



**UNIVERSITÀ
DEGLI STUDI
DI TRIESTE**

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XXXVII CICLO DEL DOTTORATO DI RICERCA IN

Biomedicina molecolare

**Cathelicidin-derived antimicrobial peptides as novel
drugs against antibiotic-resistant pathogens**

Settore scientifico-disciplinare: BIO/10 BIOCHIMICA

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INDICE

RIASSUNTO.....	3
ABSTRACT	5
1. INTRODUCTION	7
1.1 Antibiotic Resistance.....	7
1.1.1 History and statistics of antimicrobial resistance	7
1.1.2 Causes of spread of antibiotic resistance	8
1.1.3 Mechanism of action of antibiotics	10
1.1.4 Mechanisms of antibiotic resistance	12
1.2 ESKAPE pathogens	16
1.3 Antimicrobial peptides	18
1.3.1 General properties and classification	18
1.3.2 AMPs structure	20
1.3.3 AMPs mechanism of action	21
1.3.3.1 Membrane targeting mechanism	21
1.3.3.2 Intracellular targeting mechanism.....	23
1.3.4 Advantages and disadvantages of AMPs as antimicrobial agents	23
1.4 Cathelicidins	24
1.4.1 General characteristics and structures	24
1.4.2 α -helical cathelicidins	26
1.4.2.1 The porcine PMAP-36 cathelicidin	28
1.4.3 Proline-rich antimicrobial peptides (PrAMPs) of cathelicidin origin	29
1.4.3.1 General characteristics defining PrAMPs.....	29
1.4.3.2 PrAMPs uptake and mode of action	30
1.4.3.3 The bovine PrAMP Bac7 and its fragments	33
1.4.3.4 The cetacean PrAMPs Tur1A and Lip1	35
1.4.3.5 The optimized mammalian PrAMP B7-005	36
1.5 Methods and limits in studying antimicrobial mechanism of action	37
1.6 Bioorthogonal noncanonical amino acid tagging (BONCAT)	38
2. AIM OF THE THESIS	42
3. Structural and biological characterization of shortened derivatives of the cathelicidin PMAP-36	43

4. Designing New Chimeric Proline-Rich Antimicrobial Peptides to Enhance Efficacy Toward the ESKAPE+E: Beyond Sequence Extension	65
5. Testing antimicrobial peptides inhibiting protein synthesis in living E. coli and K. pneumoniae using bio-orthogonal non-canonic amino-acid tagging (BONCAT).....	82
6. GENERAL DISCUSSION	103
7. CONCLUSIONS.....	110
8. BIBLIOGRAPHY	111

RIASSUNTO

Gli antibiotici sono una delle classi di farmaci più influenti, essenziali nel combattere le infezioni batteriche e salvare milioni di vite. Tuttavia, la resistenza antimicrobica (AMR) ha reso le infezioni batteriche una rinnovata minaccia per la salute globale. Negli ultimi anni, i peptidi antimicrobici (AMP) sono stati esplorati come una nuova soluzione per contrastare l'AMR. Si tratta di piccole molecole (10-60 amminoacidi) prodotte da un gran numero di organismi, inclusi gli eucarioti, con strutture diverse. Attraverso vari meccanismi d'azione, gli AMP si sono dimostrati efficaci contro le infezioni batteriche sia *in vitro* che *in vivo*, inducendo raramente resistenza batterica. Tra questi, la famiglia delle catelicidine è una delle più studiate e comprende i sottogruppi delle catelicidine a α -elica, che agiscono danneggiando le membrane batteriche, e i peptidi antimicrobici ricchi in prolina (PrAMP), che inibiscono la sintesi proteica batterica. Nonostante la loro promettente attività antimicrobica, entrambi i tipi di catelicidine presentano limitazioni chiave che ostacolano il loro sviluppo clinico come agenti terapeutici: la maggior parte dei PrAMP mostra uno spettro di attività ristretto, essendo efficace principalmente contro alcuni batteri Gram-negativi, mentre le catelicidine ad α -elica sono piuttosto citotossiche verso le cellule eucariotiche e instabili a causa dell'attività proteolitica da parte delle proteasi presenti nel sangue e nel tratto digestivo.

L'obiettivo generale del mio progetto di tesi di dottorato è stato quello di superare alcuni limiti che impediscono la piena applicazione terapeutica delle catelicidine, riducendone la citotossicità, migliorandone la stabilità alle proteasi e ampliandone lo spettro di attività antimicrobica. Inoltre, il progetto ha mirato ad acquisire una comprensione più approfondita del meccanismo di azione antibatterica delle catelecidine, un processo cruciale per migliorare ulteriormente le loro caratteristiche terapeutiche. Il lavoro di tesi è suddiviso in tre studi distinti: due di questi sono già stati pubblicati come articoli scientifici, mentre l'ultimo è stato recentemente sottomesso.

Il primo studio mirava a ottimizzare la catelicidina a α -elica suina PMAP-36, che mostra una forte attività antimicrobica contro diverse specie batteriche Gram-negative e Gram-positive, ma che è molto lunga - rendendola costosa da sintetizzare - sensibile alle proteasi e citotossica ad alte dosi per le cellule eucariotiche. In questo studio, sono stati sintetizzati dei derivati di PMAP-36 tramite accorciamento della sequenza, modifica di residui amminoacidici chiave o inserimento di gruppi lipidici, con l'obiettivo di identificare il farmacoforo del peptide e superarne le limitazioni. Lo studio ha caratterizzato con successo peptidi più corti che mantenevano un'ampia attività antimicrobica, mostrando maggiore selettività batterica o maggiore resistenza alle proteasi. La regione centrale

(residui 12–24) di PMAP-36, che conserva le proprietà strutturali e antibatteriche, è emersa come farmacoforo del peptide, un interessante punto di partenza per ulteriori ottimizzazioni terapeutiche.

Il secondo studio si è focalizzato sul PrAMP sintetico B7-005, composto da 16 amminoacidi, dotato di elevata attività antimicrobica, principalmente contro le *Enterobacteriaceae* e pochi altri batteri Gram-negativi, ma di una potenza inferiore rispetto ai suoi omologhi nativi più lunghi. Lo studio ha mirato a potenziare l'efficacia di B7-005 estendendone la sequenza con 6 o 11 amminoacidi derivati dalle sequenze C-terminali di altri tre PrAMP più lunghi di origine bovina o cetacea: Bac7(1-35), Tur1A e Lip1. Queste estensioni hanno portato a identificare sei nuovi composti chimerici, con attività antibatterica migliorata e spettro d'azione più ampio, superando le prestazioni di B7-005, Bac7(1-35) e Tur1A. Tutti e sei i nuovi peptidi hanno mantenuto bassa citotossicità e la stessa capacità di B7-005 di inibire la traduzione proteica in *E. coli*, con solo un modesto effetto perturbante sulle membrane batteriche. Lo studio ha evidenziato i benefici e alcune limitazioni dell'allungamento di B7-005, permettendo di caratterizzare PrAMP chimerici promettenti per lo sviluppo di nuovi antimicrobici.

Il terzo studio ha cercato di superare i limiti dei metodi tradizionali per lo studio del meccanismo d'azione intracellulare *in vivo* degli AMP, compresi i PrAMP, studiati principalmente in *E. coli* con tecniche complesse e spesso basate su analoghi radioattivi. Questo studio ha valutato l'efficacia del metodo BONCAT (bioorthogonal noncanonical amino acid tagging), basato su fluorescenza, rapido, economico e che impiega analoghi molecolari per rilevare AMP che inibiscono la sintesi proteica nei batteri vivi, in particolare in due specie Gram-negative. Il metodo è stato validato utilizzando tre PrAMP: Bac7(1-35), Bac7(1-16) e B7-005, confermando una loro forte e specifica inibizione della sintesi proteica non solo nel batterio modello *E. coli*, ma anche nel patogeno *K. pneumoniae*. BONCAT ha anche permesso di capire se questo effetto fosse dovuto direttamente all'azione dei peptidi antimicrobici sulla sintesi proteica, oppure se fosse un effetto indiretto causato dal danneggiamento della membrana. Ciò è stato dimostrato testando AMP noti per agire sulla membrana, incluso un derivato litico di catelicidina caratterizzato nel primo studio. Inoltre, il metodo ha permesso di valutare la cinetica d'azione, rivelando che i PrAMP derivati dal Bac7 bloccano quasi del tutto la traduzione in *E. coli* entro 10 minuti dall'incubazione. L'applicazione di BONCAT si è dimostrata molto efficace nel chiarire i meccanismi molecolari d'azione degli AMP e nel fornire informazioni preziose sulla loro cinetica, rendendolo un potente strumento per lo screening di nuovi antimicrobici che bersagliano la sintesi proteica, con l'obiettivo finale di contrastare la resistenza antimicrobica.

I risultati di questa tesi di dottorato hanno ampliato la conoscenza sulle catelicidine, contribuendo allo sviluppo di AMP più sicuri, stabili ed efficaci. Inoltre, il lavoro ha fornito nuovi strumenti per studiare i loro meccanismi d'azione contro patogeni batterici clinicamente rilevanti.

ABSTRACT

Antibiotics are one of the most influential classes of drugs, essential for fighting bacterial infections and saving millions of lives. However, antimicrobial resistance (AMR) has made bacterial infections a renewed global health threat. In recent years, AMPs (antimicrobial peptides) have been explored as a new solution to contrast AMR. They are small molecules (10-60 amino acids) produced by a wide range of organisms, including many eukaryotes, and show diverse structures. Through various mechanisms of action, AMPs proved to be effective against bacterial infections *in vitro* and *in vivo*, being less likely to induce bacterial resistance. Among them, the family of cathelicidins is one of the best studied, including the subgroups of α -helical cathelicidin-derived peptides, which act by damaging bacterial membranes. Proline-rich antimicrobial peptides (PrAMPs), some of which are classified as cathelicidins, constitute a distinct group that inhibits bacterial protein synthesis. Despite their promising antimicrobial activity, both α -helical cathelicidin-derived peptides and PrAMPs exhibit key limitations that hinder their clinical development as therapeutic agents: most PrAMPs show a narrow activity spectrum, being effective mainly against some Gram-negative bacteria, while α -helical cathelicidins are quite cytotoxic towards eukaryotic cells, and are unstable due to proteolytic activity by blood and digestive tract proteases.

The general aim of my PhD thesis project was to overcome some obstacles preventing the full therapeutic application of cathelicidins by reducing their cytotoxicity, improving their stability to proteases, and broadening their antimicrobial activity. Furthermore, the project aimed at gaining a deeper understanding of their mechanism of antibacterial action, a critical process to further improve their therapeutic features. The thesis work is divided in three different studies: two of which have been already published as research paper, and the last-one has been recently submitted.

The first study focused on optimizing the porcine α -helical cathelicidin PMAP-36, which shows potent antimicrobial activity against several Gram-negative and Gram-positive bacterial species, but which is very long — making it expensive to synthesize — protease-sensitive, and also cytotoxic to eukaryotic cells at high doses. In this study, a series of PMAP-36 derivatives were synthesized by shortening its sequence, modifying key amino acid residues or inserting lipid groups, with the aim of identifying the peptide's pharmacophore and overcoming its limitations. The study successfully characterized shorter peptides that retained a broad-spectrum antimicrobial activity while exhibiting enhanced bacterial selectivity or increased protease resistance. The central region (residues 12 to 24) of PMAP-36, which retained its structural and antibacterial properties, was identified as a

pharmacophore of the peptide, making it an interesting starting point for further therapeutic optimization.

The focus of the second study was the 16-amino-acid synthetic PrAMP B7-005, endowed with high antimicrobial activity, mainly against *Enterobacteriaceae* and a few other Gram-negative bacteria, although it remains less potent than its longer native peptides. This study aimed to enhance the potency of B7-005 by extending it with 6 or 11 amino acids derived from the C-terminal sequences of three other longer PrAMPs: the bovine Bac7(1-35) and the cetacean TurlA and Lip1. These extensions led to the identification of six new chimeric compounds, with improved antibacterial activity and wider spectrum of action, outperforming the natives B7-005, Bac7(1-35) and TurlA. All six new peptides retained low cytotoxicity and maintained the same ability as B7-005 to inhibit protein translation in *E. coli*, with only a modest perturbation of bacterial membranes. The study highlighted the benefits and some limits of B7-005 elongation, leading to the characterization of promising chimeric PrAMPs, for new antimicrobial developments.

The third study addressed the limitations of traditional methods for studying the *in vivo* intracellular mechanism of AMPs, including PrAMPs, mainly studied in *E. coli* using complex techniques, often involving radioactive analogues. This study aimed to evaluate BONCAT (bioorthogonal noncanonical amino acid tagging) as a fast, cost-effective and fluorescence-based method using molecular analogues to detect AMPs inhibiting protein synthesis *in living bacteria*, specifically across two Gram-negative species. The method was validated using three PrAMPs, Bac7(1-35), Bac7(1-16), and B7-005, confirming a strong and specific inhibition of protein synthesis by PrAMPs not only in the model organism *E. coli* but also in the pathogenic *K. pneumoniae*. BONCAT also distinguished whether this effect was a direct consequence of the AMP acting on the translational machinery, or an indirect, secondary effect resulting from membrane disruption. This distinction was demonstrated using AMPs known to affect membrane integrity, including a lytic cathelicidin-derived characterized in the first study. Additionally, the method enabled to assess the precise kinetics of action, revealing that Bac7-based PrAMPs nearly fully blocked protein translation in *E. coli* within 10 minutes of incubation. The application of BONCAT was very effective in elucidating the molecular mechanism of action of AMPs and in providing valuable kinetics insights. This makes BONCAT a powerful tool for the screening of new antimicrobials that target protein synthesis, with the final aim to fight AMR.

In conclusion, the results of this PhD thesis expanded the knowledge on cathelicidins, contributing to the development of safer, more stable and more effective AMPs. Furthermore, the work provided new tools to study their mechanisms against clinically relevant bacterial pathogens.

1. INTRODUCTION

1.1 Antibiotic Resistance

1.1.1 History and statistics of antimicrobial resistance

In the twenty-first century, antibiotics have been and are undoubtedly one of the most influential classes of drugs among medical inventions (1), proven to be essential in fighting bacterial infections and saving millions of lives (2). Antibiotics also play a fundamental role in the advancement of medicine and surgery, preventing and treating infections in individuals undergoing chemotherapy, surgical procedures such as transplants, or suffering from chronic conditions such as diabetes, kidney failure, or rheumatoid arthritis. This class of drugs contributed to increasing human life expectancy. In the United States alone, average life expectancy has risen from 56.4 years in 1920 to nearly 80 years today (3). These beneficial effects can be seen throughout the world, where antibiotics play an important role in reducing morbidity and mortality related to bacterial infections, including those linked to poverty and poor sanitation in developing countries (4). The word “antibiotic” was coined by Selman Waksman to define small molecules of microbial origin with properties that inhibit the growth of other microbes (5). The modern era of antibiotics began in 1928 (**Figure 1.1**) with Alexander Fleming's discovery of penicillin, which was used successfully until the 1940s, to treat infections in soldiers during World War II (3).

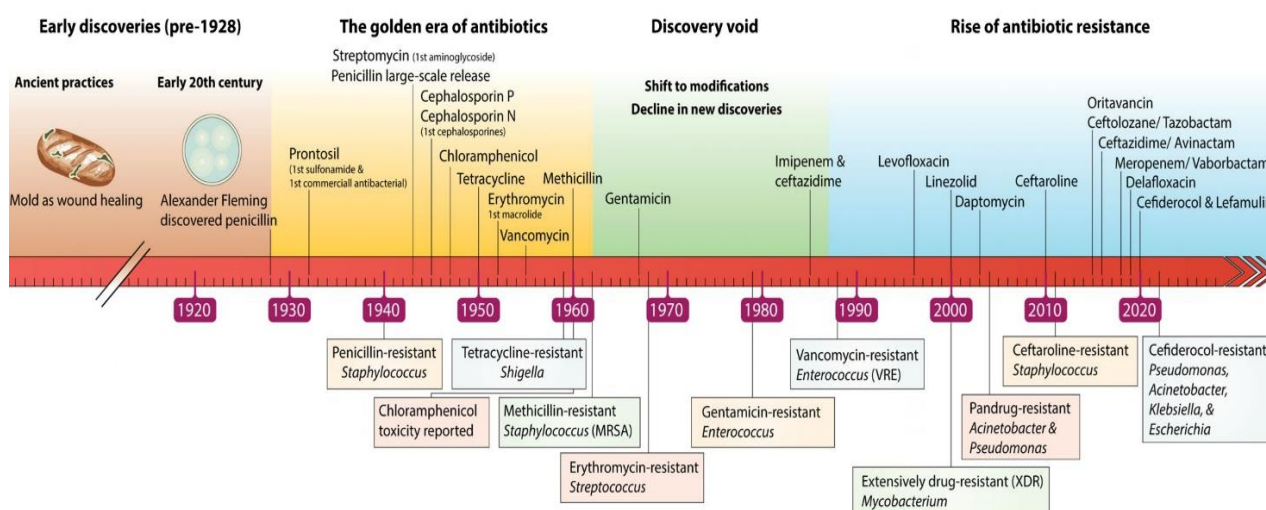


Figure 1.1 History of antibiotic evolution and its significant events, from their discovery to the emergence of various types of resistance. Image from Tahmasebi et al., 2025 (6).

Subsequently, until the 1960s-1970s, there was the so called “golden era” of antibiotics, with the discovery, development, and marketing of classes of antibiotics that are still in use today. During those years, it was commonly believed that the health problem of bacterial infections was now under control and destined to disappear (2). Despite this confidence, for almost all antibiotics on the market, an increasing presence of resistant bacteria has been observed over the years (3). The rapid and relatively simple development of different antibiotics in a short period of time has led to both excessive use of these drugs and a slowdown in antibiotic research, resulting in the current situation where few new antibiotics are in clinical trials (1). For instance, the last truly new class of antibiotics to be approved and reach the market was Lefamulin (Xenleta), which was approved by the U.S. Food and Drug Administration (FDA) in August 2019 (7,8), ending a period of nearly 30 years without new antibiotic classes (1).

As an unfortunate consequence of the described situation, decades after the first antibiotic was used, the problem of bacterial infections has once again become a major public health concern (9). Infections that were previously treatable with standard antibiotics are now resistant to them and much more difficult to cure (6). Antimicrobial resistance (AMR) is in fact defined as “the inability or reduced ability of an antimicrobial agent to inhibit the growth of a bacterium, which, in the case of a pathogenic organism, can lead to therapy failure” (10). Addressing infections caused by antimicrobial-resistant bacteria requires an investment of 2–3.5% of gross domestic product (11) and AMR was linked to an estimated 4.71 million deaths in 2021. Despite an encouraging 50% decline in deaths from bacterial infections in children under 5 years old between 1990 and 2021, deaths from antibiotic-resistant bacteria increased by 80% in adults aged 70 and older. Forecasts estimate up to 8.22 million deaths associated with AMR in 2050, with South Asia, Latin America, and the Caribbean as areas most at risk. Better access to antibiotics and the discovery of better treatments for serious infections could prevent approximately 92 million deaths of all ages between 2025 and 2050 (12).

1.1.2 Causes of spread of antibiotic resistance

Bacteria are microorganisms capable of adapting over time to survive, replicating and spreading as quickly as possible (13). To this end, if an antibiotic prevents them from surviving, genetic modifications may occur, resulting in the development of resistance (1). Antibiotic resistance has been intensified and accelerated by the improper and excessive use of antibiotics in recent decades, when, in addition to being therapeutically used for incorrect diagnoses or inappropriate prescriptions, they have also been overused in agriculture, veterinary medicine, animal husbandry, and industry (14).

There are several contexts that mainly contribute to the emergence and spread of antibiotic-resistant bacteria (**Figure 1.2**). Hospitals, farms, wastewater treatment plants (WWTP), and agricultural environments are the main places where these bacteria are transmitted between the environment, humans, and animals, and are therefore an important target in the management and prevention of antibiotic resistance (15).

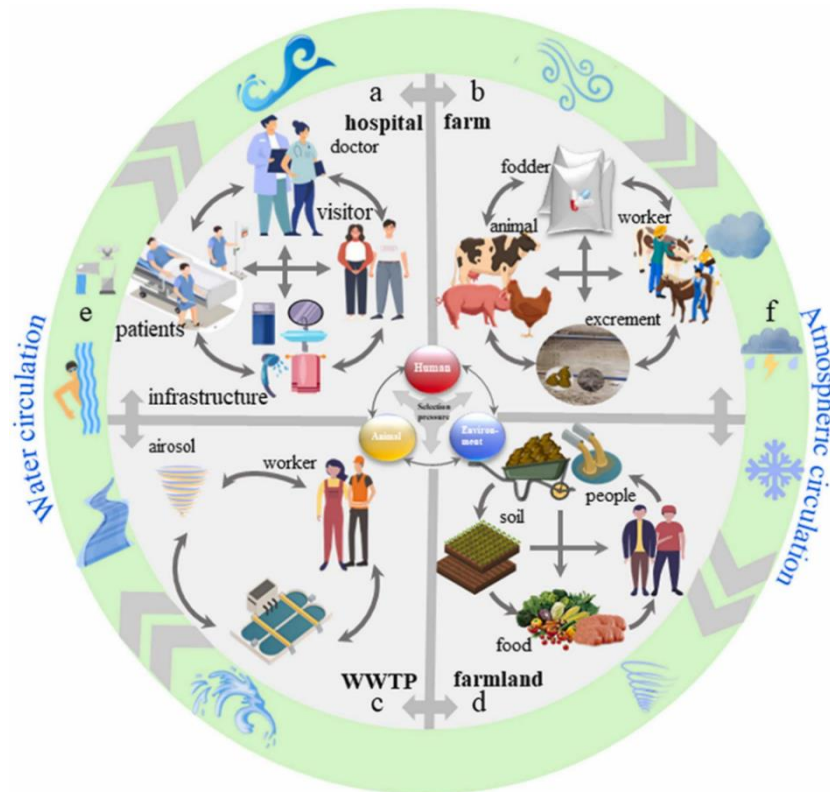


Figure 1.2 The main environmental sources of antibiotic-resistant bacteria for human. Hospital environment (a), farm environment (b), plant (WWTP) environment (c), agriculture environment (d), water environment (e) and atmospheric circulation (f). Image from Ding et al., 2023 (15).

More specifically: the hospital environment is a place with a high bacterial presence due to the rooms, work surfaces, and water systems, which are inevitably contaminated by various pathogens (15). It is also common for bacteria isolated from a hospital environment to be resistant to antibiotics (16). If hospitals do not have appropriate procedures and protocols for cleaning spaces, these bacteria can spread, promoting the emergence and spread of AMR (17). Additionally, in many parts of the world, farms with intensive livestock operations heavily rely on antibiotics to promote animal growth, due to the high bacterial presence in the animal environment, including manure, wastewater, soil, and air (15). As in humans, this usage causes the emergence of antibiotic-resistant bacteria, which can easily circulate in the ecosystem with animal waste and enter the food chain, resulting in resistant infections in humans (18). Furthermore, wastewater treatment plants (WWTP), processing municipal, hospital,

and slaughterhouse waste, contain high levels of antibiotic-resistant bacteria (ARB), being potential health risks to workers and nearby populations through inhalation, skin contact, or ingestion (15). The methods used to treat wastewater are not entirely effective in removing these pathogens, resulting in contamination of the soil and aquatic environments where it is discharged (19,20). Moreover, in agriculture, the use of irrigation water and manure leads to the contamination of agricultural products with bacteria that can survive on the surface and inside food, entering the food chain and carrying pathogens that are resistant to humans and animals (15). Finally, freshwater resources can be contaminated by domestic, industrial, hospital, or agricultural wastewater, transmitting resistant bacteria to humans through showering or drinking water, particularly in regions with inadequate water treatment systems (15).

In addition to these factors, there are phenomena such as poor people's hygiene and sanitation, self-medication, loss of sensitivity to antibiotics in patients, and antibiotic therapies that are not correctly completed by the patient; all of this conduct to the emergence and spread of antibiotic resistance (1).

1.1.3 Mechanism of action of antibiotics

The antibacterial activity of antibiotics can be classified into at least five different mechanisms of action (see specific examples in **Figure 1.3** and **Table 1.1**) (1).

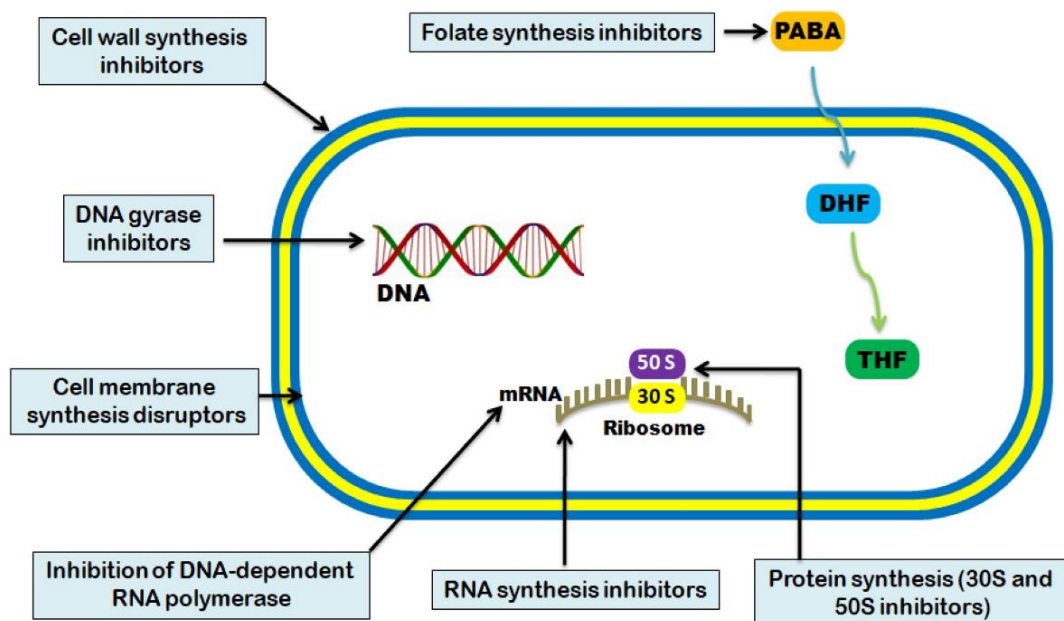


Figure 1.3 Graphical representation of the different mode of action of antibiotics. Image from Uddin et al., 2021 (1).

Table 1.1 Mode of action of the five classes of antibiotics. Table adapted from Uddin et al., 2021 (1).

Mode of action	Targets	Drug class	Specific drugs examples
Cell wall synthesis inhibition	Penicillin-binding protein	β -lactams	Penicillin G, amoxicillin, and cephalosporin C
	Peptidoglycan subunits	Glycopeptides	Vancomycin
Inhibition of protein synthesis	30S subunit	Aminoglycosides and tetracyclines	Streptomycin, gentamicin, neomycin, tetracycline, and doxycycline
	50S subunit	Macrolides, chloramphenicol, and oxazolidinones	Erythromycin, azithromycin, chloramphenicol, and linezolid
Inhibition of nucleic acid synthesis	RNA	Rifamycin	Rifampin
	DNA	Quinolones and Fluoroquinolones	Nalidixic Acid, Ciprofloxacin and ofloxacin
Anti-metabolites	Folic acid synthesis enzymes	Sulfonamides and trimethoprim	Sulfamethoxazole, dapsone, and trimethoprim
Disruption of membranes	Lipopolysaccharides and phosphatidylglycerol	Polymyxins and cyclic lipopeptides	Polymyxin B, colistin and daptomycin

- 1) **Cell wall synthesis inhibition.** Bacterial cell walls are composed of cross-linked peptidoglycan (21). Antibiotics such as glycopeptides (vancomycin) or β -lactams inhibit peptidoglycan biosynthesis, and therefore cell wall synthesis, making bacteria susceptible to osmotic pressure and autolysis. In particular, β -lactams act by binding to penicillin-binding proteins (PBPs), enzymes involved in the final stages of peptidoglycan cross-linking. This mechanism of action is selective for bacteria because peptidoglycan is absent in animal cells (22–24).
- 2) **Inhibition of protein synthesis.** The 70S ribosome of bacteria is composed of two subunits: the 30S and the 50S. Some antibiotics target these subunits to inhibit protein synthesis in bacteria. Specifically, aminoglycosides and tetracyclines target the 30S subunit, while chloramphenicol, macrolides, and oxazolidinones target the 50S subunit (25). Together with the final target, also the path to the ribosome can variate among them. For example, tetracycline passively enters the bacterial cell membrane through porin channels and

reversibly binds to the 30S ribosomal subunit, blocking the binding of tRNA to the mRNA-ribosome complex and consequently protein synthesis (26). Chloramphenicol, on the other hand, crosses bacterial membranes because it is lipid-soluble and reversibly binds to the L16 protein of the 50S ribosomal subunit; in this way, it prevents the transfer of amino acids during the elongation step and therefore the expansion of peptide chains, blocking the protein synthesis of bacteria (27).

- 3) **Inhibition of nucleic acid synthesis.** Other antibiotics act on bacteria by inhibiting the synthesis of RNA (rifamycin) or DNA (quinolones and fluoroquinolones) (1). For example, rifampin inhibits DNA-dependent RNA polymerase (RNAP) and acts specifically on it, blocking the synthesis of bacterial RNA and causing the death of bacteria (28). In contrast, nalidixic acid blocks bacterial DNA replication by inhibiting the activity of DNA gyrase and inducing bacterial death (29).
- 4) **Anti-metabolites.** Other synthetic antibiotics act as antimetabolites, *i.e.*, they act as inhibitors of metabolic enzymes in bacteria, keeping infections under control. For example, sulfonamides and trimethoprim can inhibit two different stages of folic acid metabolic pathway (30).

Disruption of membranes. Finally, antibiotics such as polymyxins target the outer membrane of Gram-negative bacteria by interacting with lipopolysaccharides (LPS), disrupting its structure and integrity and increasing therefore permeability (1). For example, the cationic antibiotic colistin binds electrostatically to the lipid A portion of LPS in the outer membrane, displacing Ca^{2+} and Mg^{2+} cations and destabilizing the membrane. This disruption facilitates colistin's penetration to the inner membrane, where it compromises membrane integrity, ultimately causing cell lysis (31). Another example is daptomycin, a cyclic lipopeptide antibiotic, that kills Gram-positive bacteria by forming a Ca^{2+} -dependent tripartite complex with phosphatidylglycerol and lipid II at the division septum. This sequesters lipid II and causes delocalisation of key peptidoglycan synthesis enzymes such as MurG and other divisome components, thereby blocking cell wall biosynthesis and ultimately leading to cell death (32,33).

1.1.4 Mechanisms of antibiotic resistance

The sensitivity or resistance of bacteria to an antibiotic is measured based on its minimum inhibitory concentration (MIC), defined as the minimum concentration of the antimicrobial agent capable of inhibiting bacterial growth. Bacteria may be naturally resistant to a drug or become resistant by

acquiring resistance genes from other organisms (24). In the former case, natural resistance is divided into intrinsic, which is always expressed within a bacterial species, or induced, when bacteria already naturally possess genes that are only expressed after exposure to an antibiotic, giving resistance; for example, reduction of outer membrane permeability and the natural activity of efflux pumps are among the most common mechanisms of intrinsic antibiotic resistance (34). In the latter case, acquired resistance can be temporary or permanent and is due to the acquisition of genetic material that confers resistance. This process occurs primarily through horizontal gene transfer (HGT), which includes conjugation, transduction, and natural transformation. Among these, plasmid-mediated conjugation is considered the most frequent mechanism for the acquisition of external resistance genes, but both transduction (mediated by bacteriophages) and natural transformation also play significant roles and should not be underestimated. In fact, while all bacteria are capable of natural transformation, some species, such as *Acinetobacter spp.*, are particularly efficient at doing so. Additionally, bacteria may also develop resistance through spontaneous mutations in their chromosomal DNA (estimated at one mutation per 10^6 – 10^9 cell divisions), which can be triggered or accelerated by stressors such as UV radiation or chemical exposure (24). Among the mutations that most promote AMR are those affecting genes that code for: drug transporters, drug targets, drug transport regulators, and enzymes that modify antibiotics (35).

