug.

The other cell lines showed variable sensitivity to treatment with the peptide. The data indicated that the cytotoxic effects of B7-005 were observed for the A549 and HaCaT cell lines at lower concentrations than for HUVECs (around 64 μM), with the MEC-1 cell line being the most sensitive.

In comparison, Bac7(1-16) in HaCaT and MEC-1 cells showed a toxic effects at a concentration slightly higher (100 μM) than 64 μM[116,148]. This small increase in cytotoxicity of B7-005 compared to natural Bac7(1-16) may be related to the stronger membrane destabilizing effect of the first peptide.

However, it should be noted that these cell lines are tumorigenic or immortalized cells, and the more pronounced cytotoxicity of B7-005 for cells of this type could also be considered as a result of anti-tumor activity of the peptide rather than a strictly cytotoxic effect. Indeed, anti-tumor activity

of certain antimicrobial peptides against tumoral cells has been extensively reported in the literature [149] in agreement with our results.

By analysing cells at short times, we gained insights into the mechanism of toxicity of B7-005. Although differences between cell lines emerged also in this case, the mechanism of damage appears to be generally membranolytic. Thus, the membrane of A549 cells was found to be strongly affected by B7-005. This effect appears to be less evident in HaCaT and MEC-1 cells, where membrane permeabilisation and morphological changes observed on the cells after 1h indicate that the overall effect of damage by B7-005 is small in the short term.

The use of different cell lines was also necessary to give us an indication of the possible mode of administration of B7-005 as a life-saving drug. The low toxicity to HUVEC endothelial cells led us to hypothesise a possible systemic application of the peptide, because in this case the first cells with which B7-005 would come into contact with would be endothelial cells in which the peptide has not shown major side effects.

Following this indication, we performed cytotoxicity experiments in a more complex system such as the Zebrafish embryos model. We chose Zebrafish embryos for our experiments because, in addition to its experimental advantages, such as rapid development, ease of breeding and manipulation, it also allows systemic injections and its developed immune system can give us a more complete indication of the side effects of administering B7-005.

B7-005 was then injected into the duct of Cuvier of the Zebrafish larvae, so that it entered the bloodstream directly, reflecting the systemic administration of the peptide that we had hypothesised. The results of this experiment were indeed unexpected but optimistic, because B7-005 proved safe for the fish up to very high concentrations (24mg/kg, i.e. a millimolar dose, far from the micromolar doses defined as toxic to eukaryotic cells in vitro, but even more from bactericidal doses).

In the literature, toxic doses for other peptides are given at much lower concentrations than for B7-005. For example, bacteriocin AS-48 produced by *Enterococcus* strains, whose in vitro antimicrobial efficacy is well documented, at a concentration of 6,4 µM causes 100% lethality in zebrafish larvae after 24 hours[150].

The low toxicity of B7-005 observed in a live system compared with the toxicity observed in vitro assays on eukaryotic cells leads us to suggest that the correlation between these two assays is not obvious or direct. Indeed, in a complex system, B7-005 provided more promising evidence than the in vitro system. This does not change the fact that toxicity may still be different in another living system such as mice or humans, but this aspect allows us to make observations about the methods that are used to determine the toxicity of a molecule, which therefore should not stop at single assays, but should be extended to more cell lines and more living systems to provide more correct and complete indications. In this context , a recent study by Greco et al. (2020) highlighted how

toxicity tests of antimicrobial peptides carried out in vitro and in vivo often give very different results[151]. Promising in vivo toxicity tests have prompted us to develop an infection model in Zebrafish, to test B7-005 in the future and observe whether it is able to resolve an infection in a complex organism while being a safe drug.

Moreover, the model of infection in a complex organism could not only allow evaluation of the efficacy of peptide B7-005 as an antimicrobial drug, but also provide insight into the effects of biological fluids on B7-005. Thus, it would be interesting to observe whether the peptide forms a complex with macromolecules in biological fluids or whether its activity is affected by serum components when the latter are highly concentrated.

6. Conclusion

The positive results presented in this work highlight the potential of B7-005 as a candidate for further drug development to treat infections caused by ESKAPE pathogens, against which there are increasingly fewer effective solutions. These properties make B7-005 a potential broad-spectrum drug that could be used in clinical situations such as bacteremia, where antibiotics that act against multiple pathogens are needed. We have also shown that B7-005 could potentially be a drug that could be used in the clinical setting for a longer period of time than conventional antibiotics due to its low resistance development. Biocompatibility tests with eukaryotic cells and in vivo provided us with good evidence for the future applicability of B7-005 as a drug. We believe that the characterization of B7-005 could be further expanded. The effect of B7-005 in synergistic combination with other antimicrobial compounds could further expand its utility in various clinical scenarios. The anti-biofilm activity of B7-005 on several bacterial species should be further investigated to find a new approach against persistent bacterial infections, which are a serious health problem, especially in severe chronic diseases such as cystic fibrosis. Studies on the antimicrobial activity of B7-005 against foodborne pathogens or plant-infecting pathogens could expand the application range of B7-005. In addition, studying the toxicity of B7-005 in other animal models could provide further clues to its potential area of use.

Subsequent studies on the efficacy of B7-005 to resolve infections in vivo should definitively prove that this peptide can be a life-saving drug.

7. Bibliography

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8. Acknowledgments

First of all, I would like to thank Prof. Marco Scocchi, my PhD supervisor, who trusted me and gave me the opportunity to grow both as a person and as a researcher during this PhD period.

I am very grateful to Dr. Mario Mardirossian, the co-supervisor of this PhD, for being my mentor, supporting me throughout the years and teaching me not to give up in front of the difficulties that this work can bring.

I thank Prof. Sabrina Pacor who supervised me in the cytotoxicity experiments on eukaryotic cells, which would not have been possible without her collaboration. I thank her for giving me the opportunity to learn new techniques, but most of all for her patience and great willingness to accompany me on this journey.

I thank Dr. Sara Capolla. The bacterial assays by flow cytometry were performed in collaboration with her at the University of Trieste.

I would like to thank Dr. Sara Bozzer. The use of zebrafish as a model organism was possible thanks to her experience and collaboration in this work.

I also thank Dr. Luigi de Pascale for his organisational and moral support and for sharing laboratories, experiments, scientific discussions and intense working days.

Finally, my thanks also go to Prof. Alessandro Tossi for his helpful comments on my work and his confidence in this project.