Antimicrobial resistance (AMR) mechanisms can be grouped into four main categories (**Figure 1.4**): (1.) limiting drug uptake, (2.) drug inactivation, (3.) active drug efflux, and (4.) modification of the drug target. Intrinsic resistance typically involves limited uptake, drug inactivation, and efflux, whereas acquired resistance more commonly relies on drug inactivation, efflux, and target modification. Due to structural differences, such as the absence of an outer membrane, Gram-positive bacteria are generally less efficient in preventing drug entry via permeability barriers. In contrast, Gram-negative bacteria, that possess an outer membrane and a wide variety of efflux systems, are capable of employing all four mechanisms of resistance (24).

- 1) Limiting uptake of a drug:** the structure of pathogens affects intrinsic antibiotic resistance. For example, gram-negative bacteria, with their LPS layer, have a natural barrier that confers them innate resistance to certain large antimicrobials, while bacteria without a cell wall (*Mycoplasma*) are naturally resistant to all drugs that act on it (24). In Gram-negative bacteria, hydrophilic molecules often enter the cell through porin channels. Therefore, as resistance mechanism, bacteria can limit the absorption of drugs by reducing the number of porins or by acquiring mutations that alter the size or charge properties of these channels, thereby restricting drug entry (36). Finally, among the factors that limit drug uptake, biofilm formation

plays an important role. Bacteria can form a biofilm that protects them from antimicrobial agents, thanks to its dense and sticky composition (as it contains polysaccharides, proteins, and DNA from the bacteria themselves). The biofilm makes it difficult for antimicrobials to target the bacteria, requiring a significant increase in drug concentration (24)

- 2) **Inactivation of a drug:** bacteria can inactivate drugs either by degrading them or by modifying them by the transfer of a chemical group to them. For example, tetracycline can undergo hydrolysis, as can β -lactams by β -lactamase enzymes (24,37). Among the chemical groups most transferred to drugs are acetyl, phosphoryl, and adenylyl groups. For example, chloramphenicol can undergo acetylation by transferases which inhibit the drug (24,38).
- 3) **Efflux pump:** bacteria possess genes encoding efflux pumps, some of which are constitutively expressed and potentially confer intrinsic resistance, while others are induced or overexpressed in response to specific substrates or environmental stimuli. These pumps transport a wide variety of compounds, being called multidrug resistant (MDR) pumps, and their primary function is to rid the bacterial cell of toxic substances. Almost all bacteria have multiple types of efflux pumps with different structures and energy sources and, in many bacteria, antibiotic efflux is one of the most common resistance mechanisms (24,39). Furthermore, efflux pumps can make a bacterium resistant to multiple antibiotics because they can expel several structurally different compounds, as in the case of the extrusion of β -lactam drugs (24,40)
- 4) **Modification of drug target:** bacteria have several targets for antimicrobials (**Table 1.1**), which can undergo changes without lose their function and generating AMR. Gram-positive bacteria, for example, can alter the structure and/or number of penicillin-binding proteins (PBPs), effectively inhibiting the binding of β -lactam drugs and their effect on peptidoglycan biosynthesis (24). On the other hand, the previously described lipopeptide antibiotic daptomycin acts by depolarizing the cell membrane through binding to calcium. Mutations in certain genes make the cell membrane surface positively charged, reducing the binding of calcium and therefore of the antibiotic (24,41,42). For drugs that act by inhibiting protein synthesis, certain mutations or methylations of ribosomal subunits, as well as the phenomenon of ribosomal protection, induce AMR by altering the ability of antibiotics (aminoglycosides, tetracyclines, oxazolidinones, macrolides) to bind to the ribosome (24,43). Resistance to drugs that inhibit nucleic acid synthesis (such as fluoroquinolones) often arises through structural modifications of their target enzymes. Mutations in the *gyrA* gene, encoding DNA gyrase in Gram-negative bacteria, or in the *grlA* gene, encoding topoisomerase IV in Gram-positive bacteria, alter the drug-binding sites, preventing effective binding and inducing AMR (24,44). Finally, for drugs

that inhibit metabolic pathways, such as sulfonamides and trimethoprim, antimicrobial resistance occurs through mutations in the active sites of enzymes involved in the folate biosynthesis pathway (DHPS - dihydropteroate synthase for sulfonamides, DHFR - dihydrofolate reductase for trimethoprim) or through the overproduction of resistant DHPS and DHFR enzymes. These changes interfere with the competitive binding of the antibiotics while still allowing the natural substrates to bind (24,45).

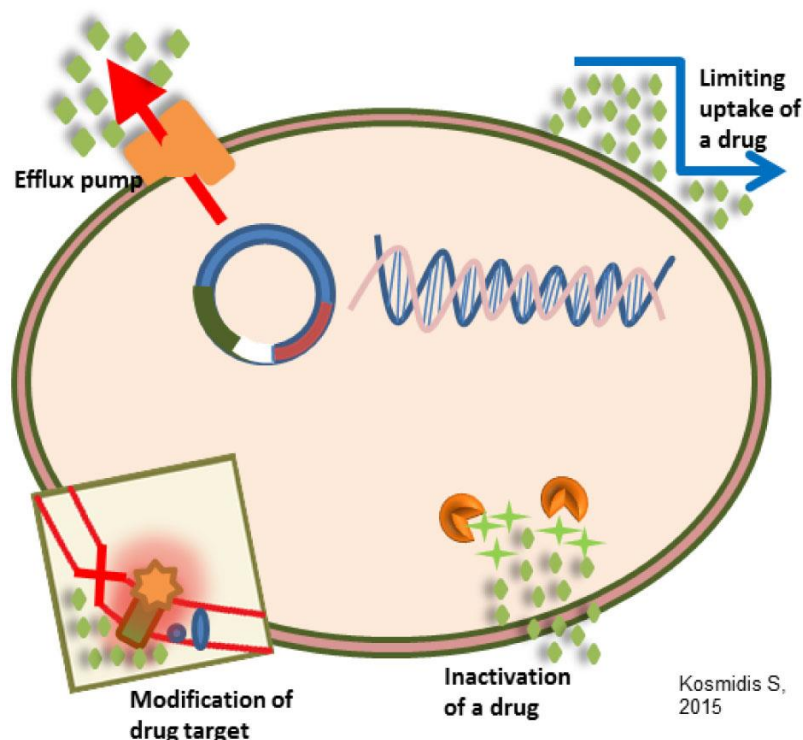


Figure 1.4 Illustration of the four main categories of AMR onset mechanisms: efflux pump, limiting uptake of a drug, modification of drug target and inactivation of a drug. Image from Reygaert, 2018 (24).

A prominent example of bacteria that employ these antibiotic resistance mechanisms are the ESKAPE pathogens, which exhibit reduced susceptibility to major antibiotic classes - including macrolides, tetracyclines, β -lactams, oxazolidinones, lipopeptides, and even last-resort antibiotics such as polymyxins and glycopeptides - and therefore represent a major challenge in the treatment of healthcare-associated infections (46).

1.2 ESKAPE pathogens

In 2017, the World Health Organization (WHO) published a list of antibiotic-resistant bacteria of global priority to guide research and development of new therapeutic strategies against antimicrobial resistance (AMR). Among these, six pathogens - *Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. (collectively known as **ESKAPE**) - were highlighted due to their major role in hospital-acquired infections and their ability to evade the action of multiple antibiotics (47–50). These bacteria are particularly associated with severe infections in intensive care units, including bacteraemia, urinary tract infections, pneumonia, wound infections, and meningitis. In addition to clinical settings, ESKAPE pathogens are also detected in environmental sources such as soil, food, water, urban waste, and sewage (51–53).

Enterococcus sp. primarily includes *Enterococcus faecalis* and *Enterococcus faecium*, Gram-positive and facultative anaerobic bacteria that are widely distributed. They are commensal organisms normally found in the human gut, where they help modulate the immune system (47). They are frequently associated with hospital-acquired infections such as CAUTIs (catheter-associated urinary tract infections), SSIs (surgical site infections), and bloodstream infections. Globally, antimicrobial resistance in *Enterococcus faecium* is estimated to contribute to between 100,000 and 250,000 deaths annually (54).

Staphylococcus aureus is a Gram-positive bacterium and a skin commensal that, in more susceptible patients, can become a pathogen, often with significant clinical impact (47). This bacterium is commonly found in wound infections and can cause a variety of conditions including infective endocarditis, bacteraemia, soft tissue infections, and fatal pneumonia (55). *S. aureus* is associated with approximately 1.1 million deaths worldwide, making it the leading bacterial cause of death in 135 countries and the top-cause in individuals older than 15 years (56).

Klebsiella pneumoniae is a Gram-negative bacterium from the *Enterobacteriaceae* family. It is found both in the environment and on mammals and is frequently associated with community-acquired pneumonia (47,57,58). It poses a particular threat in hospital settings due to its production of extended-spectrum β -lactamases (ESBL), and it can infect the lungs, urinary tract, brain, and bloodstream (47). In recent years, carbapenem-resistant and hypervirulent strains have been identified (59), representing a serious public health concern. Additionally, *K. pneumoniae* is intrinsically resistant to multiple classes of antibiotics, facilitating its sporadic spread worldwide (47).

Acinetobacter baumannii is a highly versatile Gram-negative opportunistic pathogen that can adapt well to harsh environmental conditions such as dryness, variable temperatures, and pH levels, allowing it to persist both in the environment and within host organisms. It is intrinsically resistant to antibiotics, as well as to metals and ammonium-based disinfectants. In severely immunocompromised or critically ill patients, it can easily cause nosocomial respiratory infections, urinary tract infections, and wound infections. Carbapenem-resistant *A. baumannii* is considered one of the most urgent and challenging clinical threats according to the WHO (47).

Pseudomonas aeruginosa is a Gram-negative, facultative anaerobic bacterium, and its resistance to carbapenems makes it a critical concern for the WHO. It is frequently isolated from burn patients or individuals with cystic fibrosis (CF), representing the leading cause of mortality in adult CF patients, as it more easily infects immunocompromised hosts from childhood (47,60,61). It can cause infections in the eyes, skin, lungs, and urinary tract, as well as nosocomial infections in healthcare settings, such as ventilator-associated pneumonia, surgical site infections, or central line-associated bloodstream infections (47,62). *P. aeruginosa* can resist many classes of antibiotics, particularly fluoroquinolones, as well as harsh environmental conditions, making its treatment extremely challenging (63).

Enterobacter sp. is a class of Gram-negative and facultative anaerobic bacteria, frequently causing bacteraemia and infections of surgical sites, the urinary tract, or those associated with medical devices (64). Among the most clinically relevant species are *E. cloacae*, *E. asburiae*, and *E. hormaechei*, all of which exhibit high levels of antibiotic resistance, particularly to carbapenems, due to the production of extended-spectrum beta-lactamases (ESBLs). These bacteria are also capable of adapting to- and surviving in harsh environments, translocating from one site to another, and more easily infecting immunocompromised patients (47).

However, although the ESKAPE pathogens represent the six priority bacteria in the fight against antimicrobial resistance (AMR), this issue also concerns other bacterial species. Among these, as included in the work of this PhD thesis, it is worth mentioning *Escherichia coli* and *Staphylococcus epidermidis*, both including multidrug-resistant strains capable of causing hard-to-treat and potentially life-threatening infections in humans (65,66).

Due to the phenomenon of ESKAPE antibiotic resistance, there is an urgent need to identify new countermeasures to contrast bacterial infections. Compounded by the low profits derived from the

development of new antibiotics, alternative avenues need to be explored, including antimicrobial peptides (AMPs).

1.3 Antimicrobial peptides

1.3.1 General properties and classification

AMPs are a class of peptides that are widespread in nature and represent an important element of the innate immune system in virtually all pluricellular organisms. They are small molecules, between 10 and 60 amino acids long (with an average of 33), with different structures and which act effectively against bacterial infections through various mechanisms of action (67). The antimicrobial activity of AMPs is related to properties such as net charge, hydrophobicity, and the ability to form amphipathic secondary structures. If these factors are well balanced, antimicrobial peptides can specifically target bacterial membranes without damaging eukaryotic ones (68). Most peptides are cationic (88%) with an average net positive charge of 3, primarily due to the presence of lysine and arginine residues, which have positively charged side chains. This positive charge is essential for the peptides to electrostatically interact with the negatively charged bacterial membranes, disrupting their integrity and thereby exerting antimicrobial action (67,69,70). There are also anionic peptides (6% of the total) which, however, often require cofactors or positive ions (such as zinc) to act effectively against bacteria (69,71). AMPs are characterized by a prevalence of hydrophobic amino acids that allow them to interact with bacterial lipid bilayers, disrupting membrane integrity or translocating into the cell to target intracellular components. The correct positioning of hydrophobic and positively charged amino acids allows most AMPs to fold into amphipathic secondary structures (68). This amphipathicity is crucial as it enables peptides to efficiently navigate aqueous environments and interact with bacterial membranes, disrupting their integrity by increasing permeability and causing dysfunction (72). While most AMPs are amphipathic, a few rare peptides consist solely of hydrophobic or hydrophilic amino acids (73).

Natural antimicrobial peptides can be classified according to four different properties (**Figure 1.5**):

1. source; 2. activity; 3. amino acid-rich species; 4. structure (67):

1. Antimicrobial peptides can be classified as either natural or synthetic. Natural AMPs are produced by a wide range of organisms across different kingdoms of life, including microorganisms (e.g., bacteriocins), fungi (e.g., peptaibols), plants (e.g., thionins, defensins, heveins, snakins), invertebrates such as insects and marine arthropods (e.g., crustins, penaeidins), and vertebrates, particularly mammals (e.g., defensins, histatins, LL-37,

indolicidin, protegrins, lactoferrins). A substantial number of AMPs also originate from amphibians, where they play a key role in innate immunity (e.g., esculentin, japonicin-2, nigrocin-2, temporin, dermaseptin, magainin, buforin II) (73–78). The scientific community has shown strong interest in increasing the potential of antimicrobial peptides by developing synthetic analogues with enhanced efficacy, stability, and selectivity. These synthetic peptides can be engineered through various modifications, such as changes in amino acid sequences, peptide length adjustments, incorporation of non-natural amino acids, and structural alterations (73), with the main goal of improving natural AMPs' antibacterial potency, selectivity, and resistance to enzymatic degradation (79). According to the ADP6 database (<https://aps.unmc.edu/>), approximately 5,680 peptides had been identified as of September 2025. However, larger repositories list more than 20,000 sequences, mainly synthetic, indicating that the true diversity of AMPs is substantially greater. In APD6, natural antimicrobial peptides account for roughly two-thirds of the entries, while about one-third have been synthetically developed. Moreover, among natural AMPs recorded in APD6, there is a strong over-representation of amphibian sequences, which may introduce a bias in the representation of the natural AMP landscape (73).

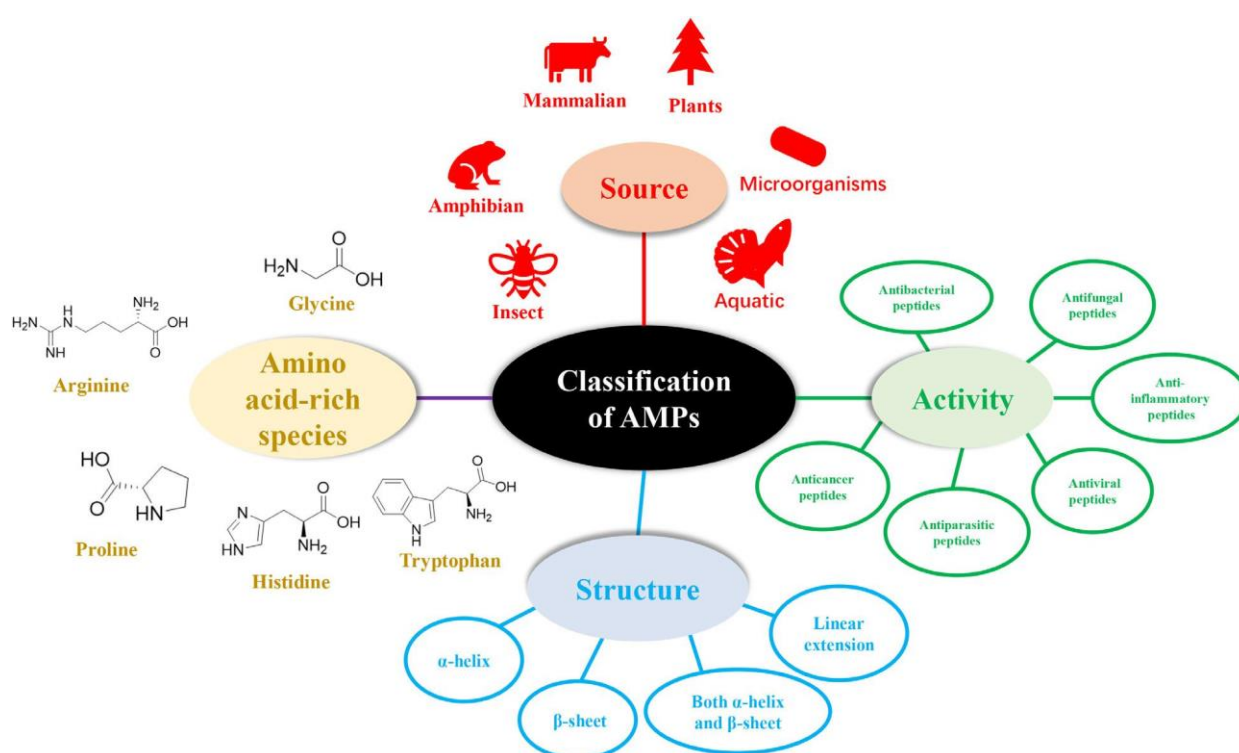


Figure 1.5 Antimicrobial peptides classification based on: source, activity, structure and amino acid enrichment composition. Image from Huan et al., 2020 (67).

2. Another classification of AMPs is based on their biological activity, depending on the microorganism they target; these peptides can act against gram-positive and gram-negative bacteria, viruses, biofilms, parasites, fungi, therefore representing a good option even for the treatment of microbial co-infections. Antibacterial peptides represent more than a half of these existing AMPs (67,73).
3. Regarding amino acid composition, AMPs can be rich in amino acids such as arginine, histidine, proline (PrAMPs), tryptophan, or glycine. To be defined as "rich" in a certain amino acid, the peptide must contain that residue in at least 25% of its sequence (80).
4. Finally, antimicrobial peptides can be classified according to their structure, an aspect deeper treated in the next paragraph.

1.3.2 AMPs structure

Antimicrobial peptides can be divided into four distinct groups based on their predominant secondary structure (**Figure 1.6**). These are respectively: α -helix, β -sheet, mixture of α and β , and without α -helical and β -sheet structures (73).

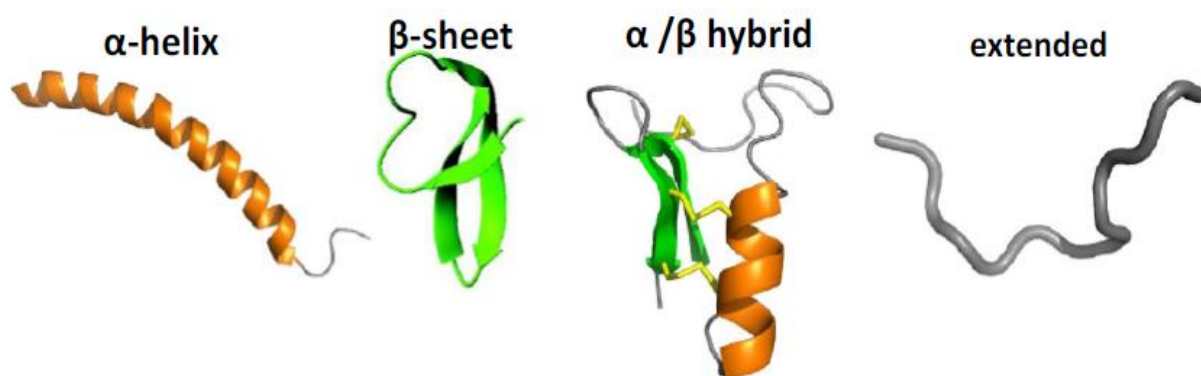


Figure 1.6 Examples of the four structural classes of AMPs: α -helix, β -sheet, α/β hybrid and extended structure. Image from Koehbach and Craik, 2019 (81), where these four classes are exemplified respectively by the peptides LL -37, human α -defensin, phormicin and indolicidin.

AMPs with an **α -helical structure** are characterized by amino acid chains that are arranged in a helical spiral, giving these peptides the ability to interact effectively with microbial membranes (82). The α -helical structure creates positively charged, hydrophilic, and hydrophobic areas on the molecular surface, promoting the interaction of the peptide with the bacterial membrane (83). Examples of this class include magainin-2, derived from the skin of *Xenopus laevis* (84) and the human peptide LL-37 (81).

Antimicrobial peptides with a **β -sheet structure** fold into a sheet-like arrangement of amino acid chains, which are often stabilized by intramolecular interactions, conferring stability and efficacy against bacteria, fungi, and viruses (73). Human alpha-defensins, which are very important members of the innate immune system, belong to this class (85).

Antimicrobial peptides with a mixed **$\alpha\beta$ structure** are characterized by a hybrid arrangement between α -helix and β -sheet, containing elements of both structures in their amino acid chains. Often these AMPs, possessing both hydrophilic and hydrophobic amino acid regions, exhibit amphipathicity (73). Phormicin belongs to this group of antimicrobial peptides (81).

Finally, some AMPs do not adopt typical α -helical or β -sheet conformation, but instead exhibit an **extended structure**, characterized by elongated or cyclic forms, differing from the previously described classes. This structural diversity provides versatility in their interactions with bacterial membranes and enhances their antimicrobial activity (73). Indolicidin is an antimicrobial peptide belonging to this class (86).

It is worth mentioning that all four structures mentioned above can further stabilize or modify their conformation upon interaction with lipid environments, such as cellular membranes. Moreover, the α -helical and β -sheet structures facilitates AMPs in their interaction with bacterial membranes, enabling them to induce membrane permeabilization. In contrast, peptides with other structural conformations may exert antimicrobial effects through intracellular targets (87). These mechanisms will be described in detail in the following paragraph.

1.3.3 AMPs mechanism of action

Antimicrobial peptides primarily kill bacteria by targeting and disrupting the bacterial membrane, which is the most common mechanism of action. On the other hand, a smaller subset of AMPs exerts their antimicrobial effect through intracellular activities (**Figure 1.7**).

1.3.3.1 Membrane targeting mechanism

Many positively charged AMPs electrostatically interact with the negatively charged bacterial envelope, facilitating their insertion into the lipid bilayer. This interaction destabilizes the membrane structure, leading to membrane collapse and leakage of intracellular ions and metabolites. Consequently, depolarization of the membrane potential, membrane dysfunction, and eventual cell

lysis occur, resulting in bacterial cell death (73,88). The mechanisms through which AMPs can insert themselves into the membrane are described by main three different models (showed in **Figure 1.7**):

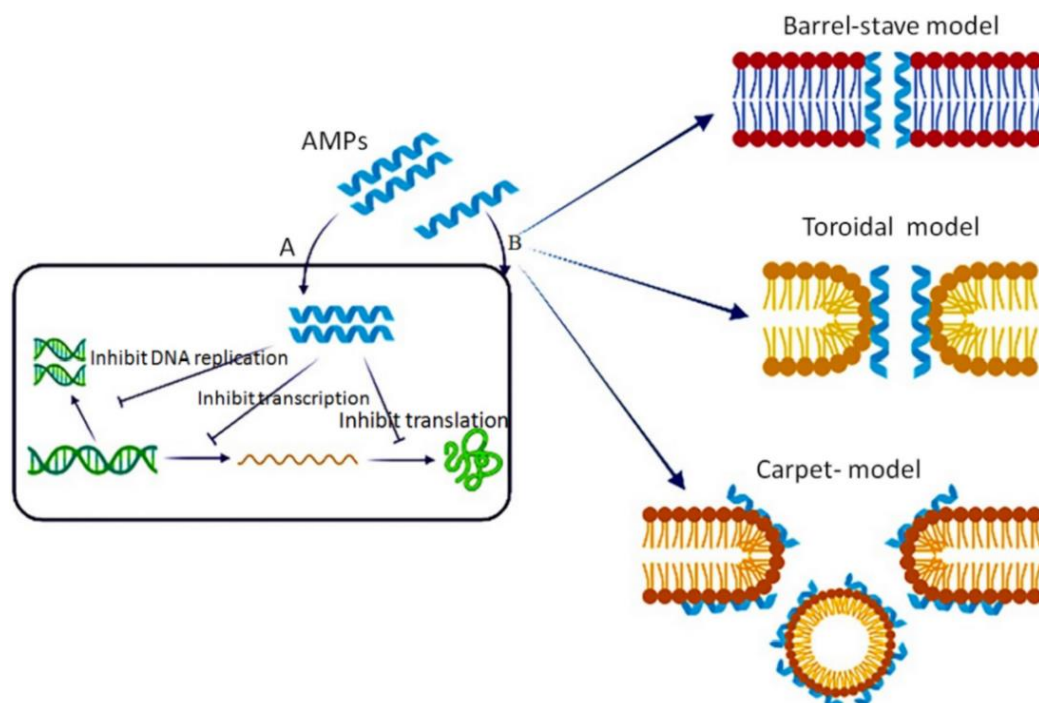


Figure 1.7 The two main antimicrobial mechanisms of action of AMPs: **A.** intracellular targeting, which mainly involves specific antibacterial action on proteins or nucleic acids; **B.** membrane targeting and disruption, which includes three distinct models (barrel-stave, toroidal, and carpet).
Image from Mabrouk et al., 2022 (89).

The **Toroidal Pore Model**, also known as the “wormhole” model, involves the vertical insertion of AMPs into the cell membrane. The peptides accumulate and induce bending of both the inner and outer membrane leaflets, forming a toroidal (doughnut-shaped) pore lined by both peptides and lipid head groups, with a diameter of 1–2 nm (90,91). In contrast, in the **Barrel-Stave Model**, AMPs aggregate to form multimers that insert perpendicularly into the membrane plane, with their helices aligned parallel to the lipid tails, creating transmembrane channels that lead to cytoplasmic leakage (92). Finally, in the **Carpet-Like Model**, AMPs align parallel to the cell membrane, with the hydrophobic end facing the phospholipid bilayer and the hydrophilic end facing the solution. In this model, the peptides cover the membrane surface like a carpet, destroying it in a detergent-like manner (93). A high concentration of AMPs is required for this mechanism to occur (67).

It should be noted that many AMPs, while interacting with the membrane, do not cause complete lysis but can penetrate the cell and exert additional intracellular effects, a phenomenon increasingly

recognized in recent studies (94). In fact, some of these peptides exhibit hybrid mechanisms of action, combining membrane disruption with intracellular targets depending on the microorganism (95).

1.3.3.2 Intracellular targeting mechanism

There is much evidence indicating a non-lytic mechanism of action for numerous AMPs, which act on specific bacterial intracellular molecular targets (**Figure 1.7**). Peptides can enter the cell thanks to their amphipathic nature, first interacting with the membrane surface and then inserting into it by forming transient pores; as these pores disintegrate, some peptides are translocated inside the membrane, which remains intact or only slightly compromised (96). A second entry mechanism is specific to proline-rich antimicrobial peptides (PrAMPs), which exploit bacterial membrane proteins such as SbmA or MdtM to efficiently enter some Gram-negative bacteria like *E. coli* (97–100). Cases have been reported of AMPs that can bind to and inhibit protein synthesis or nucleic acid DNA and/or RNA (96). More specifically, some AMPs can inhibit protein translation by binding to ribosomal subunits and blocking the mechanism of new protein synthesis from the initiation phase to the elongation phase (67). The most detailed results on this mechanism of action come from studies conducted on mammalian and insect PrAMPs, a class of peptides which will be discussed in a later specific section of the introduction of this PhD thesis. On the other hand, some antimicrobial peptides, exploiting the opposite charge of DNA and/or RNA, *in vitro* interact with these nucleic acids and inhibit their synthesis processes. Precisely because of this charge difference, sometimes the interaction between AMPs and nucleic acids may indicate a simple general electrostatic binding and not specifically related to the intracellular mechanism of action of the peptides (96). An example is the peptide buforin II, derived from the Asian toad *Bufo bufo gargarizans*, which binds to DNA after penetrating, without damage, the membrane of *E. coli* (101). In addition, indolicidin has been reported to strongly disrupt bacterial DNA replication (102), while microcin J25 (MccJ25) primarily targets and inhibits RNA polymerase (103). Furthermore, certain AMPs have been proposed to induce nucleic acid condensation (104). Notably, many antimicrobial peptides can exert multiple effects simultaneously, acting on several targets at the same time. These include the inhibition of cytokinesis, protease activity, inhibition of cell division, and inhibition of bacterial cell wall synthesis (67,96).

1.3.4 Advantages and disadvantages of AMPs as antimicrobial agents

Among the main advantages of antimicrobial peptides are: low molecular weight, high solubility, reduced cytotoxicity, good thermal stability, and a generally lower propensity for resistance development compared to conventional antibiotics (73). In fact, AMPs act primarily by interacting

with bacterial membranes, making them less likely to induce bacterial resistance, as it is difficult for bacteria to modify their membranes without compromising their viability. On the other hand, non-lytic AMPs interfere with essential cellular processes, making the emergence of resistance through genetic mutations highly unlikely, as such adaptations would demand an unsustainable metabolic cost for bacteria (105). These aspects make AMPs a valid alternative for combating infections caused by bacteria that are resistant to traditional drugs, substituting antibiotics or displaying more effectiveness by acting in combination with them; for example, many AMPs facilitate the uptake of antibiotics by bacteria, acting with a synergistic mechanism. (68,73).

Despite their promising antimicrobial properties, AMPs' clinical development as therapeutic agents is still hampered by several limitations, such as limited spectrum of activity, toxicity to eukaryotic cells, haemolytic effects, susceptibility to degradation by proteases in the blood or digestive system, and unfavourable pharmacokinetic characteristics. Specifically, the clinical use of most AMPs remains largely restricted to topical applications mainly due to their systemic toxicity, vulnerability to proteolytic degradation, and rapid elimination from the bloodstream via renal clearance. Oral administration is particularly problematic, as AMPs are broken down by digestive enzymes like trypsin and pepsin, while systemic administration results in a short half-life due to instability in serum and degradation by blood proteases, in addition to a non-negligible haemolytic activity (106–108). Commercial introduction of AMPs in medicine, for example to combat infections in surgical, dental, and ophthalmological fields or for wound care, is currently under development but, until 2020, only three of them had been approved by the Food and Drug Administration (FDA): colistin, daptomycin, and gramicidin (67).

In this context of ongoing challenges and limited clinical approvals, cathelicidins stand out as a particularly promising class of AMPs, showing promise for the development of new effective therapies.

1.4 Cathelicidins

1.4.1 General characteristics and structures

Cathelicidins are, together with alpha and beta defensins, the main family of antimicrobial peptides present in the defence systems of vertebrate animals. The cathelicidin family is estimated to be around 560 million years old and the most important distinguishing feature of these peptides, compared to other AMP families, is the presence of a large and well-conserved pro-region, called cathelin-like domain (CLD) linked to a variable C-terminal domain corresponding to the active antimicrobial

peptides. The common N-terminal pro-region of cathelicidins facilitates the exploration of an ever-increasing number of available vertebrate genomes fostering the identification of sequences for potentially new antimicrobial agents. From a mechanistic point of view, the CLD probably allows to keep the peptide domain inactive until it reaches the site of infection, thus preventing its premature degradation or sequestration. However, its precise role and the reason for its high conservation remain unknown and are still under investigation. Cathelicidin-derived AMPs are antimicrobial peptides derived from this proforms, becoming active only after their proteolytic release from the pro-region. Although the term “cathelicidins” refers to the proforms and not to the mature AMPs, it has become common also to refer to the bioactive peptides as cathelicidins (78).

Cathelicidins are expressed and secreted by cells active against infections, such as circulating immune cells or epithelial cells (109,110). Their expression can be stimulated by both exogenous and endogenous factors, such as vitamin D in primates. The genes encoding cathelicidins, located in chromosomal regions with conserved flanking genes, are composed of 4 exons (and 3 phase-0 introns): the first three encode the pro-region, while the fourth encodes the AMP domain; the fourth exon is preceded by a variable number of residues belonging to the pro-region and by an AMP cleavage site (**Figure 1.8**). After the removal of the signal sequence by a signal peptidase, the proform is stored in granulocytes or secreted by epithelial cells (78). Finally, the mature AMP is released by extracellular or granule serine proteases (depending on the organism and/or district) acting at specific cleavage sites: in other mammals, the operative protease is elastase (111), while in humans there are proteinase-3 and kallikrein (78).

Many genetic data and experimental results show that most vertebrate species possess genes encoding cathelicidins, while none have ever been reported in invertebrates. Artiodactyls are mammals that possess several cathelicidins while others, like primates, rodents, or other animals, possess only the ortholog of LL-37, the unique human cathelicidin (112). Cathelicidin peptides show many differences in size, sequence, chemical-physical properties, structure and biological activity: the only common feature among all them is the presence of the cathelin-like domain (78). They are classified into five different groups (**Figure 1.8**):

1. **Type A:** α -helical peptides (113,114) represent the largest group and are considered primarily membranolytic molecules (78); they will be described in the next paragraph of this PhD thesis.
2. **Type B:** small stapled β -harpins peptides with disulfide bridges (115,116) which are known to cross the membrane, assemble into oligomers, and create multimeric pore structures (78).
3. **Type-P:** proline-rich peptides (PrAMPs), with extended conformations (117), entering inside cells through inner membrane proteins and act on intracellular targets like ribosomes (78).

4. **Type-W**: linear peptides rich in tryptophan (118), with wedge-shaped conformations, entering inside the cytosol, without destroying membranes, and inhibiting the DNA transcription (78).
5. **Type-U**: intrinsically unstructured peptides which act on bacterial surfaces with a still not well-known mechanism of action (78).

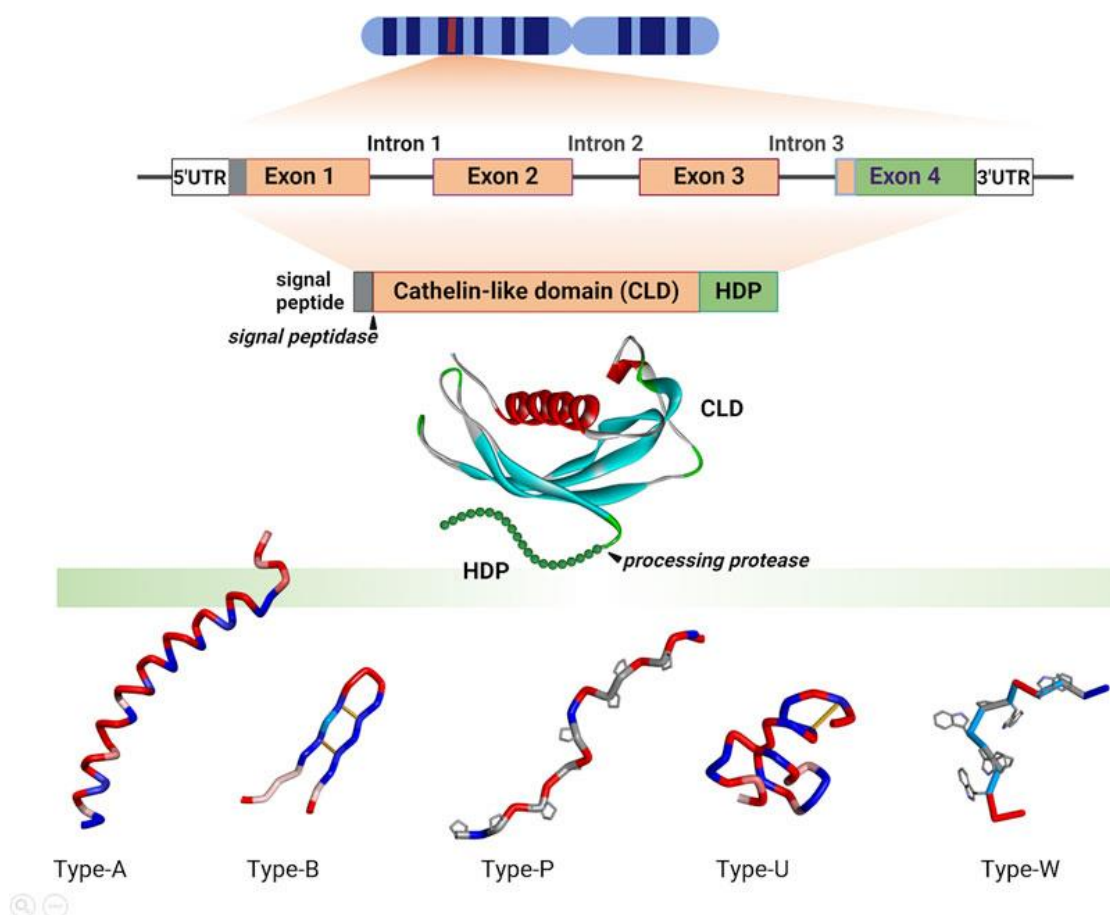


Figure 1.8 Cathelicidin genes localize in conserved chromosomal regions and are composed of four exons and three phase-0 introns. They are transcribed into proforms, containing the CLD, and then processed by signal peptidases. Finally, mature AMPs (HDP in the figure) are released by extracellular or granule proteases and assume different structures depending on species, including: α -helical (Type-A), β -hairpin (Type-B), Pro-rich (Type-P), Trp-rich (Type-U), or unstructured (Type-W) peptide forms. Image from Tossi et al., 2024 (78).

1.4.2 α -helical cathelicidins

Among cathelicidins, those with amphipathic α -helical conformations (Type-A) represent the most widespread and evolutionarily conserved subgroup, found across mammals, birds, amphibians, reptiles, and even primitive vertebrates such as hagfish. These α -cathelicidins typically adopt a

disordered structure in solution, transitioning to a helical conformation upon interaction with microbial membranes. Their amphipathic and cationic nature facilitates binding to negatively charged bacterial surfaces, where they primarily act through membranolytic mechanisms. Upon membrane association, when a critical concentration is reached, α -cathelicidins insert into the lipid bilayer and compromise membrane integrity either via a detergent-like carpet model mechanism or through the formation of toroidal pores (**Figure 1.7**). The preference for one mechanism over the other depends partly on their ability to oligomerize: for instance, human LL-37 oligomerizes and promotes pore formation, whereas the closely related rhesus monkey RL-37 remains monomeric and predominantly employs the carpet mechanism (78).

Notable α -helical cathelicidins include LL-37 (human), BMAP-27 (cattle), Cap18 (rabbit), Fowlicidins 1–3 (chicken), Crotalicidin (snake), and CathMH (frog), all of which exhibit significant structural and functional conservation. This cross-species convergence suggests a shared ancestral origin and highlights the functional advantages of the α -helical fold in innate immune defense (78). Particularly, among Type-A peptides, considerable attention has been given to LL-37, which is the only cathelicidin-derived antimicrobial peptide identified in humans. It is a 37-residue peptide with broad-spectrum antimicrobial activity against bacteria, fungi, and viruses. LL-37 is expressed by epithelial cells (skin, respiratory tract, gastrointestinal tract, testis) and immune cells such as neutrophils, monocytes, T cells, B cells, and natural killer cells (119). LL-37 has been studied both as a therapeutic agent and a biomarker for various diseases, with clinical trials showing that its topical use is generally safe and well tolerated (78). However, despite its potent antimicrobial effects and favorable tolerability profile under clinical conditions, LL-37, like many α -cathelicidins, displays toxicity toward eukaryotic cells at bactericidal or higher concentrations. This aspect, commonly assessed through their haemolytic activity, narrows their therapeutic window. To overcome this key critical issues, significant efforts have been made to develop modified Type-A cathelicidins fragments and analogues that preserve or enhance antimicrobial potency, while minimizing toxicity to host cells and improving resistance to proteolytic degradation in bodily fluids. For instance, selective truncations or amino acid substitutions in LL-37 enhance antibacterial activity without substantially altering its overall charge or amphipathic character. Central fragments such as LL-37 (7–27) or LL-37 (5–24) maintained antimicrobial effects against both Gram-positive and Gram-negative bacteria, alongside reduced haemolytic activity compared to the full-length peptide (78,119).

These optimization strategies, supported by the conserved α -helical structure, broad-spectrum antimicrobial activity, and additional immunomodulatory properties of α -cathelicidins, continue to

sustain strong interest in their therapeutic potential. In this context, the porcine α -cathelicidin PMAP-36 has emerged as a valuable model for the design of new therapeutic peptides.

1.4.2.1 The porcine PMAP-36 cathelicidin

PMAP-36 (GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG-NH₂) is a 36-amino-acid long peptide derived from a porcine myeloid cathelicidin. This peptide adopts an amphipathic α -helical structure in membrane-mimicking environments, which makes it suitable for acting through bacterial membrane interaction and permeabilization. PMAP-36 exists as a homodimer, formed by an intermolecular disulfide bridge involving the cysteine at position 35, and shows a high net positive charge with 36% of its residues being cationic amino acids (120–122). Specifically, PMAP-36 consists of a highly basic N-terminal region (residues 1 to 22), responsible for the amphipathic α -helical structure, which is interrupted by the subsequent hydrophobic tail rich in proline residues (Pro25, Pro26, and Pro32). This suggests that PMAP-36 active region is its N-terminal α -helical domain, despite the C-terminal hydrophobic tail seems to be important for a broad spectrum of activity and a higher potency (122). Despite its antimicrobial effectiveness against Gram-positive and Gram-negative bacteria, PMAP-36 also exhibits moderate haemolytic activity against murine, human, and porcine erythrocytes (120–122). Moreover, PMAP-36 exhibits significant cytotoxicity toward murine and porcine macrophage-like cells at high concentrations. This cytotoxicity is likely due to the peptide's positive charge and amphipathic nature, which confer selectivity toward bacterial membranes but can also cause interaction with mammalian cell membranes at high doses (123).

Over the years, studies have been conducted on the activity and structure of PMAP-36 with the aim of improving its antibacterial activity and reducing its cytotoxicity. It has been shown that dimerization does not affect these aspects: both the monomeric and dimeric forms of the peptide permeabilize bacterial membranes, causing rapid killing of the pathogens (122,123). Furthermore, the efficiency and mechanism of action of PMAP-36 were not altered following the truncation of 12 amino acid residues from its C-terminal tail: the N-terminal segment 1-24 exhibited strong antimicrobial activity and reduced haemolytic activity (124). However, further shortening the peptide by 4 more C-terminal amino acids to obtain the fragment 1-20, reduced antimicrobial activity against a significant number of bacteria (122). Removing the first 6 amino acids from the N-terminus of PMAP-36 yields a 7-24 fragment that retains the mechanism of action and the antibacterial activity of the original peptide, even exhibiting a better selective toxicity towards bacteria over host cells (125). Finally, in a further study, the N-terminal end of PMAP-36 was repeatedly truncated, showing that: removing up to 11 N-terminal residues of the peptide results in only a small impact on

antimicrobial activity against *E. coli*; however, removing an additional 4 residues, the 16-36 fragment shows a significant reduction in antibacterial effectiveness (123). All these studies highlight that the pharmacophore of PMAP-36 lies in its central region and encourage the development and investigation of cathelicidin-derived antimicrobial peptides as novel drugs against antibiotic-resistant pathogens.

A similar potential is observed in Proline-rich Antimicrobial Peptides (PrAMPs), another subclass of cathelicidins (Type-P) with strong potential in the fight against AMR.

1.4.3 Proline-rich antimicrobial peptides (PrAMPs) of cathelicidin origin

Among mammalian cathelicidins, a distinct group is represented by proline-rich antimicrobial peptides (PrAMPs). All the known mammalian PrAMPs belong to the cathelicidin family, and the first to be discovered were the bovine peptides bactenecins 5 and 7 (Bac5 and Bac7), from bovine neutrophils (126). However, while several PrAMPs derive from cathelicidin precursors in mammals, members of this peptide group have also been identified across a wide range of organisms, including insects, amphibians, crustaceans, and molluscs. This broader distribution highlights the evolutionary conservation and functional relevance of this unique antimicrobial compounds (87).

1.4.3.1 General characteristics defining PrAMPs

PrAMPs are characterized by a high proline content (87). The minimum proline content required to classify an antimicrobial peptide as proline-rich has been set at least 25% of its sequence. Using this threshold, approximately one hundred AMPs can be considered PrAMPs, with an average of proline content of 34%, reaching up to 49% (127). The primary sequences of PrAMPs, including mammalian ones, are characterized by repeated tetrameric motifs such as PPRX or PRPX, where X is usually a bulky hydrophobic residue or a glycine (87). The PRP motif (Pro-Arg-Pro) is involved in binding to DnaK and/or the 70S ribosome (as explained later), but it is not sufficient on its own to define a peptide as a PrAMP. In fact, although it may serve as a strong indicator of binding to these molecular targets, many other residues also contribute to the interaction of PrAMPs with DnaK or the ribosome (127,128).

All discovered PrAMPs (excluding BnPRP, the only one isolated from plants) are cationic sequences, with an average net charge of +5, with values reaching up to +11. However, since cationicity is a feature of most AMPs (80.4%), including those with lytic mechanisms, this element alone is not sufficient to distinguish PrAMPs from general AMPs. This positive charge facilitate interaction with

the bacterial membrane or cell wall and, in most PrAMPs, is provided by at least three arginine residues. However, given that some PrAMPs contain a low percentage of arginine (as low as 6%), this amino acid cannot be considered a defining factor in identifying a PrAMP, even though it represents 19% of the sequence on average (127).

Given that PrAMPs act on intracellular targets, their size is a relevant factor influencing membrane permeability. The average length of PrAMPs is approximately 25 amino acids, with a range spanning from 15 to 60 residues. However, peptide length should only be considered an indicative feature of potential PrAMP membership, rather than a strict defining criterion (127).

Finally, many PrAMPs share the ability to interact with intracellular molecular targets, most commonly the bacterial 70S ribosome and/or the DnaK chaperone (127). However, this mode of action represents a characteristic functional trend rather than a requirement for defining a peptide as a PrAMP. This distinctive mode of action will be further explored in the following paragraph.

1.4.3.2 PrAMPs uptake and mode of action

Proline residues, abundant in mammalian PrAMPs belonging to the cathelicidin family, are structurally incompatible with α -helical or β -sheet conformations, leading instead to an extended structure. This feature underlies the mainly non-lytic mechanism of action of mammalian PrAMPs, which does not involve membrane permeabilization. Studies on these cathelicidin-derived PrAMPs clearly show that they are internalized into bacterial cells and act by interacting with specific intracellular targets (87). While similar mode of action has also been identified in invertebrates PrAMPs, this section focuses deeper on proline-rich cathelicidins.

As shown in **Figure 1.9**, proline-rich cathelicidins are able to enter bacterial cells by exploiting the inner membrane proteins of Gram-negative bacteria, such as SbmA and YgdD, as well as MdtM, which plays a role particularly at high concentrations of PrAMPs (87,128,129).

Specifically, SbmA is a 406-residue protein first identified in *E. coli*, that is present in various *Enterobacteriaceae* and in other gram-negative bacteria, such as *A. baumannii*. Despite its distribution, the precise physiological substrates of SbmA remain unknown. This inner membrane transporter facilitates the import of proline-rich cathelicidins into the bacterial cytosol by utilizing in *E. coli* the electrochemical gradient across the inner membrane (99,130). The presence of SbmA correlates strongly with the antimicrobial efficacy of many PrAMPs. Bacterial mutants lacking functional SbmA exhibit reduced susceptibility to proline-rich cathelicidins, such as Bac5 and Bac7, but not to lytic antimicrobial peptides (97). A clear example of this is *P. aeruginosa*, which does not

possess SbmA and is therefore less responsive to PrAMPs treatment due to its inability to internalize these peptides, thereby preventing them from reaching their intracellular targets (131). Although internalization via SbmA is generally required for proline-rich cathelicidins to exert their primary antimicrobial activity, these peptides can also function through additional mechanisms. At elevated concentrations, well above their MICs, some PrAMPs may permeabilize the bacterial membrane directly, without needing to enter the cell. The concentration at which this shift in activity occurs varies depending on both the specific proline-rich cathelicidins and the bacterial species being targeted (132).

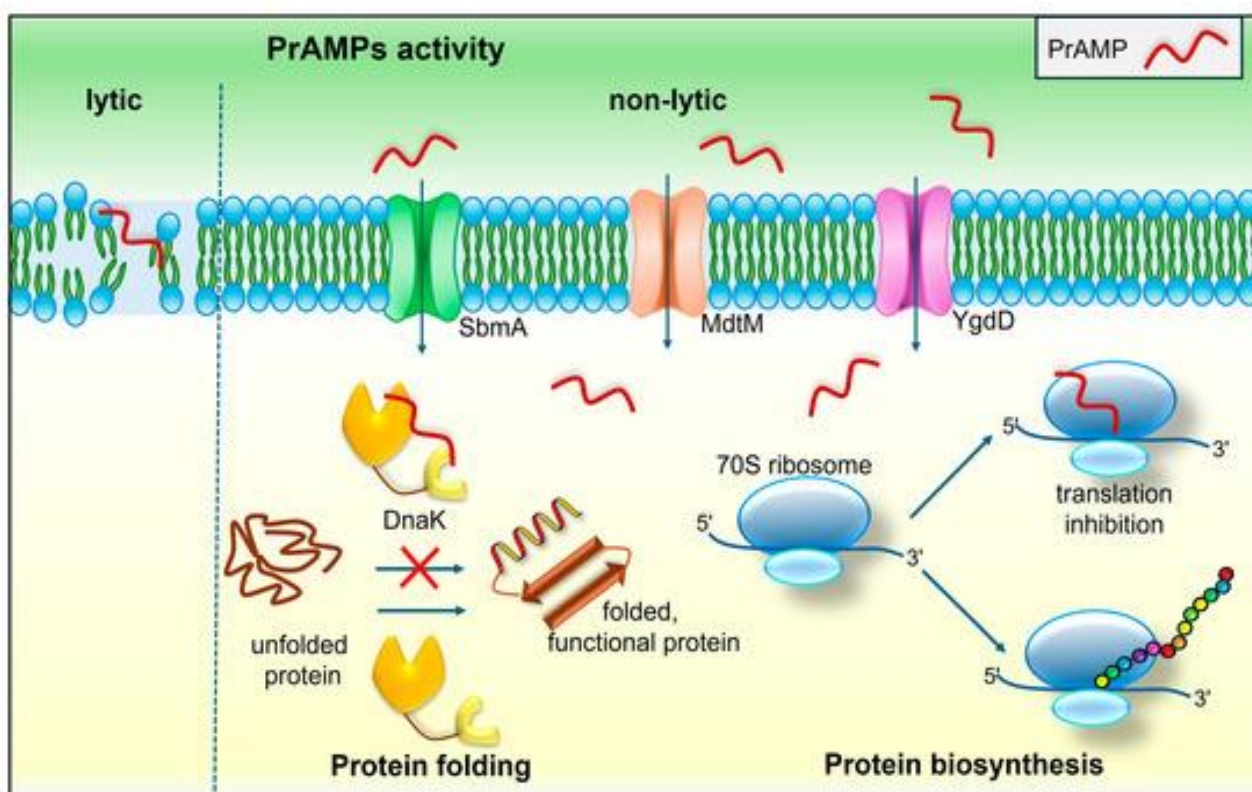


Figure 1.9 Model of the modes of action of PrAMPs. PrAMPs (right) enter bacterial cells through inner membrane transporters such as SbmA, MdtM, and/or YgdD. Inside the bacterial cell, they interact with 70S ribosomes and/or DnaK chaperone proteins, leading to the inhibition of protein production and/or misfolding of polypeptides. In contrast, lytic peptides (left) act by disrupting the bacterial membrane, causing structural damage that ultimately results in cell death. Image from Stączek et al., 2024 (133).

Regarding the mechanism of action of PrAMPs, including those from invertebrates, two distinct modes have been described once these peptides enter bacterial cells (as shown in **Figure 1.9**). Firstly, Otvos et al. (134) identified in 2000 that certain PrAMPs can interact with the heat shock protein 70 (Hsp70), known as DnaK in bacteria, and alter its function, thereby impairing the correct protein

folding process (135). Secondly, Hoffmann's group and Scocchi's group (136,137) identified, in 2014, a new different mechanism of action by which PrAMPs inhibit protein translation by interacting with the 70S ribosome. They discovered that PrAMPs, once in the cytosol, bind the ribosomes at level of the nascent polypeptide exit tunnel (NPET) blocking the first elongation step of translation (**type I PrAMPs**, e.g. Bac7 and TurlA), or act as inhibitors of the termination step of protein synthesis (**type II**, e.g. apidaecin 1b and drosocin) (**Figure 1.10**). The interaction between PrAMPs and the ribosome is stabilized by numerous hydrogen bonds and non-covalent interactions between the peptide backbone and the nucleotides of the 23S rRNA (138). Particularly, the N-terminal region is essential for antimicrobial activity, because it extends into the A-site, blocking tRNA accommodation and halting peptide elongation (138,139). Therefore, PrAMPs enable the formation of the 70S initiation complex but, despite the initiation of translation occurs, further rounds are prevented because other tRNAs cannot be accommodated in the A-site (138).

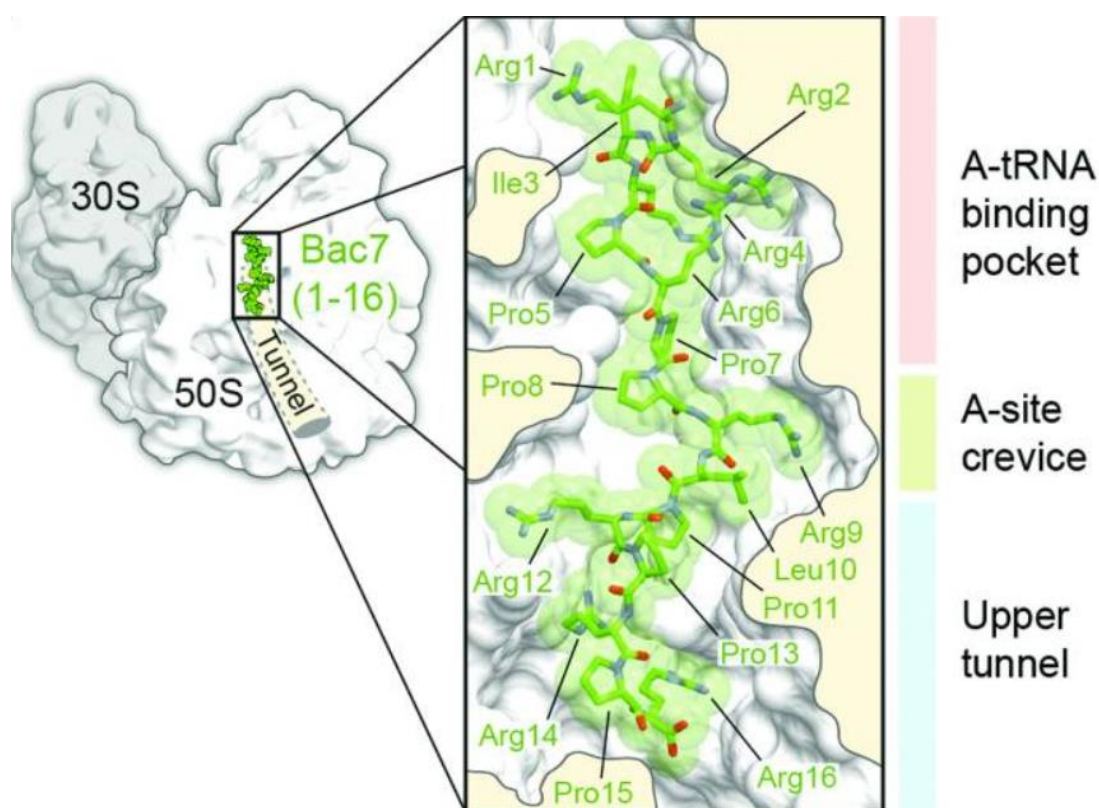


Figure 1.10 Structure and binding site of the Bac7-derived PrAMP Bac7(1–16) within the ribosomal exit tunnel. Image adapted from Seefeldt et al., 2016 (138).

Through this mechanism of action, proline-rich cathelicidins exhibit strong antibacterial activity *in vitro* and good potency *in vivo*, being selectively active against Gram-negative bacteria, particularly

against *Enterobacteriaceae*, already at low micromolar concentrations. However, they do not affect Gram-positive microorganisms at the same concentrations, therefore showing a narrower spectrum of action compared to membranolytic AMPs (87). Furthermore, PrAMPs are biocompatible with eukaryotic cells, generally showing low cytotoxicity or side effects in animal models even at concentrations higher than those required for antimicrobial activity (140,141). To date, various strategies have been developed to improve proline-rich cathelicidins, including the use of large-scale peptide libraries, such as those that led to the discovery of derivatives bactenecin 5 and 7 fragments with an enhanced spectrum of activity (142,143). Additionally, some PrAMPs have also been shown to cross the blood-brain barrier, increasing their relevance for future therapeutic development (144). Furthermore, modifications aim to enhance their efficacy and reduce proteolytic degradation, thereby improving their stability and activity in biological fluids for medical applications. All these aspects make PrAMPs, and particularly mammalian ones, suitable and promising for the development of new antimicrobial agents (87).

The next sections will focus on selected proline-rich cathelicidins from bovine and cetacean origin, which stand out for their potent activity and favourable properties, making them attractive candidates for therapeutic application and rational peptide design against the over-growing phenomena of AMR.

1.4.3.3 The bovine PrAMP Bac7 and its fragments

Bac7 is a natural 60-residue PrAMP isolated from the large granules of bovine (*Bos Taurus*) neutrophils. Its 23.5 kDa pre-proform, produced by bovine bone-marrow cells, is processed into a 20 kDa preform, stored in the large granules of neutrophils until infections, and released, through elastase's cleavage, as the mature 7 kDa Bac7 (145). Fragments of this cathelicidin are known to interact with LPS (146) and to translocate across the bacterial membranes blocking the intracellular protein synthesis. The capability of Bac7 fragments to inhibit translation on both bacterial and mammalian ribosomes likely explains why this peptide is stored as an inactive pro-peptide (138). This PrAMPs is very effective in killing *E. coli* and *K. pneumoniae* and in blocking *E. cloacae* growth at micromolar concentration, but without affecting the Gram-positive pathogen *S. aureus* (126). As visible in **Table 1.2**, Bac7 sequence is structured in an arginine-rich cationic N-terminal region followed by a three repetitions of 14 hydrophobic residues (132). As a PrAMP, its sequence includes 11 motifs PRPX, making Bac7 very rich in arginine, giving it a net positive charge of +17, and in proline, forcing the peptide to acquire a linear structure or at least impair in α -helix and β -sheet structures (127).

Benincasa et al. (147), showed that the cationic N-terminal region of Bac7 was sufficient to kill bacteria, with a minimum length required of 16 amino acids. More specifically, its first 35 and 16 N-terminal residues (**Table 1.2**) provided two PrAMPs, called respectively Bac7(1-35) and Bac7(1-16), which display antimicrobial activity comparable to that of the native cathelicidin Bac7, although Bac7(1-16) is slightly less effective. Specifically, both *E. coli* and *K. pneumoniae* resulted highly susceptible, already at micromolar concentrations ($\leq 1 \mu\text{M}$), to the action of Bac7(1-35) and, with a minor extent, even of Bac7(1-16). The two fragments were found also active, at low micromolar concentrations, against other Gram-negative ESKAPE species such as *A. baumannii* and *P. aeruginosa* (147). Finally, Bac7(1-35) was able to protect mice against *Salmonella typhimurium* infection, reducing their mortality and showing a promising *in vivo* result for future medical applications (148).

Table 1.2 Sequences of the PrAMP Bac7(1-60) and its shorter derivatives Bac7(1-16) and Bac7(1-35). Table adapted from Benincasa et al., 2004 (147).

Peptide	Sequence
Bac7	RRIRPRPPRLPRPR(PRPLPFPRPGPRPI) ₃ PRPL
Bac7(1-16)	RRIRPRPPRLPRPR PR
Bac7(1-35)	RRIRPRPPRLPRPR(PRPLPFPRPGPRPI) ₁ PRPLPFP

Regarding their mechanism of action, Bac7(1-35) is actively transported into *E. coli* cells through the inner membrane protein SbmA (97) and it was the first mammalian PrAMP shown to exert its antimicrobial effect by specifically inhibiting protein synthesis. Particularly, Mardirossian et al., in 2014, revealed that Bac7(1-35) can accumulate to up to 340 μM inside the cytoplasm of *E. coli* and that, despite these high levels, the peptide effectively and specifically blocks *in vitro* translation at micromolar concentrations already. Furthermore, incorporation experiments using radioactive precursors in living *E. coli* indicated that Bac7(1-35) selectively inhibits protein synthesis. Finally, ribosome co-precipitation and crosslinking assays demonstrated that the peptide binds to ribosomes, interacting with a specific subset of ribosomal proteins (137). Seefeldt et al., in 2016, presented crystal structures of the *Thermus thermophilus* 70S ribosome bound to Bac7(1-16) (**Figure 1.10**), showing that the peptide binds to a ribosomal site allowing the formation of the initiation complex but blocking

the transition to the elongation phase of translation. In line with this, Bac7(1-16) and Bac7(1-35) strongly compete with antibiotics like erythromycin, which bind the ribosomal exit tunnel (138).

1.4.3.4 The cetacean PrAMPs Tur1A and Lip1

Since mammalian PrAMPs have far been identified primarily in artiodactyls and given the close evolutionary relationship between artiodactyls and cetaceans, the genome of the bottlenose dolphin (*Tursiops truncatus*) was investigated, leading to the discovery of the PrAMP Tur1A (Table 1.3).

This peptide, which is the orthologue of bovine PrAMP Bac7, on *Escherichia coli* acts without compromising membrane integrity. The inner membrane transporters SbmA still plays a role in its uptake into the bacterial cell, although the absence of the protein affects the activity of Tur1A less evidently than that of Bac7 fragments. Similar to Bac7, Tur1A inhibits bacterial translation by binding to the ribosome and preventing the transition from initiation to elongation (149). Furthermore, in 2020, Sola et al. identified and characterized five additional peptides from different cetacean species (cePrAMPs), including Lip1 (Table 1.3), which shares high sequence similarity with Bac7(1-35) (150).

Table 1.3 Sequences and physical-chemical properties of cetacean PrAMPs (cePrAMPs) Tur1A and Lip1, identified by genome mining and compared to the active N-terminal fragment of the bovine PrAMP Bac7(1-35). Table adapted from Sola et al., 2020 (150).

Species	Peptide	Sequence ^a
<i>Bos taurus</i>	Bac7(1-35)	<u>RRIRPRPPRLPRPRRPL</u> PFPRGPRPIPRPLPFP
<i>Tursiops truncatus</i>	Tur1A	RRIRFRPPYLPRGRRPRFPFPFIPIRIPRIPI
<i>Lipotes vexillifer</i>	Lip1	<u>RRIRIRPPRLPRPRRPWF</u> PPRFPIPIRIPGKR

^a Residues conserved in >50% of sequences are shaded grey and a highly identical N-terminal stretch between Lip1 and Bac7(1-35) is underlined.

Despite Tur1A and Lip1 display a conserved N-terminal region typical of Bac7, being similar to Bac7(1-35) in inhibiting protein synthesis without disrupting membranes, variations in sequence, activity, and mode of action were observed. Interestingly, unlike Bac7(1-35), whose activity depends on the SbmA transporter, Tur1A and Lip1 retain antimicrobial efficacy even in its absence; this suggests an alternative mode of internalization and highlights a reduced susceptibility to resistance

mechanisms involving loss of SbmA. In addition, Lip1 displayed a particularly wide spectrum of activity, including ESKAPE pathogens such as *P. aeruginosa* and *E. faecium*, with minimal inhibitory concentrations (MICs) $\leq 4 \mu\text{M}$, and moderate activity even against the PrAMP-resistant *S. aureus*. However, although Lip1 shows limited membrane permeabilizing activity, it still exhibited some cytotoxicity toward human MEC-1 cells. Despite this, Lip1 and TurlA represent a diverse and promising group of cetacean-derived PrAMPs. Their relatively high sequence identity with Bac7 at the N-terminus, (up to 95% in the first 17 residues of Lip1) suggested that small sequence variations, particularly in the C-terminal region, could significantly broaden antibacterial activity and confer independence from the SbmA transporter (150).

1.4.3.5 The optimized mammalian PrAMP B7-005

To enhance the antimicrobial activity of PrAMPs starting from the shortest fragment showing full activity, the previous described Bac7(1-16) underwent systematic sequence modifications (143), leading to the synthesis of 133 new derivatives aimed at identifying key residues involved in ribosome inactivation and antimicrobial function. From this screening, five promising Bac7(1–16) variants were selected and assessed through antibacterial and membrane permeabilization assays, X-ray crystallography, and molecular dynamics simulations. Among them, B7-005 (**Table 1.4**) emerged as the most effective optimized candidate. This 16-residue synthetic PrAMP showed a broader antimicrobial spectrum than its native PrAMPs Bac7(1-35) and Bac7(1-16), proving active at micromolar concentrations (from 2 μM to 32 μM) against all ESKAPE pathogens, including Gram-positive *S. aureus* (143,151). Furthermore, B7-005 maintained its efficacy even in the presence of human serum (152).

Table 1.4 Sequence of the PrAMP B7-005. Table adapted from Di Stasi et al., 2025 (151).

Peptide	Sequence
B7-005	WRIRRRWPRLPRPWR

B7-005 exhibited bactericidal activity against both planktonic forms and biofilms of ESKAPE pathogens and *E. coli* at micromolar concentrations (151). Importantly, its antimicrobial action occurred at concentrations well below than those causing cytotoxic effects on four mammalian cell types, suggesting a safe profile for possible human application (152). The broader spectrum of B7-005 is attributed to its increased hydrophobicity with respect to Bac7(1-16) due to the presence of

three tryptophan residues (**Table 1.4**), enhancing bacterial membrane affinity and allowing it to exert mild membrane-destabilizing effects in *E. coli* cells while still inhibiting protein synthesis. This property enables B7-005 to enter bacterial cells largely independently of the SbmA transporter used by native PrAMPs, suggesting a novel bacterial uptake mechanism (143). Its reduced dependence on active transport limited the development of resistance in *E. coli*, as transporter mutations or its loss have minimal impact in decreasing the bacterial sensitivity to this peptide (143,152).

In addition, *in vivo*, B7-005 was well tolerated in a zebrafish embryo model and effectively reduced mortality of larvae pre infected with *E. coli* in a model of bacteraemia (152). However, its mechanism of action is flexible and varies depending on the target microorganism. While it consistently inhibits protein synthesis across all ESKAPE pathogens and *E. coli*, the degree of membrane permeabilization differs among species, from the absence of membrane damage to varying levels of membrane destabilization. Therefore, for many pathogens, both ribosome inhibition and membrane disruption may contribute to its antibacterial activity, suggesting a combined lytic and non-lytic mode of action. Moreover, biochemical studies of its impact on protein synthesis revealed that B7-005, while gaining membrane-disruptive features, continues to block ribosome progression into the elongation phase, in line with type I PrAMPs (151). Thus, B7-005 retains the essential features of native PrAMPs while offering a broadened spectrum and potency, making it a promising lead for antibiotic development. Anyhow, it was still unclear if B7-005 shared some limitations affecting truncated PrAMPs, which, despite showing notable antimicrobial activity, are generally less potent than their full-length native versions. This suggests that extending this already optimized shorter peptide could further enhance its effectiveness.

In addition, for any molecule intended for antibiotic development, it is essential to gain a clear understanding of its antimicrobial mode of action in living bacteria. The following sections of this thesis will therefore focus on the limitations of current methodologies for studying peptide mechanisms of action, and on possible new strategies to address these challenges.

1.5 Methods and limits in studying antimicrobial mechanism of action

Antimicrobial peptides affect bacteria through multiple mechanisms: while many act by damaging bacterial membranes, an increasing number are known to interfere with intracellular targets, including PrAMPs. The study of the intracellular mechanism of action of AMPs has been conducted primarily through functional (136,137,153) and structural (154–158) *in vitro* as well as *in vivo* studies

(137,159,160). Specifically, insights into the mechanism of action of PrAMPs have been primarily gained employing a wide range of methodologies such as transcription and coupled transcription–translation systems, assays for detecting protein–protein and protein–ribosome interactions, co-sedimentation techniques, protein purification, structural and crystallographic studies, molecular biology tools, and computational modeling (137,160). However, relying solely on *in vitro* systems reduces the biological relevance of the models, making it difficult to capture key cellular processes and interactions that naturally occur within living organisms.

A deeper understanding of the functional dynamics of these peptides therefore requires *in vivo* investigations, particularly in bacterial cultures. From this point of view, the antibacterial mechanism of PrAMPs has been described in living *E. coli* cells by incorporating radioactive amino acids or nucleosides to detect blockages of the major bacterial biosynthetic pathways (137). However, these studies have been mainly performed on *E. coli* (157,160), despite recent studies highlighting that the mechanism of action of AMPs, including PrAMPs, can vary from one pathogen to another (95,161). Moreover, the complexity and problematics of existing techniques often limits their application (including legal and environmental concerns in using isotopes), hindering the study of antimicrobial peptide mechanisms and slowing down the characterization of new candidates. To address these challenges, innovative approaches are needed. A promising strategy explored in this thesis is the BONCAT method, which will be introduced in the following section.

1.6 Bioorthogonal noncanonical amino acid tagging (BONCAT)

Bioorthogonal noncanonical amino acid tagging (BONCAT) is a novel click chemistry approach for fluorescently tracking protein synthesis in individual microorganisms within environmental samples. This technique involves the *in vivo* incorporation of amino acid analogues to visualize protein production in natural prokaryotic communities. Specifically, microbial communities are incubated with methionine analogues, containing azide or alkyne groups, which are incorporated by bacteria in newly synthesized proteins. After incorporation, these methionine surrogates are subsequently labelled with fluorophores bearing complementary azide or alkyne groups through a copper(I)-catalyzed azide-alkyne cycloaddition (click reaction). This fluorescently tagging process enables the detection of translationally active cells. BONCAT has been validated across a variety of archaeal and bacterial cultures and enrichments, and applied to complex environmental samples including oral biofilms, freshwater, and anoxic sediments (162–165). However, this technique has never been applied to study AMPs.

As summarized before, BONCAT relies on the substitution of natural methionine with non-canonical amino acids which are incorporated into proteins during translation. The field is still open to innovation, and it has still to be explored in order to optimize protocols for targeted applications. Several bioorthogonal amino acids containing azide or alkyne groups effectively compete with native amino acids during translation (162). This is possible due to the relaxed specificity of methionyl-tRNA synthetase, the enzyme that attaches methionine to its tRNA but can also misrecognize these analogues (166). However, only a limited number of these analogues are efficiently incorporated without requiring genetic modification of the host (167). Among these, L-azidohomoalanine (AHA) and L-homopropargylglycine (HPG), both showed in **Figure 1.11**, show the highest translational activity, with activation rates approximately 390 and 500 times lower than methionine, respectively, in *E. coli*, compared to norleucine, which has an activation rate 1050 times lower (166). The azide or alkyne functional group in AHA or HPG allows selective detection of proteins synthesized during incubation with these analogues, through the copper(I)-catalyzed azide-alkyne cycloaddition with labelled fluorophores (164). This click reaction is particularly suited for biomolecule detection due to its rapid kinetics, high chemo- and regioselectivity, formation of a single product, and excellent yields (168,169).

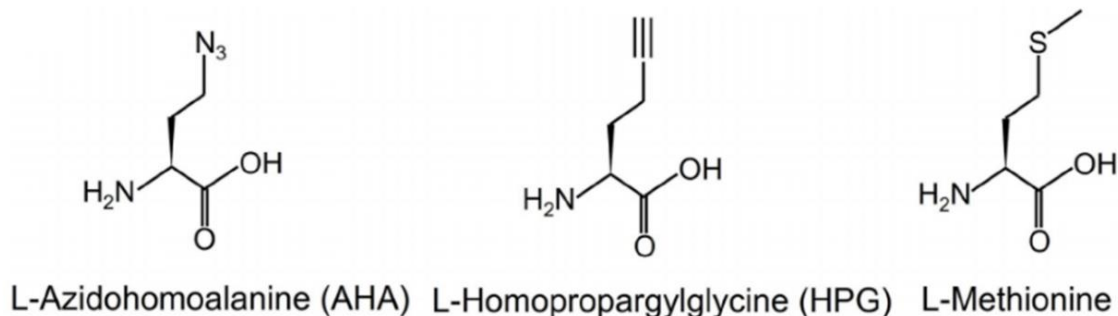


Figure 1.11 Structures of the methionine (Met) analogues L-azidohomoalanine (AHA) and L-homopropargylglycine (HPG), compared to that of the natural amino acid L-methionine. Image adapted from Kalesh e Denny, 2019 (170).

The click reaction is easy to implement and can be performed in aqueous or other solvent conditions. For the current PhD project copper sulfate (CuSO_4) served as the catalyst for the click reaction, enabling fluorescent detection of newly synthesized proteins. Its catalytic activity depended on maintaining copper in the Cu(I) oxidation state, which was achieved by adding a reducing agent like sodium ascorbate. To stabilize Cu(I) and prevent its rapid oxidation, the copper chelator THPTA was used in large excess. Finally, aminoguanidine was used to minimize protein cross-linking and

precipitation during the reaction (165). For completeness' sake, two types of azide-alkyne click reactions exist (**Figure 1.12**):

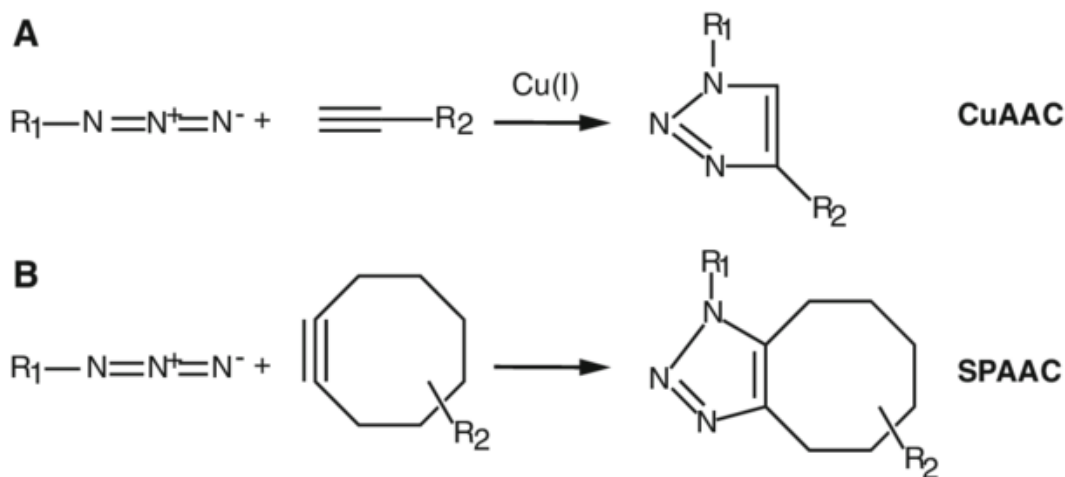


Figure 1.12 Two different azide-alkyne cycloaddition reactions. **(A)** CuAAC: azide-alkyne cycloaddition catalyzed by Copper(I), **(B)** SPAAC: cycloaddition of azides and strain-promoted alkynes (cyclooctynes). Image adapted from Saleh et al., 2019 (171).

(A) a Cu(I)-catalyzed cycloaddition (CuAAC) between an azide and a terminal alkyne (172–174) just described before; and **(B)** a strain-promoted variant using a reactive strained cyclooctyne (SPAAC), which proceeds without copper catalyst (175,176). Despite the first one was used in this PhD thesis, both enable selective conjugation of azides and alkynes within complex biological and chemical environments (162).

Regarding bacterial applications, BONCAT has been successfully used to monitor activity in about 30 cultured and uncultured prokaryote phyla (177), indicating broad applicability across diverse microorganisms. The method has also been applied to prokaryote communities from freshwater, freshwater sediments, marine systems, and deep-sea sediments (162,165,178–180). Furthermore, BONCAT tracked protein synthesis during viral infections in bacteria and marine phytoplankton, including *E. coli*/T7, *Synechococcus* sp. WH8101/Syn1, and *Emiliana huxleyi*/EhV207 (181). This approach has also been used to study soil microbial activity (182) and prokaryote responses to starvation in bathypelagic zones (183). Finally, BONCAT has been widely utilized in eukaryotic cells (164) and bacteria to study pathogenesis and virulence mechanisms (184), quorum sensing (185), and antibiotic responses (186). Furthermore, BONCAT has been successfully combined with flow cytometry (FCM), demonstrating that this approach enables fluorescence-based visualization of cells actively synthesizing proteins, specifically in marine prokaryotes. The method, graphically

summarized in **Figure 1.13**, was optimized on *E. coli* monocultures before applying it to natural marine samples (163).

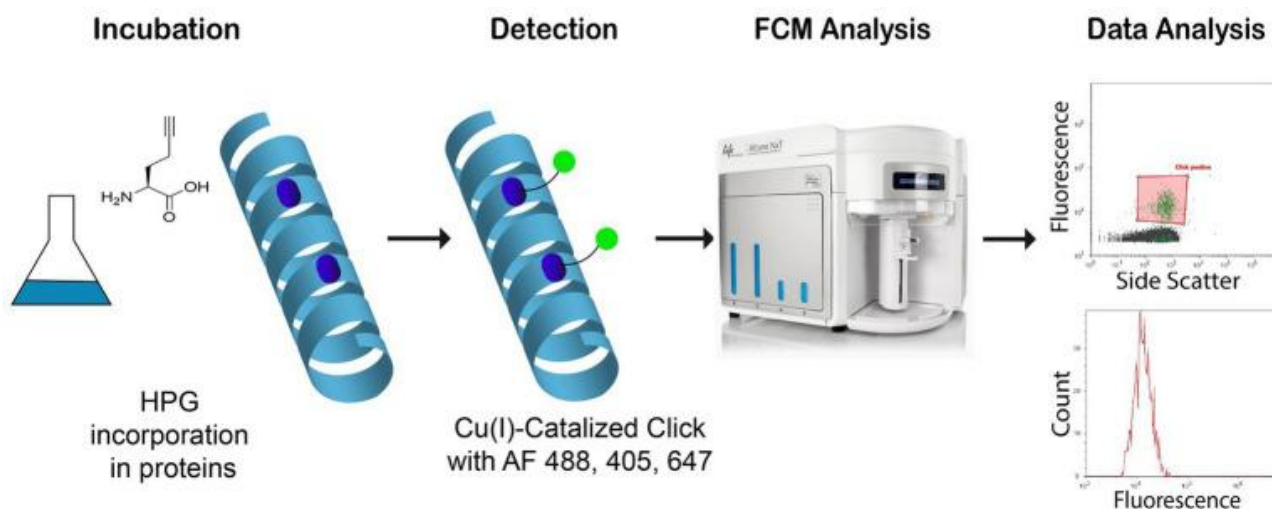


Figure 1.13 Graphical summary of BONCAT method to study newly synthesized proteins after L-homopropargylglycine (HPG) incorporation into new bacterial proteins. Samples are analysed by flow cytometry, after labelled by click chemistry with Alexa Fluor®, azide 488, 405, and 647.

Image from Lindivat et al., 2020 (163).

Additionally, BONCAT-FCM can detect, quantify, and differentiate bacterial cells following physical water treatments like UV irradiation and heating, offering reliable vitality assessments of single cells. One major advantage of BONCAT is its analysis of all newly synthesized proteins within cells, compared to assays focused on specific enzymes (187). Therefore, the combined BONCAT-FCM method was found to be fast, reproducible, and capable of processing large sample numbers, with positive results.

2. AIM OF THE THESIS

Antimicrobial peptides (AMPs) are considered potential alternative or supportive compounds to antibiotics to contrast the ever-growing problem of antibiotic resistance (AMR), particularly against ESKAPE pathogens. Among AMPs, the family of cathelicidins is one of the best studied, including the subgroups of α -helical cathelicidin-derived peptides, acting by damaging bacterial membranes, and the Proline-rich Antimicrobial Peptides (PrAMPs), mainly inhibiting bacterial protein synthesis. Despite their promising antimicrobial efficacy, these molecules present significant limitations that hinder their clinical development as therapeutic agents. These issues include, in some cases, a narrow spectrum of action, low biocompatibility towards eukaryotic cells, and susceptibility to blood and/or digestive tract proteases, which reduce their stability. Additionally, current methodologies used to investigate *in vivo* the intracellular mechanisms of AMPs, especially PrAMPs, are often technically demanding, frequently rely on radioactive labelling, and typically restricted to *E. coli* as the model organism. On the other hand, a deep characterization of the molecular mechanism of action is mandatory for any compounds to be proposed as lead compound to develop new drugs.

Considering these limitations, the overall **aim** of my PhD research was to address some of the key barriers impeding the clinical translation of cathelicidins. Specifically, my work focused on reducing their cytotoxicity, enhancing their resistance to proteolytic enzymes, and expanding their antimicrobial spectrum. A further goal was to achieve a more comprehensive understanding of their antibacterial mechanism of action, which is essential for therapeutic optimization.

This thesis is structured in three different research projects, each addressing a different challenge in the development and characterization of AMPs. All of them have already been published as research articles. The first study aimed to optimize the porcine α -helical cathelicidin PMAP-36 by designing shorter and structurally modified analogues, in order to reduce their cytotoxicity and improve stability while retaining antimicrobial potency. The second project focused on enhancing the activity of the synthetic proline-rich peptide B7-005 by generating chimeric variants with extended C-termini derived from cetacean and bovine PrAMPs, aiming to broaden the antimicrobial spectrum without compromising biocompatibility. The third study explored the use of BONCAT, a non-radioactive fluorescence-based technique, to investigate the intracellular mechanism of action of PrAMPs in living bacteria, providing an advancement on the knowledge on these compounds, as well as the further development of a versatile tool for studying AMPs as protein synthesis inhibitors in multiple Gram-negative bacterial species.

3. Structural and biological characterization of shortened derivatives of the cathelicidin PMAP-36

This chapter introduces a research article (and its supplementary materials) published on Scientific Reports in 2023:

Barbara Biondi, **Luigi de Pascale**, Mario Mardirossian, Adriana Di Stasi, Matteo Favaro, Marco Scocchi & Cristina Peggion. **Structural and biological characterization of shortened derivatives of the cathelicidin PMAP-36**. *Sci Rep* **13**, 15132 (2023). <https://doi.org/10.1038/s41598-023-41945-1>

The first study of my PhD thesis aimed to optimize the porcine α -helical cathelicidin PMAP-36, a peptide with strong antimicrobial activity against a range of Gram-negative and Gram-positive bacteria. However, its considerable length makes it costly to synthesize, and it is prone to degradation by proteases. Additionally, at higher concentrations, PMAP-36 is significantly toxic toward eukaryotic cells, including mouse and pig macrophage-like cells (123) and moderately haemolytic against murine, human, and porcine erythrocytes (120–122). To address these issues, in this study we designed a series of PMAP-36 derivatives by shortening the peptide sequence, altering specific amino acid residues, or attaching lipid moieties. The goals were to further characterize the essential pharmacophore of PMAP-36, provide compounds with reduced toxicity, explore the role of the α -helical structure in activity, and determine which modified version offered the best balance between antimicrobial potency and low cytotoxicity.

My contributions to this study combined both experimental and conceptual aspects. Experimentally, I was primarily responsible for the following core activities (which are described in detail in the *Methods* section): bacterial culture handling; Minimum inhibitory concentration (MIC) assay; cytotoxicity assay on human cells; haemolysis of ram red blood cell; evaluation of peptide solubility and aggregation. Complementing this experimental work, I played a major role in the analysis, interpretation, and curation of data, as well as in drafting and critically revising the manuscript.



OPEN

Structural and biological characterization of shortened derivatives of the cathelicidin PMAP-36

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Cathelicidins, a family of host defence peptides in vertebrates, play an important role in the innate immune response, exhibiting antimicrobial activity against many bacteria, as well as viruses and fungi. This work describes the design and synthesis of shortened analogues of porcine cathelicidin PMAP-36, which contain structural changes to improve the pharmacokinetic properties. In particular, 20-mers based on PMAP-36 (residues 12-31) and 13-mers (residues 12-24) with modification of amino acid residues at critical positions and introduction of lipid moieties of different lengths were studied to identify the physical parameters, including hydrophobicity, charge, and helical structure, required to optimise their antibacterial activity. Extensive conformational analysis, performed by CD and NMR, revealed that the substitution of Pro25-Pro26 with Ala25-Lys26 increased the α -helix content of the 20-mer peptides, resulting in broad-spectrum antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* strains. Interestingly, shortening to just 13 residues resulted in only a slight decrease in antibacterial activity. Furthermore, two sequences, a 13-mer and a 20-mer, did not show cytotoxicity against HaCat cells up to 64 μ M, indicating that both derivatives are not only effective but also selective antimicrobial peptides. In the short peptide, the introduction of the helixogenic α -aminoisobutyric acid forced the helix toward a prevailing 3_{10} structure, allowing the antimicrobial activity to be maintained. Preliminary tests of resistance to Ser protease chymotrypsin indicated that this modification resulted in a peptide with an increased in vivo lifespan. Thus, some of the PMAP-36 derivatives studied in this work show a good balance between chain length, antibacterial activity, and selectivity, so they represent a good starting point for the development of even more effective and proteolysis-resistant active peptides.

Antimicrobial resistance (AMR) is a serious threat to human health worldwide. Therefore, the interest in new drug candidates with antimicrobial activity, as new agents that could substitute or complement conventional antibiotics, is steadily increasing¹. Antimicrobial peptides (AMPs) are an ancient class of molecules that form a first line of defence in eukaryotes to combat microorganisms. Attention on this class of compounds has risen because their most common mechanism of action targets bacterial membranes or other general structures, in a manner different to most antibiotics, that typically target specific proteins or membrane components². This specific mode of action makes the development of bacterial resistance less likely to occur, thus making AMPs promising antibacterial drugs³. Thousands of AMPs have now been isolated from a variety of natural sources comprising microorganisms, plants, and animals including mammals⁴. The vast pool of natural antimicrobial peptides offers a broad spectrum of biological activities to choose from, but often these compounds are not stable, with a short half-life, and when used out of their innate immune context are often quite toxic to host cells. To allow for clinical applications, it would be important to develop new artificial, optimised and long lived AMP analogues that overcome the drawbacks of the natural peptides⁵.

Cathelicidins are a family of antimicrobial peptides in vertebrates that plays an important role in the innate immune response^{6,7}. They contain highly variable C-terminal domains displaying direct antimicrobial activity

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against a variety of bacteria, fungi, and some virus and parasites⁸. Porcine myeloid PMAP-36 is a highly cationic, 36-amino acid long cathelicidin displaying antimicrobial activity (in vitro and in vivo)^{9,10} as well as immunomodulatory properties^{11,12}. The N-terminal region is characterised by several charged residues and is organised as an α -helical structure, interrupted by three proline residues (Pro25, Pro26 and Pro32) located at the C-terminal end^{12,13}. Helicity was predicted by helical wheel projections and then verified by circular dichroism measurements in phosphate buffer pH 7, with addition of 25% TFE. The presence of a cysteine (Cys35) determines the formation of peptide homodimers with a total net charge of + 26. Dimerization probably occurs in storage form of the peptide's precursors in peripheral white blood cells¹³.

The peptide displayed a broad spectrum of antibacterial activity against Gram+ and Gram- bacteria at μ M concentrations and against *Candida* as well^{10,12–15}, but it also exhibited a significant cytotoxicity which is likely associated to its membranolytic activity^{13–16}.

Structure-activity studies have been performed to improve the antimicrobial activity and reduce the cytotoxic effects of this peptide. Monomerization did not affect the antibacterial properties of PMAP-36^{10,12–15}. On the other hand, the N-terminal segment 1–24 fully retained a comparable antimicrobial activity to the entire peptide and increased its selectivity for prokaryotic cells by showing lower haemolytic activity¹⁵, while the shorter fragment 1–20 exhibited decreased potency¹³. Further truncation of the first six N-terminal residues of the peptide 1–24 to obtain the 18-mer peptide encompassing residues 7–24 of the native peptide instead resulted in a fully active peptide, suggesting that hydrophobic residues upstream of the two prolines are more important for activity than the six N-terminal residues¹⁴. In addition, Scheenstra et al. showed that the N-terminal truncation of 11 residues in the PMAP-36 monomer had little effect on killing of *E. coli*, while further truncation of the peptide completely blocked its antibacterial activity¹². All these data are consistent with the indication that the central part of the molecules is the pharmacophore.

In this work, we designed and tested a series of shortened PMAP-36 analogues focusing on the 12–31 and 12–24 regions, which constitute the central and most active portion of PMAP-36.

The aim was to clarify the importance of the α -helical structure and to find the most active while least cytotoxic derivatives. The structures of these new analogues were analysed by CD and NMR and their antibacterial effects, cytotoxicity and stability were studied. The results provide interesting new data for further optimization of these molecules for future use as therapeutics.

Results

Design, synthesis, and physicochemical parameters of PMAP-36 derivatives. The peptides designed for this study are shortened analogues of PMAP-36, lacking the dispensable N-terminal portion 1–11 and covering the region between Lys12 and Ile31. The C-terminal portion 32–36 of the original PMAP-36 was also removed to prevent kinking of the helical structure due to Pro32 and to avoid dimerization caused by Cys35.

The remaining 20-mer (12–31) (compound **a**, Table 1) contains two prolines that interrupt the helical structure (Fig. 1). To evaluate the effects of extending the α -helical conformation to the entire sequence, a new compound was designed in which Pro25 was replaced by Ala25 and Pro26 by Lys26 (compound **b**, Table 1), to also increase the charge, which is important for antimicrobial activity. The hydrophobic and the cationic residues were inserted in a manner that would increase the amphiphilic character of the resulting α -helix, as perceived in the helical wheel projections (Fig. 1).

Furthermore, we prepared a series of analogues based on compound **b** ([A25,K26]-PMAP12–31) modified by linking an acyl chain of different length. Analogues **c**, **d**, and **e** were prepared by respectively linking octanoic, lauric, and palmitic acid to the N-terminus of the sequence. Thus, we investigated whether variations in the lipid moiety could modulate the insertion of the derivatives into bacterial membranes, and if it could also influence their antimicrobial potency. To investigate if the position of the lipid chain impacts the biologic effects, 11-aminoundecanoic acid (Aun) was linked to the C-terminus of compound **b** giving the analogue **f**. This analogue was intended to be compared with analogue **d** to evaluate the different effects of lipidation at the N- and C-termini.

	Peptide sequence	Theoretical MW	Experimental MW	Retention time ^a	Hydrophobicity ^b	Helicity
		[M + H] ⁺ _{calcd}	[M + H] ⁺ _{exp}	t _R (min)	Δt_R	(%) ^c
PMAP-36	*KRLKKIGKVLKWIPIVGSIPLGCG-NH ₂					
a	Ac-KRLKKIGKVLKWIPIVGS-NH ₂	2314.5	2314.8	13.2	0	18
b	Ac-KRLKKIGKVLKWIPIVGS-NH ₂	2319.5	2319.7	17.2	4.0	94
c	Oct-KRLKKIGKVLKWIPIVGS-NH ₂	2404.2	2403.8	22.3	9.1	71
d	Lau-KRLKKIGKVLKWIPIVGS-NH ₂	2460.3	2459.7	22.8	9.6	47
e	Pal-KRLKKIGKVLKWIPIVGS-NH ₂	2516.4	2516.9	25.8	12.6	53
f	Ac-KRLKKIGKVLKWIPIVGS-Aun-NH ₂	2503.3	2503.8	20.3	7.1	82
g	Ac-KRLKKIGKVLKWI-NH ₂	1650.2	1650.5	12.1	– 1.1	ND ^d
h	Ac-KRLKKIGKVUKWI-NH ₂	1623.1	1623.4	11.7	– 1.5	ND

Table 1. List of PMAP-36 analogues and of their physico-chemical properties. *Omitted N-terminal sequence = GRFRRLRKKTR. ^a Retention time refers to 5%–95%B (CH₃CN/H₂O, 9:1) over 30 min gradient, C18 column; relative to compound **a**: ^b $\Delta t_R = t_R(\mathbf{x}) - t_R(\mathbf{a})$, where **x** stays for peptides **b–h**; ^ccalculated from the $[\theta]_{222}$ value in SDS solution¹⁷; ^dND, not determined for mixed helices. Aun: 11-aminoundecanoic acid; Aib, U: α -aminoisobutyric acid.

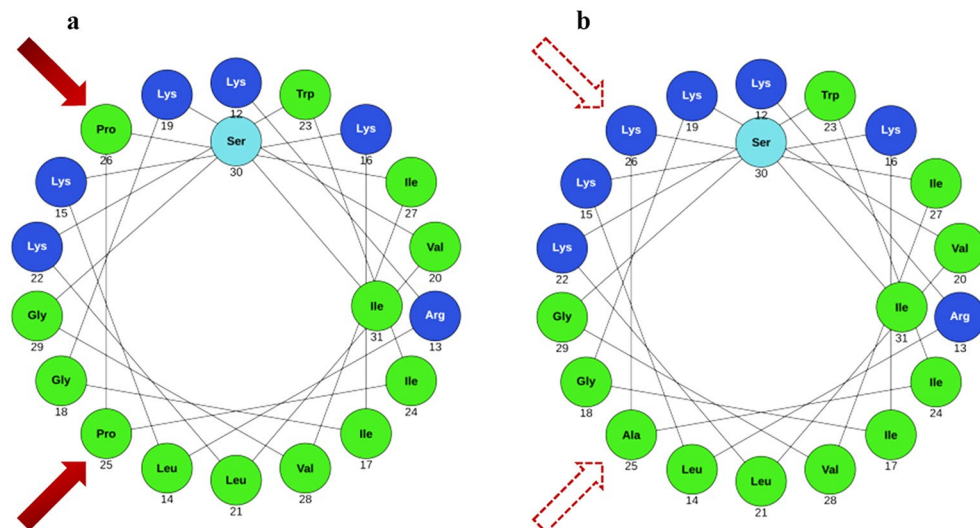


Figure 1. Helical wheel projections of [P25,P26]-PMAP12-31, compound **a**, (left) and [A25,K26]-PMAP12-31, compound **b** (right). Cationic residues are in blue, hydrophobic residues in green. (<https://www.donarmstrong.com/cgi-bin/wheel.pl>).

To exclude the possibility that any difference between lipidated and non lipidated peptides might be due to the lack of the free positively charged N-terminal amino group, all non-lipidated analogues were acetylated at their N-terminus. In this way the number of amides in peptides of the same length remained unmodified. Furthermore, all peptides were amidated at their C-terminus as is the natural PMAP-36¹³.

A shorter analogue with only 13 amino acids (derivative **g**) was also designed to test whether a further shortening of the 20-mer derivative (**a**) could produce a compound capable of maintaining antibacterial activity. Since peptide shortening often leads to a decrease in helix stability, derivative **h** was also prepared, in which Leu21 was replaced by α -aminoisobutyric acid (Aib, U). This non-proteinogenic amino acid is known for its strong ability to promote helix folding in synthetic and natural sequences^{18–20}. The complete series of PMAP-36 analogues is described in Table 1.

Conformational analysis of PMAP-36 derivatives. The secondary structure of the peptides was investigated by circular dichroism (CD) spectroscopy in various environments, including 0.2 mM potassium-phosphate buffer (aqueous solution, pH 7), 100 mM sodium dodecyl sulphate (SDS), and 2,2,2-trifluoroethanol (TFE). As these peptides are active on membranes, we chose SDS, as SDS micelles represent a model mimicking the prokaryotic cell membrane. TFE, on the other hand, is a solvent that induces the formation of helical structures²¹.

All analogues showed an unordered conformation in aqueous solution with a negative peak in the region around 200 nm (Fig. 2A).

Peptide **a** showed a modest helical structure (18% helicity) in the presence of SDS micelles, which are a first approximation of the amphiphilic environment of biological membranes, while the substitution of Pro25 and Pro26, respectively, by Ala and Lys, significantly increased the helical content (94% helicity) (Fig. 2A). The CD spectrum of peptides **b–f** in SDS showed the typical three-band shape with two negative maxima at 208 and 222 nm, assigned to the $n \rightarrow \pi^*$ transition and to the parallel component of the $\pi \rightarrow \pi^*$ transition respectively, and a positive maximum around 195 nm attributed to the antiparallel component of the $\pi \rightarrow \pi^*$ transition (Fig. 2A). The presence of an acyl chain of different lengths at the N-terminus in the analogues **c**, **d**, and **e** resulted in a perturbation of the helical structure in the form of a decrease in helicity. In contrast, the introduction of Aun at the C-terminus (peptide **f**) did not significantly affect the conformation of the peptide (Fig. 2A). A similar behaviour was observed for the analogues **c–f** analysed in TFE, condition that however evidenced an anomalous CD spectrum for peptide **b** (Fig. 2A), in which the observed ratio between molar ellipticities at 222 and 208 nm was > 1 , suggesting an aggregation phenomenon compatible with the presence of a coiled-coil structure^{16,22}.

The short peptides **g** and **h** showed an ordered conformation in SDS and TFE (Fig. 2B). CD spectra deviated from those of a pure α -helix in both peptides. We observed a shift of one of the two negative maxima to around 205 nm, accompanied by a shoulder centred near 222 nm. Furthermore, the value of the ratio $R([\Theta]_{222}/[\Theta]_{204-206})$ moves away from the ideal 1 of the α -helix with values around 0.4–0.5 typical of the 3_{10} -helix, values that were first theorised²³ and then determined in the first unequivocal experimental CD spectrum reported for an ideal right-handed 3_{10} -helical peptide^{24,25}. The contribution of the 3_{10} -helix conformation was different for peptides **g** and **h**, and in the two different membrane mimetic environments studied.

CD spectra of peptide **g** in SDS showed a contribution, albeit modest, of the 3_{10} -helix, as evidenced by the $R([\Theta]_{222}/[\Theta]_{204-206})$ value of 0.55 and by the shift of the negative maxima. At the same time, there was still a relevant contribution of α -helix as evidenced by the very intense positive band at 195 nm. The contribution of 3_{10} -helix appeared to be more significant in peptide **h**. Furthermore, to a R value of 0.55, the ellipticity at about

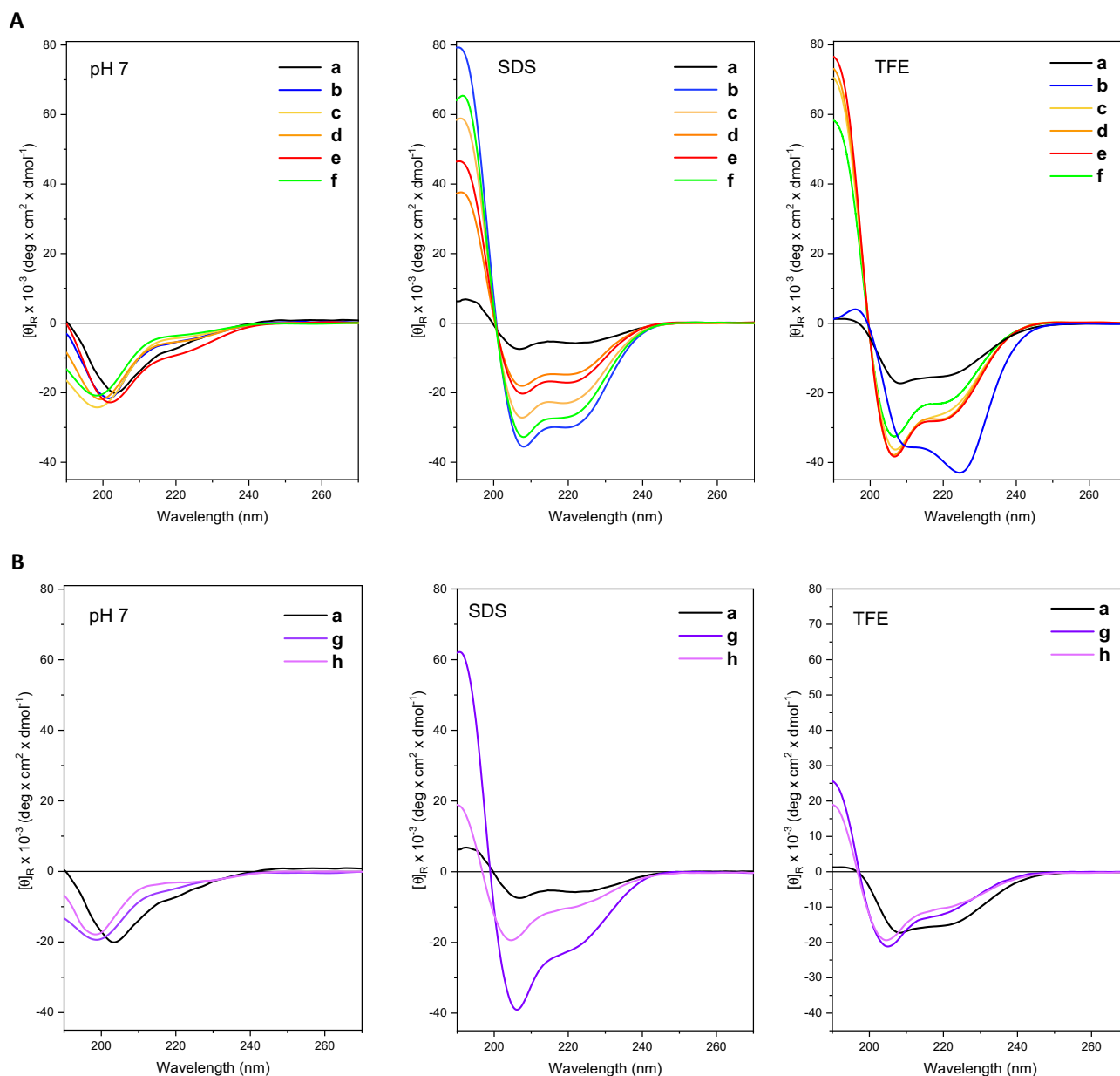


Figure 2. (A) CD spectra of PMAP12-31 **a**–**f** analogues in PBS, SDS and TFE, at 25 °C. (B) CD spectra of peptides **a**, **g** and **h** in PBS, SDS and TFE, at 25 °C.

195 nm was only slightly positive in the case of peptide **h**. Both **g** and **h** showed low ellipticity at 195 nm in TFE and *R* ratios of 0.55 and 0.58, respectively. These results could be due to an equilibrium of 3_{10} - and α -helices and/or from 3_{10} and α -helix segments that coexist in the same molecule.

We performed a 2D-NMR analysis in TFE-*d*₂ solution to obtain a more in-depth view of the conformational preference of some of the analogues, focussing on peptides **a**, **b**, **g**, and **h**.

The NMR spectra of **a** evidenced little dispersion of the proton resonances and extensive overlapping, suggesting poor organisation in helical structure (data not shown).

In contrast, peptides **b**, **g**, and **h** exhibited greater signal dispersion, indicating well-organised structures. Complete assignment of proton resonances was established based on TOCSY and NOESY spectra, using the Wüthrich's procedure²⁶ (Table S1–S3 and Figs. S1–S5 in Supporting Information).

In general, helical structures are readily identified by a series of consecutive NN(*i*, *i* + 1) NOEs, concomitant with α N(*i*, *i* + 3) NOEs^{26–28}. These connectivities, evident for **b**, **g** and **h**, reveal short interproton distances (< 3.5 Å) that demonstrate helical structure, without distinguishing between the different helix types²⁷.

3_{10} -helix and α -helix are distinguished by the relative intensities of the intermediate range α N(*i*, *i* + 2) and α N(*i*, *i* + 4) NOEs. It is known that 3_{10} -helices should display α N(*i*, *i* + 2) and α N(*i*, *i* + 3), whereas α -helices display only α N(*i*, *i* + 3) and α N(*i*, *i* + 4) NOEs^{26, 27, 29}.

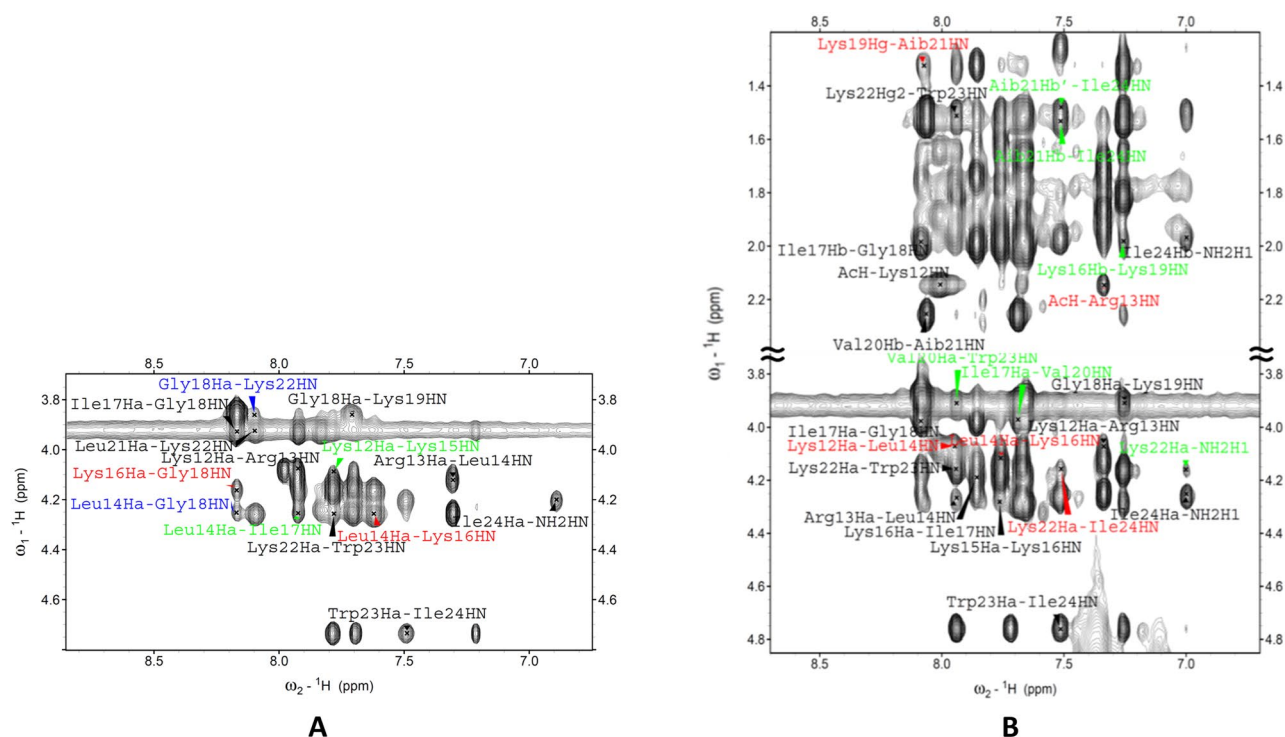


Figure 3. (A) Fingerprint region of the NOESY spectrum (600 MHz) of peptide **g** in TFE solution ($c = 1.2$ mM, $T = 308$ K). $\alpha\text{N}(i, i + 2)$, $\alpha\text{N}(i, i + 3)$, and $\alpha\text{N}(i, i + 4)$, cross-peaks are highlighted in red, green, and blue, respectively. (B) Regions of the NOESY spectrum (600 MHz) of peptide **h** in TFE solution ($c = 1.2$ mM, $T = 308$ K). $\alpha/\beta\text{N}(i, i + 2)$ and $\alpha/\beta\text{N}(i, i + 3)$ cross-peaks are highlighted in red, and green, respectively.

In Fig. 3A the fingerprint region of the NOESY spectrum of peptide **g** is reported. Together with all sequential cross peaks $\alpha\text{N}(i, i + 1)$, we observed two $\alpha\text{N}(i, i + 2)$, Leu14/Lys16 and Lys16/Gly18, several long range correlations involving $\alpha\text{N}(i, i + 3)$, Lys12/Lys15 and Leu14/Ile17, and $\alpha\text{N}(i, i + 4)$, Leu14/Gly18 and Gly18/Leu22. These findings confirmed the adoption of a mixed 3_{10} -/ α -helical conformation for **g**.

Figure 3B shows regions of the NOESY spectrum of **h**. In the fingerprint region we observed all sequential cross peaks $\alpha\text{N}(i, i + 1)$, three $\alpha\text{N}(i, i + 2)$, Lys12/Leu14, Leu14/Lys16 and Lys22/Ile24, and several intermediate-range correlations involving $\alpha\text{N}(i, i + 3)$, Ile17/Val20, Val20/Trp23 and Lys22/NH(C-terminus amide). The $\beta\text{N}(i, i + 3)$ signals involving Aib21 are observed in the aliphatic region. The absence of the long range $\alpha/\beta\text{N}(i, i + 4)$ correlations is indicative of a more pronounced 3_{10} -helical character for peptide **h**, with respect to peptide **g**.

Antibacterial and cytotoxicity of PMAP-36 derivatives. All derivatives were tested on a representative panel of reference strains of Gram-positive and Gram-negative bacteria (Table 2). For this purpose, the minimum concentration needed to inhibit bacterial growth (MIC) was measured. Peptide **a** effectively inhibited all tested bacterial strains (MIC = 1–4 μM) with the sole exception of *S. aureus* (MIC = 64 μM). The substitution of the two prolines in peptide **b** markedly changed the spectrum of activity, making the peptide active against *S. aureus* but, at the same time, decreasing its antimicrobial activity against all other bacterial species by 2–8 times (Table 2).

Lipopeptides **c**, **d**, and **e** generally decreased their antibacterial potency proportionally to the length of the carbon chain. Interestingly, lipopeptide **f** with Aun at the C-terminus showed better activity than compound **d**, bearing a lipid tail of the same length but at its N-terminus. The overall results with these lipopeptides suggest that the addition of a lipid moiety to the peptides at the C-terminus of PMAP12-31 is less detrimental than at its N-terminus, but does not improve antibacterial activity. The low solubility of compounds **c**, **d** and **e** in Müller-Hinton broth (see Supporting Information Fig. S6) also limited the MIC assay to peptide concentrations below 64 μM and may have contributed to the scarce antimicrobial activity of these compounds.

Interestingly, the shorter derivatives **g** and **h** still resulted in active peptides with a comparable activity spectrum to that of peptide **a** and only a slightly reduced activity (MIC = 2–8 μM , except against *S. aureus* MIC > 64 μM). The substitution of Leu21 with Aib in peptide **h** did not substantially modify its antimicrobial activity compared to peptide **g** (Table 2).

The effects of PMAP-36 derivatives were also observed in eukaryotic cells. To determine the viability of the cells in the presence of the peptides, the metabolic MTT assay was performed using the HaCaT cell line incubated with different concentrations of each compound (Fig. 4A). Peptides **a** and **g** did not decrease cell viability even at 64 μM after 24 h of incubation. On the contrary, peptide **b** affected cell viability, which dropped to 30%

Peptide	MIC (μ M)*					
	<i>E.coli</i> ATCC 25922	<i>S.aureus</i> ATCC 25923	<i>A.baumannii</i> ATCC 19606	<i>K.pneumoniae</i> ATCC 700603	<i>P.aeruginosa</i> ATCC 27853	<i>S.epidermidis</i> ATCC 12228
a	2	64	1	1	4	2
b	4	4	2	8	16	4
c	16	16	8	16	32	8
d	>32	32	16	32	>32	32
e	>32	>32	>32	>32	>32	>32
f	8	8	4	16	16	8
g	4	>64	2	8	8	4
h	4	>64	2	8	8	8

Table 2. Antibacterial activity (MIC) of PMAP-36 derivatives against Gram-negative and Gram-positive bacteria. *MIC, minimum inhibitory concentration is the lowest peptide concentration causing no visible growth after 18 h incubation in Mueller–Hinton broth at 37 °C. Values indicate the mode of three independent tests. Coloured boxes indicate different antimicrobial activity: high activity (green) low or no activity (red). Lipopeptides have not been tested above 32 μ M due to their low solubility (see *Supporting Information* for evaluation of aggregation).

(compared to an untreated control) at 16 μ M and even further at higher concentrations of the compounds. Lipopeptides **c**, **d**, **e**, and **f** showed significant cytotoxicity in the same range of concentrations required for antimicrobial activity (8–16 μ M), with HC50 values < 32 μ M. Surprisingly, peptide **h** containing Aib, decreased cell viability at a concentration of 8 μ M to just 70% (compared to an untreated control) and is at or above the 50% viability value up to 32 μ M. To determine whether the mechanism of cytotoxicity was due to membrane disruption, a haemolysis assay was performed on red blood cells (RBCs). It was confirmed that peptides **a**, **g**, and **h** had no membranolytic effect on erythrocytes (Fig. 4B). On the other hand, peptides **b**, and lipopeptides **c**, **d**, **e**, and **f** showed a significant lytic effect at all concentrations tested, suggesting that the cytotoxicity observed in eukaryotic cells were mainly due to membrane damage. Interestingly, peptide **h**, albeit slightly metabolically cytotoxic to HaCaT cells, did not appear to damage erythrocytes (Fig. 4B). Therefore, further studies will be needed to understand the mechanism of cytotoxicity of peptide **h** on the HaCaT cells.

Resistance to protease. The stability of the peptides in the presence of chymotrypsin was monitored by HPLC–MS, analysing the fragments derived from the degradation of the peptide. All 20-mer analogues (**a**–**f**) are sensitive to chymotrypsin. The rate of peptide degradation was calculated as the percentage of intact peptide detected at defined time points. All peptides were mostly degraded within the first 15 min of incubation, with the exception of the lipopeptide **e** and the short peptide **h**. After 90 min, 65% of the peptide **e** was still intact, indicating that palmitoylation protects against hydrolysis. For peptide **h**, which was designed with an Aib residue to improve proteolytic resistance, 50% of the peptide was still intact after 90 min (Fig. 5A). The other peptides were cleaved on the C-terminal side of Leu21 or Trp23 as the first cleavage observed. Successive degradations involve Leu14 for all peptides (with the exception of **a** and **h**). Concerning 13-mer peptides (**g** and **h**), degradation at position 21 occurred only for **g** and not for the Aib containing peptide **h**, for which Leu14 is the sensitive point for chymotrypsin attack. A summary of the fragments detected for each peptide is shown in Fig. 5B.

Discussion. It is well known that the transition of AMP use from the laboratory to real applications is hampered by their poor pharmacokinetic properties. An effective way to overcome this weakness of AMPs is to introduce structural changes that increase resistance to proteases, while maintaining or improving activity and, at the same time limiting toxicity.

In this study, we synthesised and tested a series of PMAP-36 derivatives by shortening the sequence length, changing amino acid residues at critical positions, and inserting lipid moieties. This helped to identify physical parameters, including hydrophobicity, charge, and helicity, required to optimise the antibacterial activity and selectivity of this peptide.

We first showed that the shortened PMAP12-31 peptide **a**, which is the monomeric central part of the natural PMAP-36, exhibited extensive broad-spectrum antibacterial activity against *E. coli*, *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, and *S. epidermidis* strains with MIC values of 1–4 μ M. Interestingly, we found that this peptide could be further shortened to 13-mer 12-24 (compound **g**) with only a 1–8-fold decrease in antibacterial potency, depending on the pathogen. Interestingly, both compounds **a** and **g** were not cytotoxic against HaCaT cells up to 64 μ M and not haemolytic, indicating that both derivatives are effective and selective antimicrobial compounds (see Table 3 and Figs. S9, S10 in *Supporting Information*) and useful leads for further development. In addition, *A. baumannii* and *K. pneumoniae*, which are among the most worrying pathogens due to their high degree of resistance³⁰, and have never been tested against PMAP-36 derivatives, were found to be highly sensitive to both compounds **a** and **g**. Peptide **g** is almost identical to the 12-mer peptide RI12 reported in Lyu et al.¹⁴ and Lv et al.¹⁵, however, it contains an additional acetylated lysine at the N-terminus. Both peptides are not haemolytic and are active against Gram-negative but are only scarcely active against Gram positive bacteria. The addition of a charged residue in **g** increased the antimicrobial activity against the *S. epidermidis* strain.

Pro25 and Pro26 of peptide **a** were replaced by the two helical promoting residues Ala and Lys (compound **b**) attempting to increase the amphipathic α -helix content of the peptide. CD analysis and NOESY spectrum

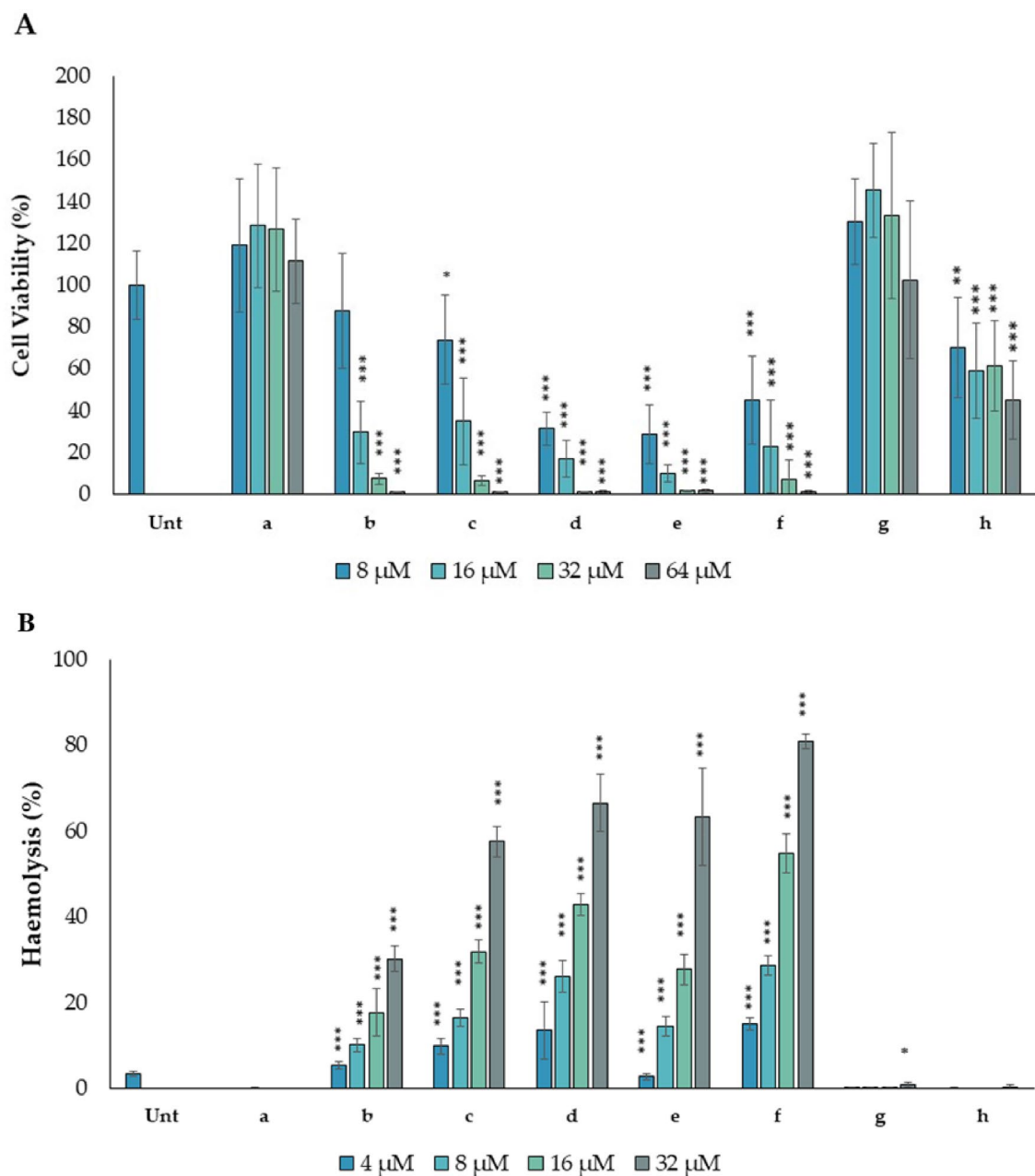


Figure 4. Effects of PMAP-36 derivatives on the viability of the HaCaT cell line (A) and red blood cells (B). (A) Cell viability was measured as absorbance at 570 nm after 24 h from the exposure to the peptides. Results are reported as percentages of viable cells with respect to the untreated control cells (Unt) set as 100% of viability. Data are the average $\pm$ SD of at least three independent experiments in internal duplicate ($n=6$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus the untreated control (Test t-student). (B) Haemolysis assay was performed against 4% v/v suspension of ram red blood cells (RBCs) in PBS. Absorbance of released haemoglobin (540 nm) were measured after 1 h exposure to the peptides. RBCs suspension with 1% Triton or without peptide treatment were used to achieve 100% haemolysis and as negative haemolysis control (Unt), respectively. The results are shown as a percentage with respect to of RBCs treated for 1 h with 1% Triton X-100. Data are the average $\pm$ SD of three experiments ($n=3$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ versus the untreated sample (Test t-student).

confirmed the significant increase in peptide structuration with 94% α -helical conformation in the presence of membrane-mimetic environments. In addition, compound **b** displayed hydrophobicity higher than that of peptide **a** likely due to the enhanced amphipathicity of the helix, acquiring biological activity against *S. aureus* (MIC 4 μ M), but at the same time decreasing activity 2–8-fold against the other strains. Furthermore, this compound also showed an increase in cytotoxicity compared to compound **a**. Because compounds **a** and **b** have a similar charge, the main differences are the increased helicity and hydrophobicity of **b**, which did not result in a more selective peptide (Table 3). These results are consistent with those of other studies^{32,33} in which high hydrophobicity and

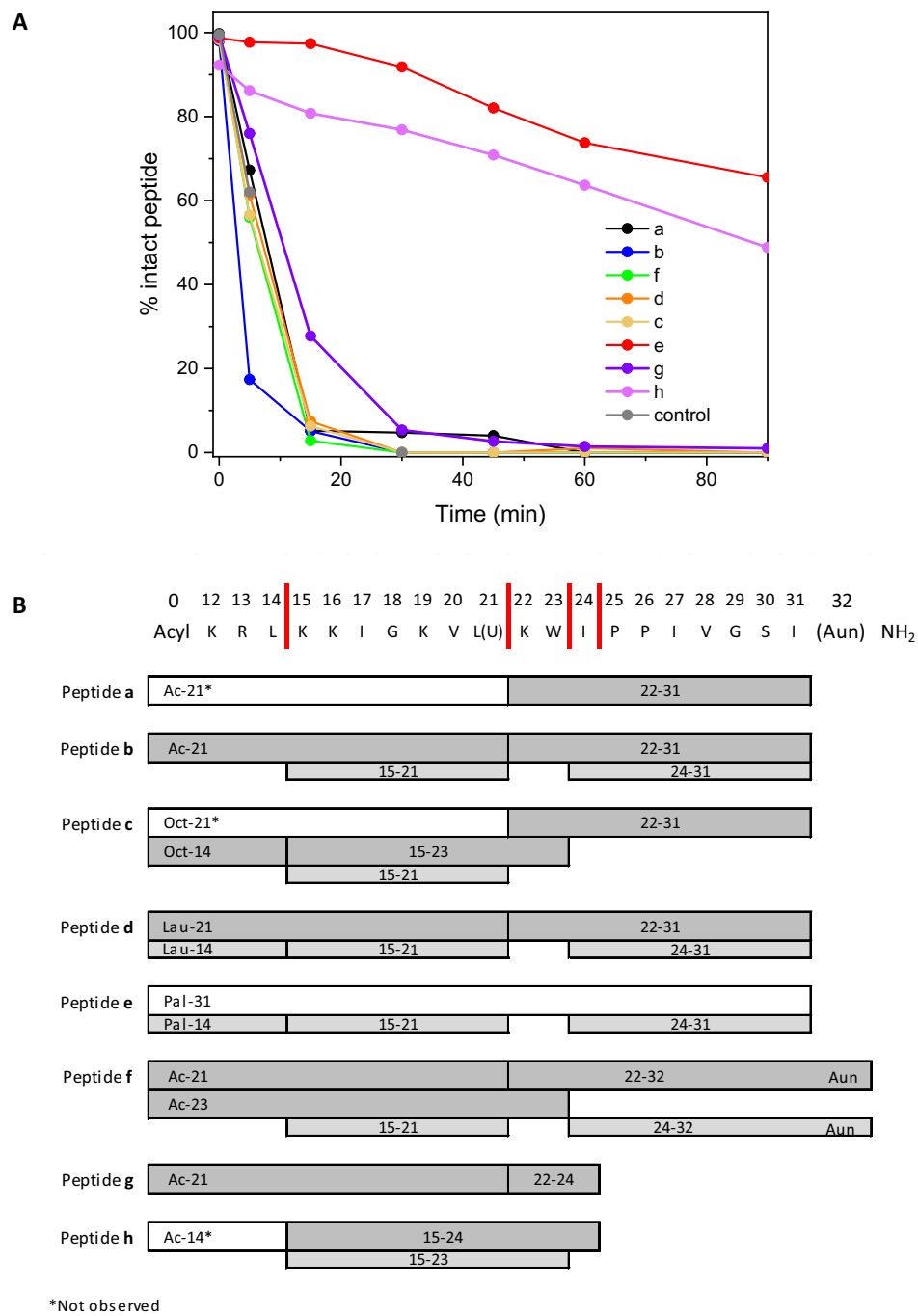


Figure 5. (A) Chymotrypsin degradation of compounds **a–h**. The percentage of preserved peptide is represented over time. We estimated the residual amount of intact peptide in a sample as the HPLC peak area. (An example of the HPLC profiles with labelled peaks corresponding to hydrolysis products is reported in the Supporting Information, Fig. S8). As a positive control we used the C-terminal portion of natural Endonuclease V involved in the DNA repair mechanism that occurs after DNA damage caused by ultraviolet radiation. (B). General sequences of peptides **a–h** and their degradation products by chymotrypsin. All peptide fragments were detected by HPLC–MS (see Materials and Methods). Fragments obtained within a 30-min incubation with chymotrypsin are indicated by dark grey colour; fragments obtained after longer incubation times are indicated by light grey colour. White boxes indicate hypothetical fragments not detected. Vertical red bars in the general sequence (top) indicate the position of observed cleavage sites.

amphipathicity (hydrophobic moment) were observed to be correlated with increased haemolytic activity, while antimicrobial activity was found to be less dependent on these factors.

Peptide	Minimum selectivity index*	
	IC ₅₀ /MIC	MHC/MIC
a	> 16	> 8
b	≥ 1.6	≥ 1
c	≤ 1.6	< 0.5
d	< 0.2	≤ 0.1
e	< 0.2	< 0.2
f	< 1	< 0.5
g	> 8	> 4
h	≥ 6.8	> 4

Table 3. Selective antibacterial activity of PMAP-36 derivatives. *Minimum selectivity index is calculated as ratio between cytotoxicity/haemolytic activity and MIC on at least five out of six bacterial species. The IC₅₀ was considered as the peptide concentration at which cell viability is reduced by 50% compared to the untreated control. MHC was taken as the lowest concentration of peptides which induced 10% of haemolysis of red blood cells³¹.

Structure–activity comparison was also performed on short 13-mer **g** and **h** compounds. Despite the short sequences, both peptides exhibited a helical structure. To increase the helicity of peptide **g**, which may be hampered by end-effects due to its short length, we replaced Leu21 with the helicogenic residue Aib. Short peptides containing Aib tend to adopt the 3₁₀-helical conformation^{17–19}, and, in fact, we could show, although the substitution did not have a significant effect on hydrophobicity, the presence of Aib induced the helix to adopt the prevailing 3₁₀ conformation, as demonstrated by CD analysis and the NOESY spectrum^{18–20}. Similar to what was observed above, the substitution of Leu21 increased helicity but again did not affect antimicrobial activity although it somewhat increased cytotoxicity against HaCaT cells (even remaining non-haemolytic, see Fig. 4). This result was unexpected and differs from the temporin-1Dra results obtained with the membranolytic frog skin peptides, in which Aib substitutions increased both its antimicrobial and cytolytic activities³⁴.

We also tested the effects of elongating the acyl chain at the N-terminus of compound **b** (peptides **c**, **d**, and **e**) and a lipid chain insertion at the C-terminus (peptide **f**) of length similar to that of compound **d**. The lipopeptides showed a gradual decrease in helicity related to their lipid chain length compared with compound **b** under membrane-like conditions (SDS micelles). At the same time, all lipidated derivatives tended to aggregate. Moreover, the lipidated derivatives showed lower antimicrobial activity and increased cytotoxicity with respect to the compound **b**. However, in this respect, C-terminal lipidation was significantly less deleterious than N-terminal modification.

Lipidation and acylation have been shown to promote the antimicrobial activity of several AMPs^{35,36} and to boost or modulate the antibacterial potential and the properties of already active AMPs³⁷, also including some cathelicidins^{38,39}. However, this is not the case with our derivatives. The additional lipid chain has been shown to promote aggregation (see Supporting Information) even at low concentrations, and the acylated peptides are poorly active. The gradual decrease in helicity as a function of the length of the lipid tail length suggests that the alkyl chains interfere with helical structuration reducing the antimicrobial activity of the peptides. This hypothesis is also supported by the behaviour of peptide **f**, in which the addition of a hydrophobic tail at the C-terminus (Aun) did not affect helicity and partially affected its antibacterial potency. It resulted in higher antimicrobial activity and lower cytotoxicity than that of peptide **d** endowed with an acyl chain (lauric acid) of comparable length. These differences in activity are probably due to the higher helical content of compound **f** (82% versus 47%) compared to that of the related analogue **d**. However, in all cases, modification of compound **b** with lipid tails did not improve the biological activity of the peptide.

The proteolytic degradation of bioactive peptides shortens their in vivo lifespan and may alter their pharmacological profile³. The tests of resistance to the serine protease chymotrypsin presented here indicated that most PMAP-36 derivatives were susceptible to degradation and were hydrolysed by this protease within 10–20 min. The only exceptions were palmitoylated peptide **e** and Aib-containing peptide **h**, which were less than 50% degraded after 90 min.

The proteolytic fragment profile suggests that cleavage occurred at canonical sites⁴⁰ between Leu14 and Lys15 and between Leu21 and Lys22. The introduction of Aib into the 13-mer compound **h** significantly reduced susceptibility to chymotrypsin. In the future, Aib or other non-proteinogenic residues could be introduced to obtain more stable peptides that retain antimicrobial activity and selectivity, ensuring that the peptides are not cytotoxic. As a preliminary assessment of the pharmacokinetic potential of the peptides, we also performed proteolytic degradation tests in serum for the most promising peptides **g** and **h**. Peptide **g** is rapidly degraded, as already observed in the presence of chymotrypsin. On the contrary, peptide **h** is capable of withstanding up to 180 min. Although this period is shorter than that observed in the presence of chymotrypsin, the result is still interesting, since the composition of the serum places the peptide in a more unfavourable environment (Supporting Information, Fig. S10). The observed resistance is reasonably attributable to the presence of the Aib residue. To obtain a more detailed profile of peptide stability, this preliminary study in serum needs to be extended to all peptide series.

In conclusion, we demonstrate that the α -helical cathelicidin PAMP-36 can be shortened to either side and that its central part (residues from 12 to 24) substantially retains its structural characteristics and antimicrobial activity against a wide spectrum of bacteria. We have also shown that the onset of a stable helical conformation is an essential prerequisite for the maintenance of antibacterial activity, but also that an additional increase in helicity and/or hydrophobicity does not improve the biological activities of peptides, as also indicated in the literature³³. The derivatives **g** and **h**, which show the best ratio between activity/selectivity and peptide length, emerged as an interesting starting point for further optimisation to make them more resistant to proteolysis.

Methods. *Peptide synthesis.* The solid phase peptide synthesis of the PAMP-36 analogues was performed on the Rink Amide MBHA resin, using standard Fmoc chemistry protocols^{41, 42}. Deprotection of the Fmoc group was carried out with a 20% piperidine solution in *N,N*-dimethylformamide (2×10 min), and for the activation of the carboxylic groups, a mixture in a three-fold molar excess of amino acid and coupling reagent was used in the presence of a six-fold excess of DIPEA. Reaction time for each coupling was 50 min. On-resin *N* α -acetylation was achieved using an Ac_2O /DIPEA/DMF (1:0.5:20 ratio) mixture in DMF, reaction time 30 min. For the lipidated analogues, the introduction of the acyl chain was carried out on resin and required the pre-activation of octanoic acid, lauric acid and palmitic acid in the presence of HBTU, HOBT, and DIPEA. Fmoc-Aun-OH is commercially available (Iris Biotech GmbH, Marktredwitz, Germany) and requires the same activation protocol as the other Fmoc-amino acids.

At the end of the synthesis, each peptide was cleaved from the resin using a mixture of TFA, TIS, and water in a 95:2.5:2.5 ratio. The filtrates were collected and concentrated under a flow of nitrogen, and the crude peptide was precipitated by the addition of diethyl ether. The crude peptides were purified by flash chromatography on an Isolera Prime chromatographer (Biotage, Uppsala, Sweden) using a SNAP Cartridge KP-C18-HS 12g or preparative RP-HPLC on a Phenomenex C18 column ($22.1 \text{ mm} \times 250 \text{ mm}$, $10 \mu\text{m}$, $300 \text{ \AA}$) using an Akta Pure GE Healthcare (Little Chalfont, U.K.) LC system equipped with an ultraviolet detector (flow rate of 15 mL/min) and a binary elution system: A, H_2O ; B, $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ [9:1 (v/v)]; gradient from 25 to 55% B in 30 min. The purified fractions were characterised by analytical HPLC-MS on a Phenomenex Kinetex XB-C18 column ($4.6 \text{ mm} \times 100 \text{ mm}$, $3.5 \mu\text{m}$, $100 \text{ \AA}$) with an Agilent Technologies 1260 Infinity II HPLC system and a 6130 quadrupole LC/MS instrument. All compounds were $> 95\%$ pure.

The lyophilised peptides were then resuspended in DMSO (Sigma-Aldrich) and quantified using an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Amersham, UK). Peptide concentrations were calculated spectrophotometrically using the absorbance at 280 nm and the molar extinction coefficients of tryptophan ($\epsilon = 5500/\text{M cm}$). All peptides were stored frozen at -20°C .

Circular dichroism. The electronic CD curves in the far-UV region (below 260 nm) were obtained on a Jasco (Tokyo, Japan) model J-1500 spectropolarimeter with a fused quartz cell of 0.02 cm pathlength (Hellma, Mühlheim, Germany). The values are expressed in terms of $[\theta]_R$, the molar ellipticity per residue ($\text{deg} \times \text{cm}^2/\text{dmol}$). Spectra were recorded at room temperature in water, in spectrograde TFE (99.9% Acros Organics, Geel, Belgium) and 100 mM SDS solution at $3 \times 10^{-4} \text{ M}$ peptide concentration. The final molar peptide-to-lipid ratio is 1:300.

NMR. The monodimensional and correlated spectroscopy (COSY)/total correlation spectroscopy (TOCSY) and NOESY 2D NMR spectra of the selected analogues were obtained at 308 K by the use of a Bruker AVANCE NEO-600 spectrometer from an about 1.5 mM concentration sample dissolved in approximately 700 μL of TFE, d_2 solution. Suppression of the solvent signal was achieved by the use of an excitation sculpting program⁴³. All homonuclear spectra were acquired by collecting 512 experiments, each one consisting of 64–80 scans and 2 K data points. The spin systems of the coded amino acid residues were identified using standard double-quantum filtered-COSY⁴⁴ and clean TOCSY⁴⁵ spectra. In the latter case, the spin-lock pulse sequence was 70 ms long. NOESY experiments were utilized for sequence-specific assignments⁴⁶. To avoid the problem of spin diffusion, the build-up curve of the volumes of the NOE cross peaks as a function of the mixing time (50–500 ms) was obtained first (data not shown). The mixing time of the NOESY experiment used for interproton distance determination was 150 ms (i.e., in the linear part of the NOE build-up curve).

Bacterial culture. The bacterial strains used were *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 700603, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus epidermidis* ATCC 12228. The reference bacterial strains used were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). They were grown overnight (o/n) at 37°C in complete Müller-Hinton broth (MHB, Difco) medium with shaking (130 rpm). The day after, the overnight bacterial cultures were diluted in fresh MHB (300 μL o/n culture in 10 mL) and incubated at 37°C with shaking (130 rpm) for approximately 2 h (mid-log phase) until reaching an optical density (OD) at 600 nm ($\text{OD}_{600} \approx 0.3$).

Minimum inhibitory concentration (MIC) assay. Peptides diluted in MHB to a concentration of 128 μM were added to the first column of wells of a 96-well round-bottom microtiter plate (Sarstedt, Milan, Italy) and serially diluted (1:2) in a final volume of 50 μL MHB was added to the next wells. Then, a mid-log bacterial cultures ($\text{OD}_{600} \approx 0.3$) were diluted to 5×10^5 colony-forming units (CFU)/mL in MHB and 50 μL of this bacterial suspension was added to each well. In this way, the final bacterial concentration was reduced to 2.5×10^5 CFU/mL and the peptide concentration in the wells was also halved in a final volume of 100 μL (final bacteria load was 2.5×10^4 CFU/well). The same amount of untreated bacteria in MHB was used as a control for bacterial growth.

MHB (100 µL) was used as a negative control to check the sterility of the medium. The plate was sealed with parafilm to minimize evaporation and incubated overnight at 37 °C (approximately 18 h). MIC was determined by visual inspection at the end of incubation as the first clear well. Data are reported as mode of independent experiments repeated three times or four times in case of uncertain values.

Cytotoxicity assay on human cells. Cytotoxicity was measured by the MTT assay on human immortalized epidermal keratinocyte HaCaT cell line (DKFZ, Eppelheim, Germany). The HaCaT cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Milan, Italy). DMEM medium was supplemented with 10% foetal bovine serum (FBS; Sigma, Milan, Italy), 2 mM glutamine (Sigma, Milan, Italy), 100 U/mL penicillin (Sigma, Milan, Italy) and 100 µg/mL streptomycin (Sigma, Milan, Italy). The cells were maintained at 37 °C in the presence of 5% CO₂ and in a humidified incubator.

Cells were counted in a Bürker-Türk chamber to prepare a suspension of 2×10^5 cells/ml in DMEM High Glucose Complete Medium (Sigma-Aldrich, Milan, Italy). 100 µL of this suspension was added to the wells of a 96-well flat-bottom transparent microtiter plate (Euroclone, Milan, Italy) specially treated for tissue culture. The plate was incubated for 24 h at 37 °C and 5% CO₂ to allow the cells in each well to reach a semiconfluent state. The following day, serial dilutions (1:2) of the peptides, starting with 64 µM, were prepared in DMEM medium, and 100 µL of these solutions were used to replace the medium in the plate with HaCaT cells. The same amount of untreated DMEM high-glucose medium was used as a cell growth control. DMEM high-glucose complete medium (100 µL) was used as a negative control to check the sterility of the medium. The plate was sealed with Parafilm to minimize evaporation and incubated at 37 °C and 5% CO₂ for 20 h. Then, 25 µL MTT (Sigma-Aldrich, Milan, Italy) dissolved in phosphate-buffered saline (PBS) was added to each well to a final concentration of 1 mg/mL MTT, and the plate was incubated for 4 h in the dark at 37 °C and 5% CO₂.

At the end of incubation, 100 µL 10% (w/v) Igepal (Sigma-Aldrich, Milan, Italy) in 10 mM HCl was added to each well, and the plate was incubated overnight in the dark at 37 °C and 5% CO₂. The absorbance of MTT at 570 nm was measured using a Nanoquant Infinite-M200Pro plate reader (Tecan, Männedorf, Switzerland). Student's t-test was performed for all samples.

Haemolysis of ram red blood cell. The lytic potential towards eukaryotic cell membranes was assessed by measuring the release of haemoglobin from commercial ram red blood cells (RBCs) (Microbiol s.r.l., Cagliari, Italy) after exposure to the peptides. First, erythrocytes were isolated by centrifugation, washed with PBS and then suspended at 8% (v/v) in PBS. Secondly, 100 µL erythrocytes suspension was added into each well of a 96-well microplate. Then, 100 µL peptide solution was added to each well at different concentrations (the final concentration of erythrocytes suspension was 4% v/v), and the plates were incubated for 1 h at 37 °C and centrifuged at 1000 g for 10 min. Finally, 100 µL aliquots of supernatant were transferred to fresh 96-well microplate, and the release of haemoglobin was monitored by measuring the absorbance at 540 nm with Tecan microplate reader (Tecan Trading AG, Switzerland). 0% and 100% haemolysis were determined in PBS and 1% Triton X-100, respectively.

Proteolytic stability. The proteolytic stability of synthetic peptides was assessed using chymotrypsin (Sigma-Aldrich) and human serum (Sigma-Aldrich)⁴⁶. The peptide (0.125 mg) was dissolved in the minimum amount (5 µL) of DMSO (dimethylsulfoxide) and then diluted (final volume: 50 µL) with Tris-HCl 50 mM, pH 7.8. Then, it was incubated with and without the enzyme solution (1.25 µg of enzyme in 150 µL buffer) at 37 °C for 24 h. After the workup procedures, the samples were analysed by RP-HPLC as described in the 'peptide synthesis and characterization' experimental section. A peptide not resistant to chymotrypsin was used as a positive test. As a control, we used the 128-137 C-terminal portion of natural Endonuclease V. The peptide sequence is as follows: WYKYGKAIYA. All the degradation experiments were executed in triplicates.

Hydrophobicity. Peptide hydrophobicity was calculated as difference in retention times between peptide analogues **b-h** and peptide **a**. Each peptide was dissolved in MeOH to a concentration of 0.5 mg/mL and then subjected to RP-HPLC on an analytical Phenomenex Luna C18 (100 Å, 5µ) column using 0.1% TFA in 9:1 water:acetonitrile as solvent A, and 0.1% TFA in 9:1 acetonitrile:water as solvent B. The peptides were eluted using a linear gradient as follows: 5%B to 95%B over 30 min. The flow rate was 1 mL/min and the absorbance was measured at 215 nm and 280 nm. The retention time was recorded for each peptide.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

C.P. and M.S. conceived and designed the experiments; B.B. L.D.P., A.D.S. and M.F. conducted the main experiments; B.B. and M.M. analysed the data; C.P. and M.S. wrote the main manuscript text. M.M. and B.B. critically revised the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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SUPPORTING INFORMATION

Structural and biological characterization of shortened derivatives of the cathelicidin PMAP-36

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1- NMR data of peptide **b**

Table S1. ¹H-NMR chemical shifts of peptide **b**.

2- NMR data of peptide **g**

Table S2. ¹H-NMR chemical shifts of peptide **g**.

Figure S1. Figure S1. Fingerprint region of the NOESY spectrum (600 MHz) of peptide **g** in TFE solution (c= 1.2 mM, T=308 K)

Figure S2. Figure S2. Amide NH region of the NOESY spectrum (600 MHz) of peptide **g** in TFE solution (c= 1.2 mM, T=308 K)

3- NMR data of peptide **h**

Table S3. ¹H-NMR chemical shifts of peptide **h**.

Figure S3. Amide NH region of the NOESY spectrum (600 MHz) of peptide **h** in TFE solution (c= 1.2 mM, T=308 K)

Figure S4. Fingerprint region of the NOESY spectrum (600 MHz) of peptide **h** in TFE solution (c= 1.2 mM, T=308 K). C^αH_i→NH_{i+2} and C^αH_i→NH_{i+3} cross-peaks are highlighted in red, and green, respectively.

Figure S5. Region of the NOESY spectrum (600 MHz) of peptide **h** in TFE solution (c= 1.2 mM, T=308 K). C^βH_i→NH_{i+2}, C^βH_i→NH_{i+3} are highlighted in red and green respectively.

4- Evaluation of solubility and aggregation

Figure S6. Solubility of PMAP-36 derivatives at different concentrations in PBS after 4h and 24 h incubation

Figure S7. Solubility of PMAP-36 derivatives at different concentrations in completed Mueller-Hinton broth after 4h and 24 h incubation

5- Peptide stability

Figure S8. HPLC profile with labeled peaks corresponding to the hydrolysis products for peptide **d**.

Figure S9. HPLC profile for the proteolytic degradation of the control peptide.

Figure S10. Proteolytic resistance of peptide **g** and **h** in the presence of chymotrypsin or serum.

6- Peptide selectivity

Table S4. Selectivity index (SI) of the tested peptides for each bacterial species based on cytotoxicity.

Table S5. Selectivity index (SI) of the tested peptides for each bacterial species based on haemolysis.

1- NMR data of peptide b

Table S1. ¹H-NMR chemical shifts of peptide b.

Residue	NH	α	β	other
Ac ⁰				2.14
Lys ¹²	7.66	4.15	1.95	γ 1.66; δ 1.86; ε -
Arg ¹³	8.02	4.20	2.07, 2.00	γ 1.88, 1.82; δ 3.29; NHε 7.21
Leu ¹⁴	7.36	4.25	-	γ -; δ 1.05, 0.97
Lys ¹⁵	7.72	4.20	2.02	γ 1.70, 1.58; δ 1.85; ε 3.08; NHε 7.43
Lys ¹⁶	8.21	3.92	-	γ -; δ -; ε -
Ile ¹⁷	8.58	3.79	-	γ -
Gly ¹⁸	8.11	4.03, 3.87		
Lys ¹⁹	7.77	4.22	2.04	γ 1.66, 1.56; ε 3.06; NHε 7.39
Val ²⁰	7.96	3.92	2.01	γ 1.05
Leu ²¹	7.72	4.20	1.85	-
Lys ²²	-	-	-	ND for overlapping
Trp ²³	8.07	4.57	3.67, 3.50	H1 9.16; H2 7.43; H4 7.7; H5 7.15; H6 7.24; H7 7.48
Ile ²⁴	8.61	3.71	2.11	γ 1.37; γ(CH ₃) 1.10; δ 1.02
Ala ²⁵	8.27	4.09	1.57	
Lys ²⁶	7.77	4.10	-	-
Ile ²⁷	8.47	4.23	1.97	γ 1.62; γ(CH ₃) 1.15; δ 0.97
Val ²⁸	7.89	3.91	2.40	γ 1.16
Gly ²⁹	8.08	3.79		
Ser ³⁰	7.79	4.55	4.12, 3.99	
Ile ³¹	7.62	4.28	2.02	γ 1.64, 1.30; γ(CH ₃) 1.03; δ 0.94
-NH ₂				7.25, 6.14

2- NMR data of peptide g

Table S2. ¹H-NMR chemical shifts of peptide g.

Residue	NH	α	β	other
Ac ⁰				2.15
Lys ¹²	7.98	4.08	1.88, 1.79	γ 1.52; δ 1.64; ε 3.05
Arg ¹³	7.93	4.12	1.98, 1.93	γ 1.78; δ 3.26; NHε 7.00
Leu ¹⁴	7.31	4.25	1.87, 1.72	γ 1.67; δ 1.03, 0.95
Lys ¹⁵	7.78	4.18	1.99	γ 1.61, 1.50; δ 1.77; ε 3.05
Lys ¹⁶	7.62	4.16	1.93, 1.86	γ 1.38; δ 1.67; ε 3.05
Ile ¹⁷	7.92	3.93	-	γ 1.01
Gly ¹⁸	8.17	3.86		
Lys ¹⁹	7.71	4.15	1.98	γ 1.52; δ 1.79, 1.67; ε 3.06
Val ²⁰	7.49	4.21	1.92	γ 0.96, 0.91
Leu ²¹	7.83	3.93	2.30	γ 1.52; δ 1.12, 1.06
Lys ²²	8.10	4.25	1.81	γ 1.50; δ -; ε 3.06
Trp ²³	7.78	4.73	3.14	H1 9.10; H2 7.21; H4 7.18; H5 7.70; H6 7.24; H7 7.46
Ile ²⁴	7.49	4.20	1.93	γ 1.21
-NH ₂				6.89

Figure S1. Fingerprint region of the NOESY spectrum (600 MHz) of peptide **g** in TFE solution (c= 1.2 mM, T=308 K)

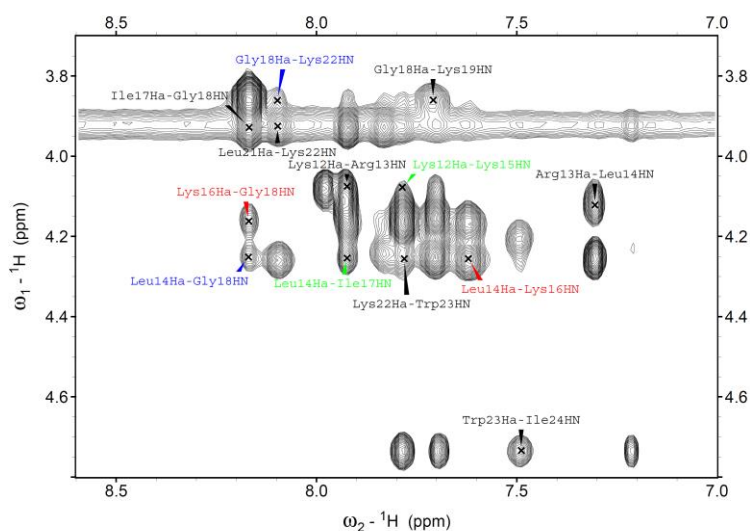
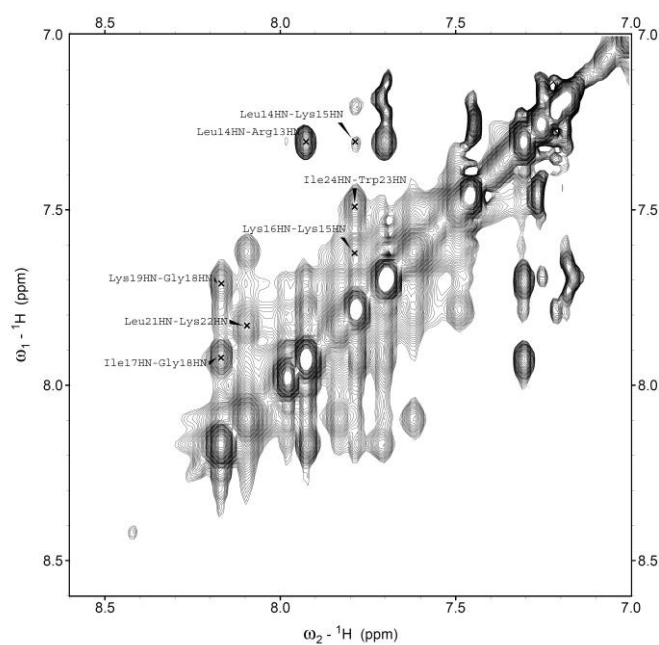


Figure S2. Amide NH region of the NOESY spectrum (600 MHz) of peptide **g** in TFE solution (c= 1.2 mM, T=308 K)



3- NMR data of peptide h

Table S3. ^1H -NMR chemical shifts of peptide **h**.

Residue	NH	α	β	other
Ac ⁰				2.14
Lys ¹²	8.01	4.07	1.88	γ 1.53; δ 1.64; ϵ 3.05
Arg ¹³	7.34	4.25	1.86	γ 1.69; δ 3.07; NH ϵ 7.31
Leu ¹⁴	7.95	4.12	1.94	γ 1.77; δ -
Lys ¹⁵	7.65	4.28	1.98	γ 1.51; δ 1.67, 1.77; ϵ 3.05
Lys ¹⁶	7.76	4.19	1.98	γ 1.60, 1.50; δ 1.77; ϵ 3.04
Ile ¹⁷	7.85	3.97	1.98	γ 1.02
Gly ¹⁸	8.08	3.91		
Lys ¹⁹	7.25	4.16	1.81	γ 1.33; δ 1.65; ϵ 3.38; NH ϵ 7.10
Val ²⁰	7.68	9.91	2.25	γ 1.11, 1.06
Aib ²¹	8.07	-	1.54, 1.48	
Lys ²²	7.66	4.16	1.95	γ 1.67, 1.51; δ 1.77; ϵ 3.06; NH ϵ 7.03
Trp ²³	7.94	4.76	3.45, 3.38	H1 9.11; H2 7.25; H4 7.13; H5 7.72; H6 7.25; H7 7.45
Ile ²⁴	7.51	4.25	1.97	γ 1.25; $\gamma(\text{CH}_3)$ 0.97; δ 0.91
-NH ₂				7.00, 6.00

Figure S3. Amide NH region of the NOESY spectrum (600 MHz) of peptide **h** in TFE solution ($c = 1.2$ mM, $T = 308$ K)

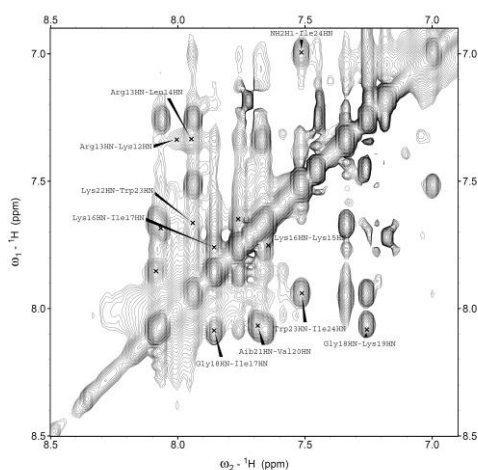


Figure S4. Fingerprint region of the NOESY spectrum (600 MHz) of peptide **h** in TFE solution ($c = 1.2$ mM, $T = 308$ K). $\text{C}^\alpha\text{H}_i \rightarrow \text{NH}_{i+2}$ and $\text{C}^\alpha\text{H}_i \rightarrow \text{NH}_{i+3}$ cross-peaks are highlighted in red, and green, respectively.

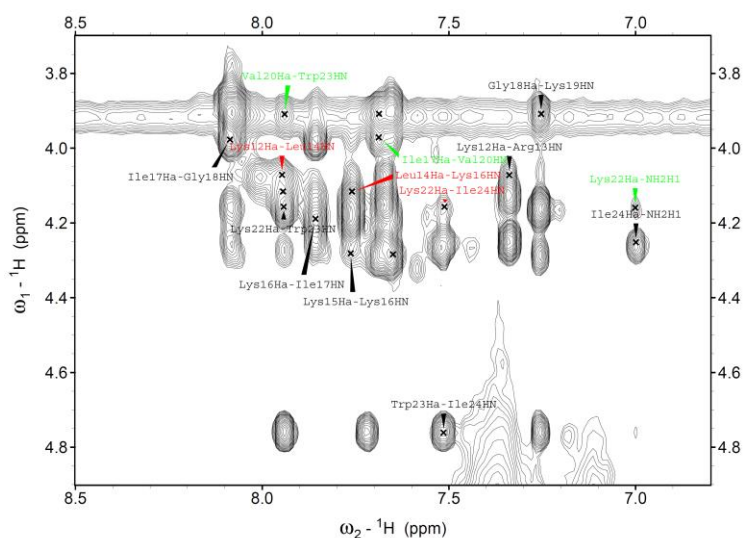
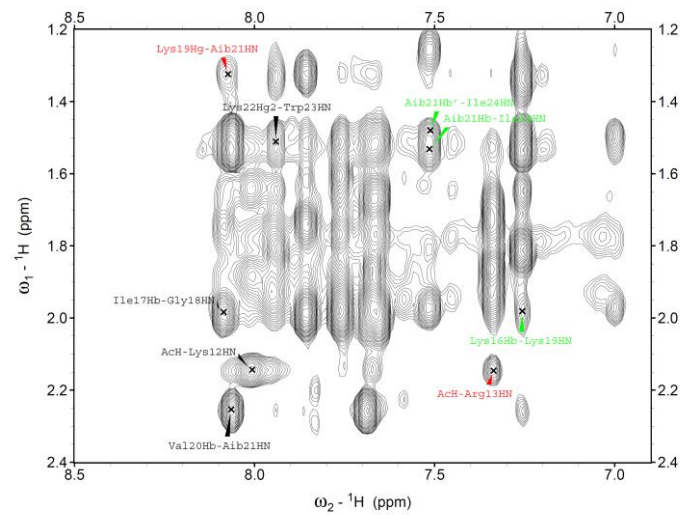


Figure S5. Region of the NOESY spectrum (600 MHz) of peptide **h** in TFE solution ($c= 1.2$ mM, $T=308$ K). $C^\beta H_i \rightarrow NH_{i+2}$, $C^\beta H_i \rightarrow NH_{i+3}$ are highlighted in red and green respectively.



4. Evaluation of solubility and aggregation

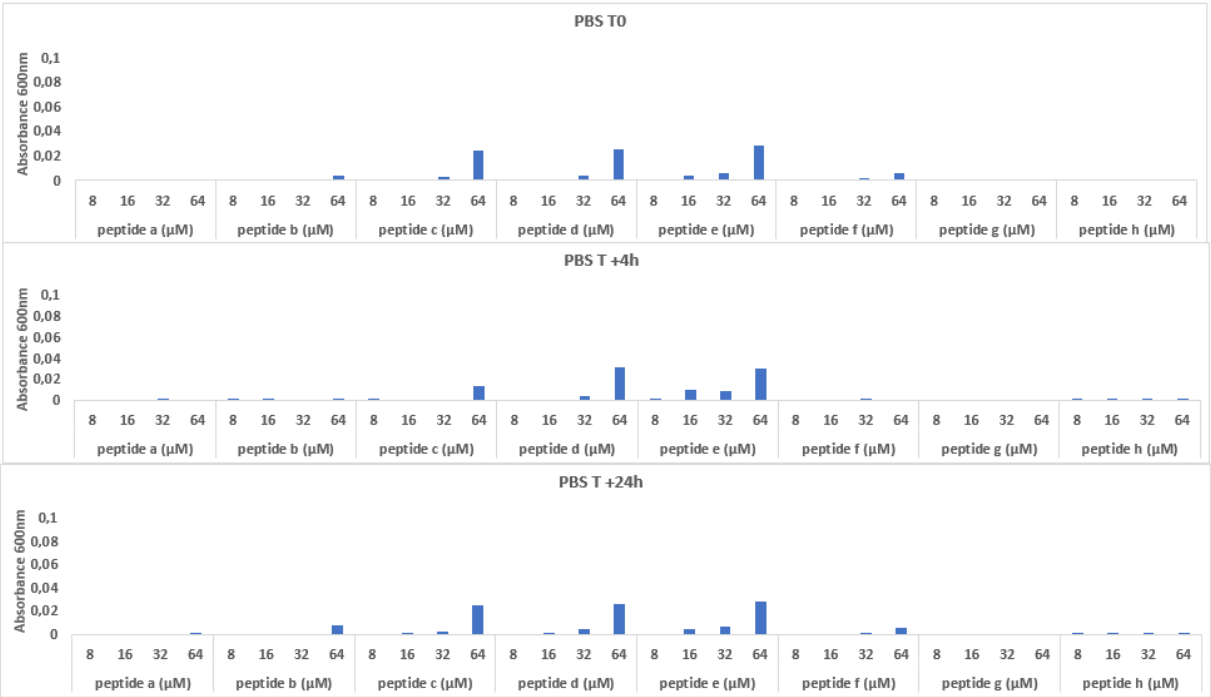


Figure S6 Solubility of PMAP-36 derivatives at different concentrations in PBS after 4h and 24 h incubation

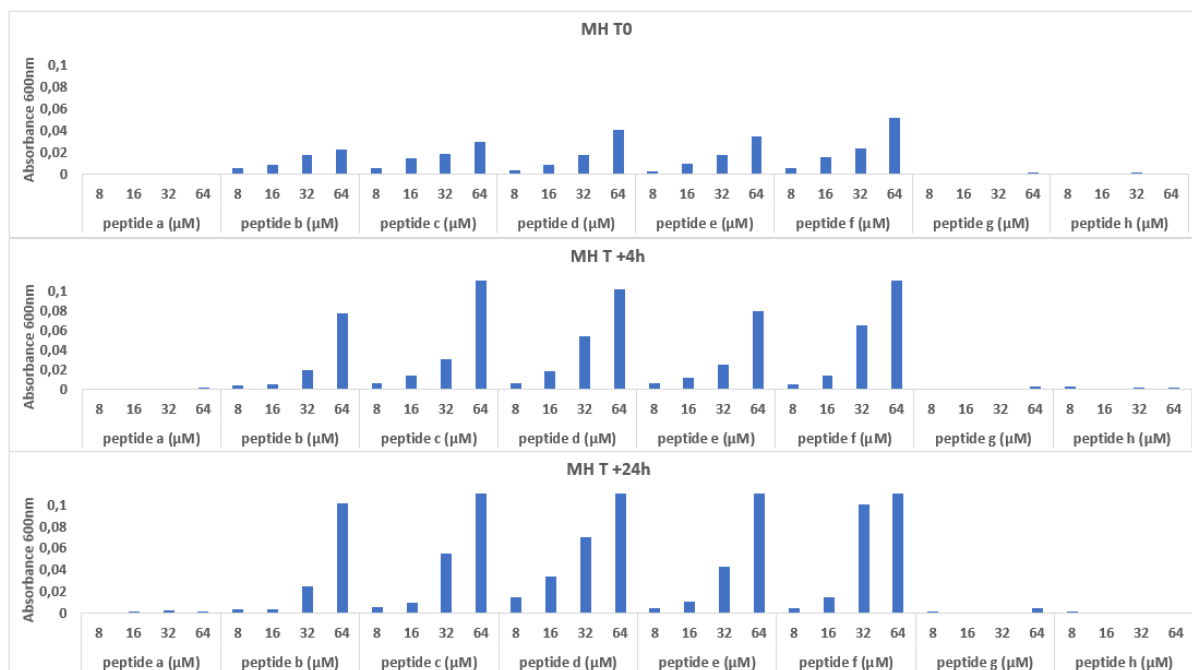


Figure S7 Solubility of PMAP-36 derivatives at different concentrations in completed Mueller-Hinton broth after 4h and 24 h incubation

5. Peptide stability

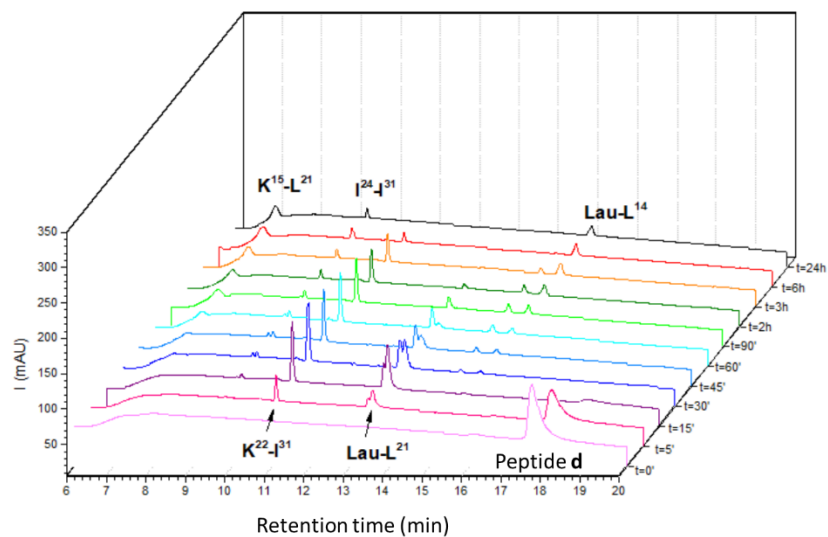


Figure S8. HPLC profile with labeled peaks corresponding to the hydrolysis products for peptide d.

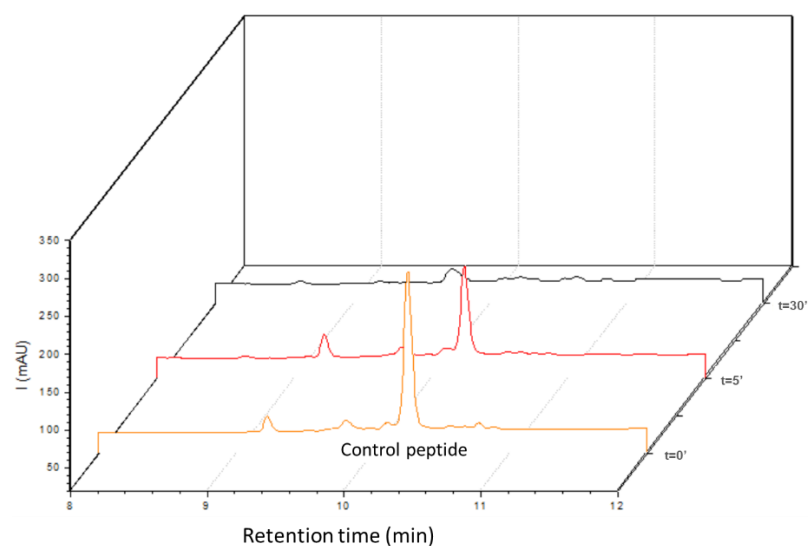


Figure S9. HPLC profile for the proteolytic degradation of the control peptide.

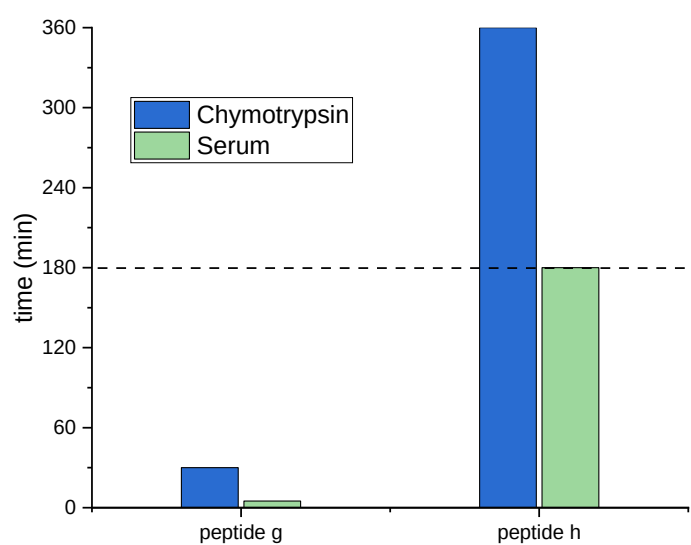


Figure S10. Proteolytic resistance of peptide **g** and **h** in the presence of chymotrypsin (blue) or serum (green). The bars indicate the final time when the HPLC-MS signal of the peptides is still detectable in solution.

6. Peptide selectivity

Table S4. Selectivity index (SI) of the tested peptides for each bacterial species based on cytotoxicity

Peptide	Selectivity index (IC ₅₀ /MIC)						
	IC ₅₀ * (μM)	<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923	<i>A. baumannii</i> ATCC 19606	<i>K. pneumoniae</i> ATCC 700603	<i>P. aeruginosa</i> ATCC 27853	<i>S. epidermidis</i> ATCC 12228
a	> 64	> 32	> 1	> 64	> 64	> 16	> 32
b	13.2	3.3	3.3	6.6	1.6	0.8	3.3
c	12.9	0.8	0.8	1.6	0.8	0.4	1.6
d	< 8	< 0.2	< 0.2	< 0.5	< 0.2	< 0.2	< 0.2
e	< 8	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
f	< 8	< 1	< 1	< 2	< 0.5	< 0.5	< 1
g	> 64	> 16	n.a.	> 32	> 8	> 8	> 16
h	54.1	13.5	0.8	27.1	6.8	6.8	6.8

SI are reported as ratio between cytotoxicity and MIC

* The IC₅₀ was considered as the peptide concentration at which cell viability is reduced by 50% compared to the untreated control.

n.a. indicates conditions where both numbers were not available but there was only the indication > than 32 μM or 64 μM.

Table S5. Selectivity index (SI) of the tested peptides for each bacterial species based on haemolysis.

Peptide	Selectivity index (MHC/MIC)						
	MHC** (μM)	<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923	<i>A. baumannii</i> ATCC 19606	<i>K. pneumoniae</i> ATCC 700603	<i>P. aeruginosa</i> ATCC 27853	<i>S. epidermidis</i> ATCC 12228
a	> 32	> 16	> 0.5	> 32	> 32	> 8	> 16
b	8	2	2	4	1	0.5	2
c	< 4	< 0.2	< 0.2	< 0.5	< 0.2	< 0.1	< 0.5
d	< 4	< 0.1	0.1	0.2	0.1	< 0.1	0.1
e	7,3	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2	< 0.2
f	< 4	< 0.5	< 0.5	< 1	< 0.2	< 0.2	< 0.5
g	> 32	> 8	n.a.	> 16	> 4	> 4	> 8
h	> 32	> 8	n.a.	> 16	> 4	> 4	> 4

SI of the tested peptides for each bacterial species are reported as ratio between minimal haemolysis concentration (MHC) and MIC.

**MHC was taken as the lowest concentration of peptides which induced 10% of haemolysis of RBC.

n.a. indicates conditions where both numbers were not available but there was only the indication > than 32 μM or 64 μM.

4. Designing New Chimeric Proline-Rich Antimicrobial Peptides to Enhance Efficacy Toward the ESKAPE+E: Beyond Sequence Extension

This chapter introduces a research article (and its supplementary materials) published on Biomolecules in 2025:

Adriana Di Stasi †, **Luigi de Pascale** †, Martino Morici, Daniel N. Wilson, Marco Scocchi, and Mario Mardirossian - († These authors contributed equally to this work). **Designing New Chimeric Proline-Rich Antimicrobial Peptides to Enhance Efficacy Toward the ESKAPE+E: Beyond Sequence Extension**. Biomolecules 2025, 15(6), 776; <https://doi.org/10.3390/biom15060776>

The second study focused on the synthetic 16-amino-acid proline-rich antimicrobial peptide (PrAMP) B7-005, which shows strong activity primarily against *Enterobacteriaceae* and other Gram-negative bacteria, though its potency is lower compared to that of other longer, naturally occurring PrAMPs. The goal of the research was to improve B7-005's antimicrobial effectiveness, particularly against ESKAPE pathogens, while preserving its favourable biocompatibility and mode of action. To achieve this, we extended the peptide at the C-terminus by adding 6 or 11 amino acids derived from cetacean PrAMPs TurlA and Lip1, as well as from the bovine PrAMP Bac7(1-35). These three peptides were selected due to their high antimicrobial activity, shared mechanism of action as translation inhibitors and close homology. Despite the high sequence similarity, a divergent C-terminal region of TurlA and Lip1 was thought to contribute to their broader antimicrobial spectrum (188). The resulting chimeric peptides were assessed in terms of their antimicrobial and bactericidal efficacy, cytotoxicity, and underlying mechanism of action.

As co-first author, I contributed substantially to both the experimental and conceptual components of this study. My experimental activities included peptide preparation, solubility testing, bacterial culture handling, antimicrobial activity assays, analysis of bacterial membrane integrity, *in vitro* cytotoxicity assays and statistical analysis, as detailed in the *Materials and Methods* section. In parallel, I was actively involved in study design and methodological planning, and contributed to data curation, analysis, and interpretation. I also participated in writing the initial draft of the manuscript and in its critical revision and editing.

Article

Designing New Chimeric Proline-Rich Antimicrobial Peptides to Enhance Efficacy Toward the ESKAPE+E: Beyond Sequence Extension

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Abstract: Proline-rich antimicrobial peptides (PrAMPs) primarily exert their antimicrobial effects intracellularly, inhibiting protein synthesis. B7-005, a synthetic 16-amino acid PrAMP, has a broader antimicrobial spectrum compared to native counterparts, despite shorter PrAMPs typically exhibiting reduced activity. This study aimed to enhance B7-005's potency by extending it with 6 or 11 amino acids derived from the C-terminal sequences of cetacean Tur1A and Lip1 PrAMPs, as well as bovine Bac7(1-35). Six chimeric derivatives were evaluated for antimicrobial and bactericidal potency, cytotoxicity, bacterial membrane permeabilization, and in vitro inhibition of protein synthesis. Extending B7-005 with sequences from other PrAMPs increased its activity against most ESKAPE+E pathogens, reducing minimum inhibitory concentration (MIC) values by 2- to 8-fold, with notable differences among bacterial species, without increasing cytotoxicity toward the A549 cell line. All chimeras retained the ability to inhibit protein synthesis in *Escherichia coli* and to modestly perturb the *E. coli* membranes like B7-005. These novel chimeric PrAMPs, particularly the 22-mer derivatives, hold promise for developing new antimicrobial agents. The study also highlights variability in bacterial responses to PrAMPs and underscores how minor sequence differences can significantly impact efficacy against specific microorganisms. PrAMPs thus represent a valuable scaffold to rationally design derivatives targeting high-priority pathogens.

Keywords: drug design; antimicrobial peptide; proline-rich peptide; ESKAPE+E; protein synthesis; antibiotic resistance



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1. Introduction

Antimicrobial peptides (AMPs) are small, bioactive proteins naturally produced by organisms across all three domains of life [1]. In prokaryotes, AMPs function as competitive inhibitors against other microorganisms [2], while in eukaryotes, they are essential components of the innate immune system, directly killing pathogens or modulating immune responses [3]. The rising interest in AMPs as potential therapeutic agents [4] is driven by the global challenges of antibiotic resistance and re-emerging infectious diseases [5,6]. Developing alternatives to traditional antibiotics could significantly enhance public health, improve healthcare systems, and yield economic benefits.

Most AMPs are short, positively charged peptides composed of 10–50 amino acids, with a net positive charge ranging from +2 to +11 [3]. They are characterized by approximately 50% hydrophobic residues, essential for their interaction with bacterial membranes, their primary target [7].

Proline-rich antimicrobial peptides (PrAMPs) are a distinct subclass of AMPs, found in arthropods and mammals, with unique features. These peptides are less hydrophobic than typical AMPs, rich in proline and arginine residues and primarily act intracellularly as protein synthesis inhibitors [8]. At low active concentrations, PrAMPs are internalized into susceptible Gram-negative bacteria via the inner-membrane transport protein SbmA/BacA and secondarily Yjil/MdtM [9]. Once in the cytosol, PrAMP target the bacterial translational machinery by binding the ribosomes at level of exit tunnel and blocking the elongation (e.g., Onc112 and Bac7 fragments) [9] or the termination (e.g., Api137 [10] and Drosocin [11]) step of the protein synthesis. This specific uptake mechanism contributes to their narrower spectrum of activity compared to membrane-targeting AMPs, as they rely on these transporters, which are unevenly distributed among bacterial species [9].

Bac7 is a natural PrAMP isolated from the large granules of bovine neutrophils [12]. Its fragment encompassing the first 35 N-terminal residues of its sequence, Bac7(1-35), was the first mammalian PrAMP demonstrated to inhibit protein synthesis [13]. Efforts to optimize Bac7(1-35) have included further shortening of the sequence that identified Bac7(1-16) as its shortest active fragment significant antimicrobial activity [14]. This short fragment retained also the ability of the longer Bac7(1-35) to inhibit protein synthesis [15], although its spectrum was limited to SbmA/BacA-producing species. With the aim to potentiate the antimicrobial activity of Bac7(1-16), this peptide underwent further optimization processes like systematic sequence modification [16]. This latter approach led to the development of B7-005. This 16-amino acid artificial derivative of Bac7(1-16) has a broadened spectrum of activity encompassing also Gram-positive bacteria and most ESKAPE pathogens [16] while still remaining not cytotoxic [17]. ESKAPE is the collective definition for a group of microorganism of major concern according to the WHO due to antibiotic resistance phenomena (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*) [18,19]. In the light of this situation, the broader antimicrobial spectrum of B7-005 with respect to Bac7(1-16) is therefore desirable and it is attributed to its higher hydrophobicity, which improves its affinity for the bacterial membranes. This feature provides B7-005 with a mechanism for internalization in bacterial cells largely independent of SbmA/BacA [16]. The minor dependency of B7-005 on active transport systems limits the development of resistance in *E. coli* [17] since the mutation or loss of the transport protein is insufficient to strongly decrease the bacterial sensitivity to this peptide [16]. However, the mode of action of B7-005 is complex and flexible. The peptide targets all the ESKAPE pathogens, although with diverse antimicrobial efficacy. Similarly, the efficacy of B7-005 in inhibiting the ribosomal activity in vitro also varied among ESKAPE microorganisms. For most of the ESKAPE the inhibition of the protein synthesis, in fact, probably concurs to various extents to the antimicrobial mechanism of B7-005, which instead relies also, and predominantly, on the destabilization of the bacterial membrane [20]. Anyhow, B7-005 probably suffers from the limitations of shortened PrAMPs that often exhibit valuable antimicrobial potency but at lower levels than that of their longer native counterparts. This, nevertheless, suggests potential for further optimization by re-elongating the already optimized shorter PrAMPs. The cetacean PrAMPs Tur1A, Bal1, and Lip1, are homologous to Bac7(1-35) and, similarly to their bovine orthologue, they are inhibitors of the protein synthesis [21]. While they share high sequence similarity in the N-terminal 18 amino acids, their C-terminal regions diverge significantly from that of Bac7(1-35) [21]. Since cetacean PrAMPs exhibit a broader

spectrum of activity compared to Bac7(1-35), this property is likely attributable to their divergent C-terminal sequences. Consequently, these C-terminal sequences could serve as valuable tools to enhance the functionality of existing PrAMPs.

This study aimed to enhance the potency and range of efficacy of the 16-amino acid B7-005 peptide by extending it with 6 or 11 amino acids from the C-terminal sequences of the cetacean Tur1A and Lip1, as well as from the bovine Bac7(1-35) PrAMPs. Six hybrid derivatives were generated and evaluated for their antimicrobial potency, killing efficiency, and cytotoxicity. Their mechanism of action was also investigated, focusing on bacterial membrane permeabilization and in vitro inhibition of protein synthesis. The extension of B7-005 with sequences from other PrAMPs led to a differential and species-specific improvement of their antimicrobial properties against both Gram-positive and Gram-negative bacteria. These results offer valuable insights for designing more effective antimicrobial agents.

2. Materials and Methods

2.1. Peptides

All peptides were synthesized by NovoPro Bioscience (Shanghai, China) using solid-phase F-moc chemistry, purified by RP-HPLC to >95% purity, and their molecular weight was confirmed via mass spectrometry. The peptides were shipped lyophilized. Upon arrival, peptides were resuspended in 500 μ L of 10 mM HCl and lyophilized three times to replace TFA with a less toxic chloride counterion. The peptides were then dissolved in sterile milli-Q water and quantified using absorbance at 214 nm and 280 nm based on Lambert–Beer’s law. The molar extinction coefficient of peptides at 214 nm was calculated according to Kuipers and Gruppen [22] with small modifications. The peptides were stored at -20°C .

2.2. Solubility Test

The solubility of the peptides in Müller–Hinton broth (MHB) (Difco Inc., Becton Dickinson, Sparks, MD, USA) was assessed by measuring any increase in the optical density (OD) over time at 600 nm, using the Nanoquant Infinite-M200Pro plate reader (Tecan Trading AG, Switzerland). Peptides diluted to several concentrations in MHB were incubated in a 96-well plate (Sarstedt, Milan, Italy) at 37°C , and OD was measured at 0, 4, and 24 h. Results are presented as the mean $\pm$ standard deviation (SD) of three independent experiments ($n = 3$).

2.3. Bacterial Cultures

Reference bacterial strains, including *Escherichia coli* BW25113, *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, were obtained from the American Type Culture Collection (ATCC) in Manassas, VA, USA. Strains were stored at -80°C in glycerol stocks until use. For all experiments, cultures were grown in sterile MHB (Difco Inc., Becton Dickinson, Sparks, MD, USA). Overnight cultures were incubated at 37°C for approximately 18 h under shaking (140 rpm, then diluted 1:30 in fresh MHB and again shaken at 140 rpm for ~ 2 h to reach the mid-log phase (OD ~ 0.3 at 600 nm). The bacterial cultures were then diluted to the desired concentration for experiments. *E. coli* BW25113 $\Delta sbmA$ (Keio collection [23]) was grown in MHB with the addition of 50 $\mu\text{g}/\text{mL}$ kanamycin (Sigma, Milan, Italy).

2.4. Antimicrobial Activity

Antimicrobial activity was assessed using the Minimum Inhibitory Concentration (MIC) assay, following CLSI guidelines. Briefly, peptides were serially diluted in MHB across a 96-well round-bottom plate (Sarstedt, Milan, Italy), starting with 50 μ L in the first well. A mid-log bacterial culture was diluted to 5×10^5 CFU/mL, and 50 μ L of this suspension was added to each well (except the MHB sterility control wells). Plates were incubated at 37 °C for 18 h, and the MIC was defined as the lowest peptide concentration that completely inhibited visible bacterial growth. MIC values were confirmed by at least three independent experiments ($n \geq 3$).

The Minimal Bactericidal Concentration (MBC) was determined by plating 25 μ L from clear MIC wells onto MH-agar Petri dishes (Difco Inc., Becton Dickinson, Sparks, MD, USA), followed by overnight incubation at 37 °C. The MBC was the lowest compound concentration, reducing bacterial viability by $\geq 99.9\%$. Results were based on at least three independent experiments ($n \geq 3$).

2.5. Analysis of Bacterial Membrane Integrity

Bacterial membrane integrity was analyzed using flow cytometry (Attune NxT[®], ThermoFisher, Waltham, MA, USA) by measuring propidium iodide (PI) uptake, with permeabilization expressed as the percentage of PI-positive cells. Bacterial cultures of *E. coli* ATCC 25922 (2.5×10^5 CFU/mL) were incubated at 37 °C for 30 min with peptides at $\frac{1}{2}$ MIC, MIC, and $2 \times$ MIC concentrations. PI (10 μ g/mL) was added 5 min before analysis. Colistin at $\frac{1}{2}$ MIC, MIC, and $2 \times$ MIC served as a positive control, while water replaced the peptide in the negative controls. Data were collected using Attune NxT software and presented as means $\pm$ SD from three independent experiments ($n = 3$).

2.6. In Vitro Translation Assays

To assess translation inhibition by the compounds in this study, an *E. coli* cell-free lysate-based translation system (RTS100 *E. coli* HY; biotechrabbit) was employed for firefly luciferase (fluc) expression, as previously described for other translation-inhibiting antibiotics [24]. Briefly, 5 μ L reactions were prepared according to manufacturer protocol, mixed with 1 μ L of the tested compound at the indicated final concentrations. Each reaction mix also contained purified fluc mRNA (16 ng/ μ L), whose translation product was exploited as a reporter. The reactions were incubated for 30 min at 32 °C at 600 rpm and were stopped by adding 3 μ L of kanamycin (50 μ g/ μ L) to each reaction tube. The stopped reactions were transferred to a black 96-well chimney flat-bottom microtiter plate and mixed with 40 μ L of fluc substrate (Promega, Madison, WI, USA). Luminescence was measured using a TECAN infinite 200Pro plate reader (Tecan Trading AG, Switzerland). Samples were normalized relative to reactions without antibiotics.

2.7. In Vitro Cytotoxicity Assay

The human lung carcinoma epithelial cell line (A549) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). A549 cells were cultured in adhesion flasks (EuroClone, Milan, Italy) with complete Dulbecco's MEM (DMEM, EuroClone, Milan, Italy) containing high glucose, supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin (Sigma, Milan, Italy), 2 mM L-glutamine, and 10% FBS (Life Technologies, Carlsbad, CA, USA). Cells were incubated at 37 °C with 5% CO₂.

Cytotoxicity in A549 cells was assessed using the MTT assay. After reaching confluence, cells were counted using a Bürker–Türk chamber and diluted to 2×10^5 cells/mL in DMEM. A total of 100 μ L of this suspension was seeded into 96-well plates (Sarstedt, Milan, Italy) and incubated overnight at 37 °C with 5% CO₂. The next day, the spent medium was

replaced with 100 μ L of fresh DMEM medium containing peptide or water at the desired concentration. After 20 h of incubation, 25 μ L of 1 mg/mL MTT (Sigma, Milan, Italy) in PBS was added, and the plates were incubated for 4 h in the dark. The solution was then removed, wells washed with PBS, and 100 μ L of IGEPAAL (10% *w/v* in 10 mM HCl) (Sigma, Milan, Italy) was added to dissolve the MTT crystals. After overnight incubation, absorbance was measured at 570 nm using a Nanoquant Infinite-M200Pro plate reader (Tecan Trading AG, Switzerland). The cytotoxicity was calculated by comparing the OD of treated samples with that of untreated controls. The results are the average $\pm$ SD of at least three independent experiments in internal triplicate ($n = 9$).

2.8. Statistical Analysis

To assess significant differences between the experimental groups, a one-way ANOVA followed by Dunnett's multiple comparisons test or a two-way ANOVA followed by Tukey's multiple comparisons test was performed. A p -value of ≤ 0.05 was considered statistically significant. The software GraphPad 10 was used for statistical tests.

3. Results

3.1. Design and Characteristics of Chimeric Peptides

To design peptides potentially endowed with stronger antimicrobial activity than B7-005 but, on the other hand, characterized by short sequences (simplifying synthesis procedures), 6- and 11-amino acid residue extensions were appended to the already rationally modified PrAMP B7-005. The 16-amino acid peptide was extended by adding C-terminal fragments derived from the internal sequences of three natural mammalian PrAMPs, Bac7(1-35), Tur1A and Lip1, selected for their antimicrobial potency and activity spectrum. Specifically, we chose 6- and 11-amino acid fragments from the 17th position onward (i.e., residues 17–22 and 17–27) of Bac7(1–35) to design B7-B6 and B7-B11, respectively. Similarly, we selected the residues 17–22 and 17–27 of the Tur1A to obtain B7-T6 and B7-T11 and the corresponding fragments from Lip1 to create B7-L6 and B7-L11 (Table 1).

Table 1. Sequences of the chimeric peptides designed for this study. The residues added to the B7-005 sequence are in bold.

Peptide	Sequence	Residues	% of Positive Charges	% Proline	GRAVY Index
B7-005	WRIRRRWPRLPRPRWR	16	50	18.8	−2.20
B7-B6	WRIRRRWPRLPRPRWR PLPFPR	22	41	27.3	−1.72
B7-T6	WRIRRRWPRLPRPRWR PRFPPP	22	41	31.8	−1.97
B7-L6	WRIRRRWPRLPRPRWR PWFPPR	22	41	27.3	−1.94
B7-B11	WRIRRRWPRLPRPRWR PLPFPRPGPRP	27	37	33.3	−1.76
B7-T11	WRIRRRWPRLPRPRWR PRFPPPFPIPR	27	37	33.3	−1.62
B7-L11	WRIRRRWPRLPRPRWR PWFPPRFPIPR	27	37	29.6	−1.59

The extensions were decided to maintain the overall chemico-physical properties conferred by the residues but modify their position. Moreover, when possible, a C-terminal Arg residue was granted to help solubility and discourage aggregation among lipophilic moieties. In fact, the overall proline content and the net positive charge were maintained consistently among the six chimeras (Table 1). All six chimeras exhibited a lower percentage of positively charged arginines (37–40 versus 50% in B7-005) and a higher percentage of proline residues (27–33% versus 18.8% in B7-005). Also, all chimeras were slightly more hydrophobic than B7-005, with the 11-amino acid-extended peptides B7-T11 and B7-L11 being the more hydrophobic derivatives (Table 1).

Some derivatives had solubility issues when dissolved in Mueller–Hinton broth (MH) at high concentrations. To assess this, we evaluated the tendency of each peptide to precipitate in MH by scattering measurements. We observed that, in a concentration-dependent manner, some peptides tended to aggregate at concentrations between 32 and 64 μM , particularly Lip-derived B7-L6 and B7-L11. However, the phenomenon diminished over time (within 24 h; Figure S1) and did not occur in PBS and in the cell culture medium DMEM (data were negative, not shown here). Consequently, the concentrations of B7-L6 and B7-L11 used in the MIC assays were prudently kept below 64 μM .

3.2. Antimicrobial Activity Against ESKAPE+E Pathogens

The antimicrobial activity of the chimeric peptides was tested toward representative ATCC reference strains of each of the ESKAPE pathogens, as well as of *Escherichia coli*, in full MH broth. Moreover, to get hints on the mode of action of the new peptides, their activity was also evaluated against an *E. coli* strain lacking the membrane transporter SbmA. Minimum inhibitory and bactericidal concentrations (MIC&MBC) were determined (Table 2, compound names are reported without the B7- suffix). In most cases, the chimeric peptides exhibited increased antimicrobial activity compared to the native B7-005. The improvement of antibacterial potency varied according to both the sequence composition and the length of the extension added to B7-005. At least one new derivative displayed a four-fold increase in antimicrobial activity compared with the native B7-005 toward every tested pathogen (Table 2). The only exceptions were the two strains of *E. coli* that were affected similarly by the chimeras and *K. pneumoniae* that displayed the same sensitivity toward all the tested compounds. Specifically, the chimeras B7-B6 and B7-B11 demonstrated a four-fold increase in activity compared to B7-005 against *A. baumannii* and *E. cloacae* and an enhanced activity also against Gram-positive *E. faecium* and *S. aureus*. Similarly, B7-T6 and B7-T11 exhibited a four-fold improvement in efficiency against *E. cloacae* and *A. baumannii*. In addition, B7-T11 showed an eight-fold increase in activity against *E. faecium*.

Lastly, the derivatives B7-L6 and B7-L11 displayed the greatest improvements, with an eight-fold increase against *E. faecium* and a four- to eight-fold increase against *E. cloacae*.

In contrast to the -B and -T derivatives, these compounds also demonstrated enhanced activity (four-fold) against *P. aeruginosa*. Notably, B7-L11 exhibited very high activity also against *S. aureus* (MIC = 4 μM). Interestingly, in all cases, the MBC values were equal to or only two-fold higher than the MIC values, suggesting that all peptides exert a bactericidal effect. The only exception was observed with *E. faecium*, which exhibited MBC values four- to eight-fold higher than the MIC (Table 2).

All the chimeras, as previously observed in B7-005 [16], displayed little or no dependence on the SbmA transporter (Table 2), suggesting that they, like B7-005, may share a similar or identical mode of internalization into the cells, at least in *E. coli* cells.

Table 2. Antimicrobial activity of B7-005 and its derivatives. Representative strains of the ESKAPE+E were used, as well as *E. coli* BW25113 and *E. coli* BW25113 ΔsbmA .

Bacterial Strain		MIC (μM) [§]						
		005 #	B6	T6	L6	B11	T11	L11
<i>E. coli</i>	ATCC 25922	1	2	0.5	4	1	2	2
<i>E. coli</i>	BW 25113	1	1	1	1	0.5	1	1
<i>E. coli</i>	BW 25113 ΔsbmA	2	2	1	2	1	1	2
<i>E. faecium</i>	ATCC 19434	32	8	16	4	16	4	4
<i>S. aureus</i>	ATCC 25923	16	8	16	8	16	16	4
<i>K. pneumoniae</i>	ATCC 700603	2	2	2	2	2	2	2
<i>A. baumannii</i>	ATCC 19606	4	1	1	2	1	1	2
<i>P. aeruginosa</i>	ATCC 27853	16	8	8	4	16	16	8
<i>E. cloacae</i>	ATCC 13047	8	2	2	2	2	2	2

Table 2. Cont.

Bacterial Strain		MBC (μ M)						
		005	B6	T6	L6	B11	T11	L11
<i>E. coli</i>	ATCC 25922	2	2	0.5	4	1	2	2
<i>E. coli</i>	BW 25113	2	1	2	1	1	1	2
<i>E. coli</i>	BW 25113 Δ sbmA	2	2	2	2	1	2	2
<i>E. faecium</i>	ATCC 19434	>64	>64	>64	32	>64	32	16
<i>S. aureus</i>	ATCC 25923	16	16	32	8	32	16	8
<i>K. pneumoniae</i>	ATCC 700603	4	8	8	4	8	4	4
<i>A. baumannii</i>	ATCC 19606	4	2	2	2	2	2	2
<i>P. aeruginosa</i>	ATCC 27853	16	16	32	8	16	16	8
<i>E. cloacae</i>	ATCC 13047	16	4	4	2	4	4	2

The suffix B7- was omitted from all compound names for clarity. § MIC and MBC were recorded after 18 h of incubation at 37 °C. Results are the mode of at least three independent experiments ($n = 3$). The geometric mean of the same results is presented in Table S1. Coloured boxes indicate different antimicrobial activity: high activity (green) low or no activity (red).

3.3. Cytotoxicity Against Human Cell Line A549

To assess whether the different extensions of B7-005 affected the biocompatibility of the new peptides with eukaryotic cells, the human pulmonary cell line A549 was selected as a model to compare the effect of the extension among the new peptides. A549 cells were therefore exposed to an increasing concentration of each peptide for 24 h, and their viability was then evaluated using the MTT assay. None of the peptides exhibited cytotoxic effects up to 32 μ M, except for B7-L11, which also showed toxicity at this concentration. In contrast, B7-B11 did not show any toxicity, even at 64 μ M (Figure 1). Overall, increasing the peptide length *per se* did not markedly decrease the biocompatibility of the peptides. Instead, cell viability was affected by the composition of the extensions.

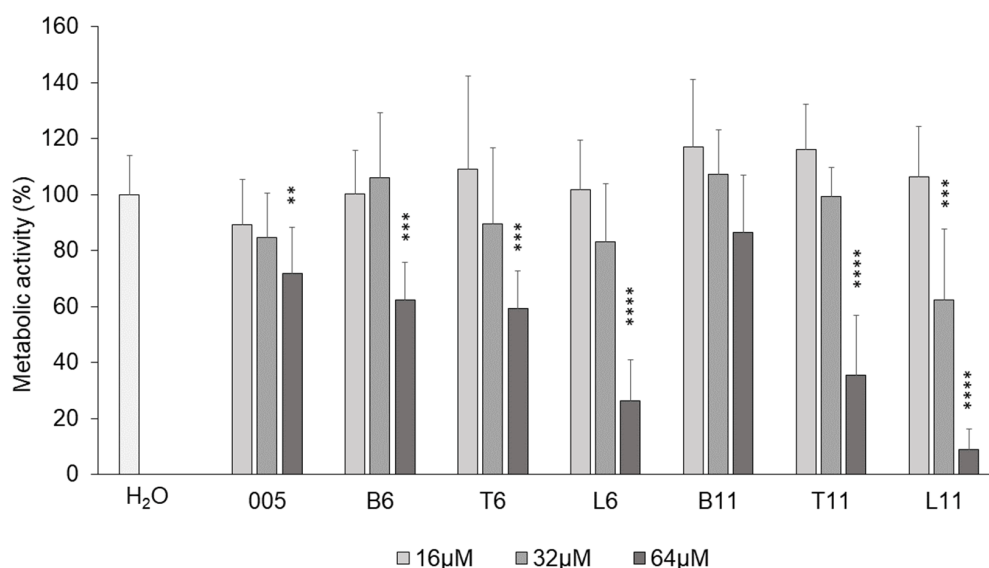


Figure 1. Viability of A549 cells measured by MTT assay in the presence of the peptides at different concentrations. MTT assay on A549 cells after treatment with different concentrations of peptides. The cell viability was measured (as absorbance at 570 nm) after 24 h from the exposure to the peptides. Samples treated with water instead of peptides were set as 100% of viability (H₂O). Error bars are the standard deviations calculated on the average of at least three independent experiments performed as internal triplicates ($n \geq 9$). ** $p \leq 0.005$ vs. H₂O; *** $p \leq 0.0005$ vs. H₂O; **** $p < 0.0001$ vs. H₂O (one-way ANOVA followed by Dunnett's multiple comparisons test).

3.4. Interaction with the Bacterial Membrane

Given the higher hydrophobicity of the chimeric peptides with respect to the B7-005, we investigated whether the new compounds exhibited enhanced membrane destabilization activity. To this aim, membrane permeabilization was evaluated by assessing the propidium iodide (PI) uptake in *E. coli* cells, chosen as a bacterial model. Overall, at both MIC and $2 \times \text{MIC}$, all peptides maintained a low membrane-destabilizing level (PI+ cells < 10–15%), not significantly different from B7-005. Notably, all the chimeric peptides displayed a permeabilization profile markedly different from that of the membranolytic antibiotic colistin, which was used as a reference (Figure 2), suggesting moderate destabilization of the *E. coli* membrane by these compounds.

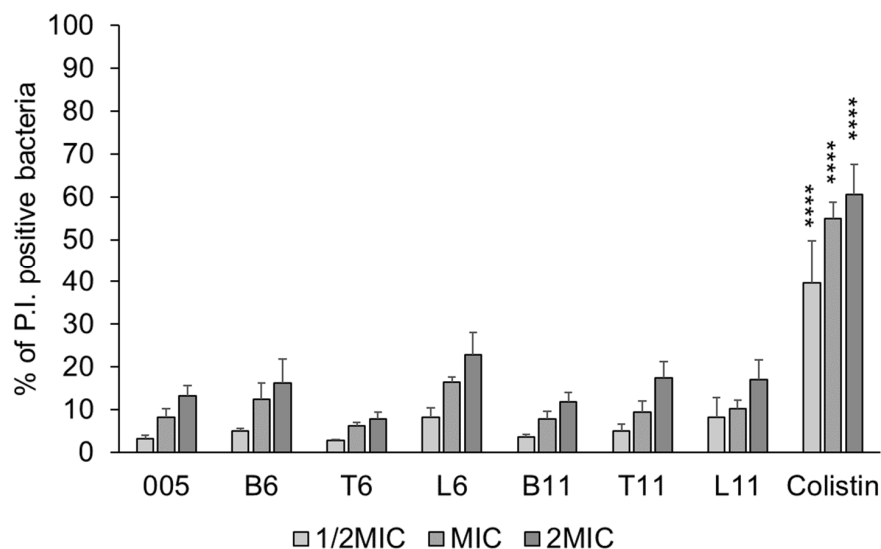


Figure 2. Membrane-damaging activity of the chimeric peptides on *E. coli* ATCC 25922. The original B7-005 and the lytic antibiotic colistin were used as comparisons. The permeabilization assay was performed in MHB, treating for 30 min 2.5×10^5 CFU/mL bacteria with the compounds at $\frac{1}{2}$ MIC, MIC, and $2 \times \text{MIC}$. The % of PI-fluorescent bacterial cells over the whole population was measured. Data are the average $\pm$ SD of three independent experiments ($n = 3$). **** = $p < 0.0001$ vs. B7-005 (two-way ANOVA followed by Tukey's multiple comparisons test).

3.5. Inhibition of Protein Synthesis

B7-005 is an efficient inhibitor of protein synthesis. To ensure that the sequence extensions did not compromise this important feature, the capability of the chimeric peptides to inhibit bacterial synthesis was checked using an in vitro translation assay with *E. coli* lysates. In vitro protein synthesis reactions were performed in the presence of the new peptides and of B7-005 as well to assess their capability to prevent the translation of a synthetic mRNA encoding firefly luciferase. Despite differences in length and composition of the C-terminal extensions appended to B7-005, all chimeric peptides showed very similar IC_{50} , indicating that they maintained the same concentration-dependent inhibitory effect of the original B7-005 toward the *E. coli* translational machinery (Figure 3).

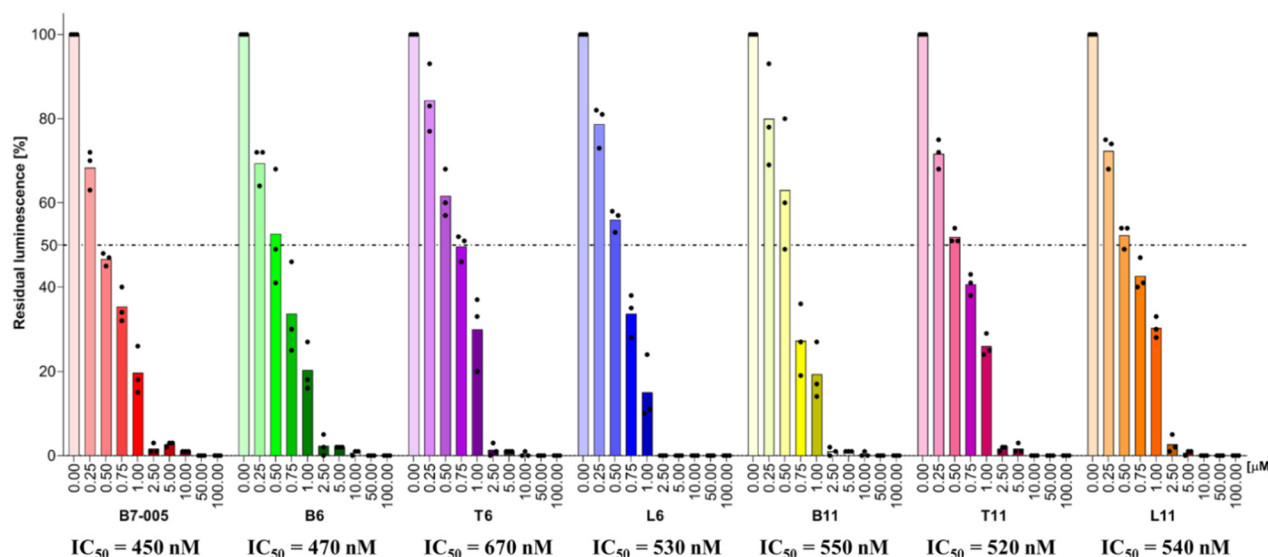


Figure 3. Inhibition of in vitro protein synthesis by B7-005 and chimeric peptides on commercial *E. coli* lysates. The inhibitory effect was evaluated as the residual luminescence signal (%) as a result of the translation of the reporter firefly luciferase. Controls received water instead of peptide and were set as 100% luciferase production. Histograms represent the mean of independent triplicates ($n \geq 3$), whose single results are reported as black dots. The dashed line indicates 50% residual luminescence.

4. Discussion

Previous studies have identified B7-005 as the best derivative among hundreds of 16-amino acid PrAMP analogues due to its broad-spectrum activity and high biocompatibility [16], despite being one of the shortest, although designed, PrAMPs [8]. To evaluate whether prolonging B7-005 could enhance its antimicrobial efficacy while preserving its favorable properties regarding the mode of action and safety, we assessed the antimicrobial activity, biocompatibility, and mode of action of chimeric molecules generated by extending PrAMP B7-005 using sequence stretches from other native mammalian PrAMPs already characterized.

Results of this study provided evidence that the chimeric molecules increase the activity with respect to B7-005 against most ESKAPE pathogens, lowering the MIC values by two- to eight-fold, though with notable differences among the different bacterial species. However, at least one chimera proved to have a greater antimicrobial activity than B7-005 toward at least each of the eight strains tested, with the only exceptions being *E. coli* and *K. pneumoniae*, which remained invariably affected. In this sense, the extension of B7-005 can be considered successful. This result aligns with the fact that most natural PrAMPs are relatively long, with sequences rarely shorter than 19–20 residues and often exceeding 30 residues [8]. One may argue that, for RW-peptides, much shorter sequence length it is sufficient to get the maximum antimicrobial activity [8]. However, on the other hand, we think that the presence of WR-residues in these peptide is not sufficient to determine their mode of action as WR-peptides, but it may just influence or slightly modify their antimicrobial mechanism as PrAMPs. Extending B7-005 to 22 amino acidic residues re-establishes a length comparable to that of several natural PrAMPs. This sequence length could be important to guarantee the efficient inhibition of protein synthesis, as reported for the fragments of the PrAMP Bac5(1-25) [25] and/or to cross efficiently the membrane barrier [8]. On the other hand, quite surprisingly, the mere increase in length from 6 to 11 residues in most cases did not result in a further predictable enhancement of antimicrobial activity.

Analyzing the activity of the single chimeric compounds, no unique trend emerged as a definitive strategy for enhancing PrAMPs potency. Among the tested compounds, we noticed the chimeras B7-B6 and B7-B11, as well as B7-T6 and B7-T11, displayed increased activity compared to B7-005 against the Gram-negative *A. baumannii* and *E. cloacae*, as well as against the Gram-positive *E. faecium* and *S. aureus*. Notably, all the chimeric peptides exhibited no cytotoxicity at concentrations up to 32 μ M, while B7-B11 remained non-cytotoxic even at 64 μ M. Differently, B7-T11, which showed significantly enhanced activity against *E. faecium*, appeared to be cytotoxic at 64 μ M. Since *K. pneumoniae* and *E. coli* are no more susceptible to these four derivatives than to the shorter B7-005, this suggests that the six-residue extension is specifically important for affecting *A. baumannii*, *E. cloacae*, *E. faecium*, and *S. aureus*. However, we cannot exclude that factors other than sequence length contribute to the increased activity of the B7-BX and B7-TX peptides against these four strains. In fact, these derivatives exhibit a higher percentage of proline residues (27–33%) compared to B7-005 (18.8%) and display greater hydrophobicity, as indicated by the GRAVY index values (−1.59 to −1.97 vs. −2.20).

The derivatives B7-L6 and B7-L11, in addition to demonstrating improved activity against *A. baumannii*, *E. cloacae*, *E. faecium*, and *S. aureus*, also exhibited enhanced potency against *P. aeruginosa*. The only peculiar feature of the B7-L series is the presence of a tryptophan (Trp) residue in position 18. The hypothesis that Trp may be critical for specifically targeting these species is supported by previous findings. The simple substitution of threonine (Thr) with Trp in the middle portion of an AMP significantly enhances its activity against *P. aeruginosa*, and this enhancement of activity was species-specific [26]. In another study, Trp end-tagged AMPs exhibited increased potency against *P. aeruginosa* [27]. Trp residues are found in high proportions in many AMPs and are consistently linked to essential functional roles in antimicrobial activity [28]. Although no clear mechanistic explanations exist for the species-specific activity of Trp enriched peptides against *P. aeruginosa* [26], our findings add valuable insights for AMP optimization and for the rational tailoring of antimicrobial agents targeting specific pathogens.

The 27-mer B7-T11 and B7-L11 exhibit the highest antimicrobial activity against *E. faecium*. These two derivatives are the most hydrophobic peptides according to the GRAVY index; therefore, it is plausible that hydrophobicity plays a role in enhancing their antimicrobial activity against *E. faecium*. This observation is in agreement with previous studies. Among some analogues of temporin-SHa with the same number of cationic residues, those displaying the highest activity against *E. faecium* were also the more hydrophobic [29]. Similarly, narrow-spectrum, highly potent, lactoferrin-derived peptides displayed selective activity against *E. faecium* [30]. These peptides are characterized by high hydrophobicity (58% of residues) and strong cationic properties (net charge +5). Since B7-005 is ineffective against *E. faecium* (Table 2 and previous publication [20]), the potent antimicrobial effect of B7-T11 and B7-L11 represents a significant achievement. Unfortunately, B7-L11 is the only peptide showing notable toxicity against the human A549 cell line at 32 μ M, making it less selective than B7-T11. This result is not surprising, as several studies confirm that highly hydrophobic antimicrobial peptides lose selectivity for bacterial cells and become more toxic toward eukaryotic membranes [31,32].

To explain the differences in antimicrobial activity and cytotoxicity of the chimeric PrAMPs against various ESKAPE pathogens, we investigated their mechanism of action in *E. coli* cells. At least toward this microorganism, all the derivatives maintained a similar ability to inhibit protein synthesis in *E. coli*, comparable to that of B7-005. Furthermore, membrane permeabilization data confirmed that all chimeras had only a modest ability to interact with or perturb *E. coli* membranes, with the extent of membrane permeabilization being similar to that of B7-005. These findings suggest a low membrane-permeabilizing

ability of the chimeras within short incubations, especially when compared with the well-known membranolytic peptide colistin. It is worth noting that the current data collected on *E. coli* cannot exclude a major (if not prominent) role of membranolysis in the mode of action of the chimeric peptides toward microorganisms different from *E. coli*, as has been previously observed for the unmodified B7-005 [20]. However, the combined data of protein synthesis inhibition and membrane permeabilization obtained in *E. coli* suggest that, regardless of the mode of action of the peptides against the specific pathogens, the extensions we have added to B7-005 are unlikely to drastically alter the molecular mechanism of the peptide, whatever it may be.

The various chimeras have antimicrobial activity against *E. coli* ATCC 25922 and *E. coli* BW 25113, which is no more than two-fold higher or lower than that of B7-005 (except for B7-L6 against *E. coli* ATCC 25922). Therefore, we conclude that the addition of C-terminal residues does not enhance the activity nor significantly alter the mechanism of action of the peptides toward *E. coli* and likely also *K. pneumoniae*. A peptide length of 16 residues appears sufficient to effectively kill these bacteria. On the other hand, the significant improvement of all the chimeras against *A. baumannii*, *E. cloacae*, *E. faecium*, and *S. aureus*, as well as for the B7-L series against *P. aeruginosa*, might be due to differences in their mechanisms of action. This hypothesis is in agreement with recent findings highlighting that B7-005 exhibits marked differences in its killing mechanisms across different ESKAPE species. Unlike in *E. coli* and *K. pneumoniae*, where B7-005 is internalized at its MIC (1–2 μ M) without relevant membrane permeabilization, the targeting of the bacterial envelope appears to play a prominent role in *S. aureus*, *P. aeruginosa*, *E. cloacae*, *A. baumannii*, and possibly *E. faecium* [20]. This suggests that the chimeras may kill these ESKAPE pathogens through membrane interactions, as often reported also for non-proline rich AMPs [33], requiring them to be longer and/or more hydrophobic to achieve greater activity than B7-005.

We do not yet know from structures how these PrAMPs interact with ribosomes from different ESKAPE organisms, thus impairing proper structure–activity relationship considerations. Circular dichroism analysis of B7-005 suggested an uncommon scenario, possibly because of Pro, Trp, and Arg in pushing the structure toward a polyproline II helix or toward turns, depending on the environment [20]. This is also reminiscent of some aspects previously observed for Indolicidin [34]. The structural scenario for the chimeric peptides of the current study may become even more complex due to the extension comprising repeats of Pro, Arg, and also aromatic residues. Further focused studies may elucidate these aspects.

However, structural considerations apart, it is evident that most chimeras, especially the 22-mer derivatives, represent an improvement in antimicrobial activity over B7-005. Notably, B7-005 already exhibits a broader activity spectrum than the natural peptides Bac7(1-35) and Tur1A, which are completely ineffective against both *E. faecium* (MIC = 64 μ M) and *S. aureus* (MIC > 64 μ M) [21]. Therefore, chimeras such as B7-B6 and B7-B11, composed of B7-005 extended with a segment of Bac7(1-35), represent an improvement over both the native peptides, maintaining biocompatibility and SbmA independence. Similarly, B7-T6 and B7-T11, which extend B7-005 with a segment of Tur1A, demonstrated superior properties compared to natural Tur1A, despite the latter being longer than both B7-T6 and B7-T11. For B7-L6 and -L11, the benefits compared to Lip1, which is the donor of the sequence used to extend B7-005, are more limited [21]. This chimeric peptide exhibits improved antimicrobial activity only against *S. aureus*. However, it is worth noting that these chimeric peptides are shorter than Lip1 (22 and 27 residues vs. 32) and exhibit decreased cytotoxicity [35].

5. Conclusions

In conclusion, the design of these novel chimeric PrAMPs has resulted in promising lead compounds for the development of new antimicrobial drugs. This study also highlights the variability in bacterial species' responses to the same PrAMPs and emphasizes how minor structural differences can significantly impact efficacy against the same microorganism. Further and systematic structure–activity relationship studies will be necessary on these (and also other) PrAMPs to clearly identify some aspects of their mode of action that may give valuable hints to the design of antimicrobial drugs. In fact, PrAMPs hold great potential for the rational design of derivatives that selectively target high-priority pathogens, which may contribute to the ongoing efforts to combat the antibiotic resistance crisis.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biom15060776/s1>, Figure S1: Peptides precipitation assay. Table S1: Geometric mean of the MIC and MBC data.

Author Contributions: Conceptualization: M.M. (Mario Mardirossian). Data curation: A.D.S., L.d.P., and M.M. (Martino Morici). Methodology: A.D.S., L.d.P., M.M. (Martino Morici), and M.M. (Mario Mardirossian). Formal analysis: A.D.S., M.M. (Martino Morici), L.d.P., and M.M. (Mario Mardirossian). Funding acquisition: M.M. (Mario Mardirossian), M.S., and D.N.W. Investigation: A.D.S., L.d.P., M.M. (Martino Morici), and M.M. (Mario Mardirossian). Project administration: A.D.S., M.M. (Martino Morici), and M.M. (Mario Mardirossian). Resources: D.N.W., M.S., and M.M. (Mario Mardirossian). Supervision: M.M. (Mario Mardirossian), M.S., and D.N.W. Visualization: A.D.S., L.d.P., and M.M. (Martino Morici). Writing—original draft: M.M. (Mario Mardirossian), M.S., and A.D.S. Writing—review and editing: all the authors. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

FBS	Fetal bovine serum
FluC	Firefly luciferase
GRAVY	Grand average of hydropathicity
IC ₅₀	Half maximal inhibitory concentration
MBC	Minimal bactericidal concentration
MHB	Mueller–Hinton broth
MIC	Minimum inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide assay
OD	Optical density
PBS	Phosphate-buffered saline
PI	Propidium iodide
PrAMPs	Proline-rich antimicrobial peptides
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
SD	Standard deviation
TFA	Trifluoroacetic acid

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Designing New Chimeric Proline-Rich Antimicrobial Peptides to Enhance Efficacy Toward the ESKAPE+E: Beyond Sequence Extension

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Supplementary materials

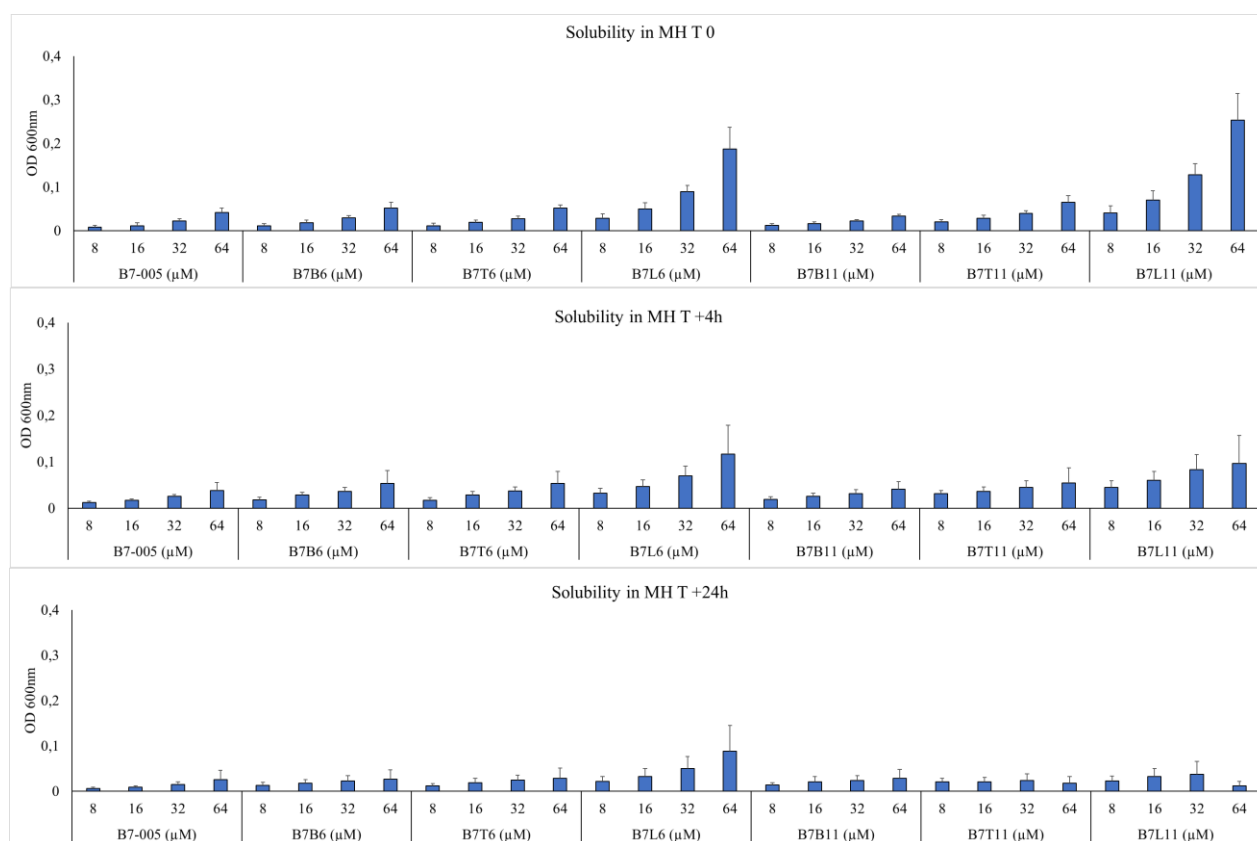


Figure S1. Peptides precipitation assay. Optical density (OD 600 nm) measurement of chimeric peptides solutions at different concentrations in MH broth incubated at 37°C up to 24 hours.

Table S1 Antimicrobial activity of B7-005 and its derivatives. Representative strains of the ESKAPE+E were used, as well as *E. coli* BW25113 and *E. coli* BW25113 Δ *sbmA*.

Bacteria strain		MIC (μ M)						
		B7-005	B7B6	B7T6	B7L6	B7B11	B7T11	B7L11
<i>E.coli</i>	ATCC 25922	1,6	1,6	0,6	3,2	0,8	1,3	2,5
<i>E.coli</i>	BW 25113	1	1	1	1	0,6	1	1,3
<i>E.coli</i>	BW 25113 Δ <i>sbmA</i>	1,6	1,6	1,3	1,6	1,0	1,3	1,6
<i>E.faecium</i>	ATCC 19434	25,4	10,1	16,0	4,0	16,0	5,0	4,0
<i>S.aureus</i>	ATCC 25923	16,0	10,1	16,0	8,0	20,2	12,7	5,0
<i>K.pneumoniae</i>	ATCC 700603	2,0	2,0	2,0	2,5	2,0	2,0	2,5
<i>A.baumannii</i>	ATCC 19606	4,0	1,0	1,0	1,6	0,8	1,3	2,0
<i>P.aeruginosa</i>	ATCC 27853	20,2	10,1	12,7	5,0	12,7	12,7	6,3
<i>E.cloacae</i>	ATCC 13047	10,1	2,0	2,5	2,0	2,0	2,0	2,0

Bacteria strain		MBC (μ M)						
		B7-005	B7B6	B7T6	B7L6	B7B11	B7T11	B7L11
<i>E.coli</i>	ATCC 25922	1,6	1,6	0,6	3,2	1,0	1,3	2,5
<i>E.coli</i>	BW 25113	2,5	2,0	2,5	1,6	1,0	1,3	1,6
<i>E.coli</i>	BW 25113 Δ <i>sbmA</i>	2,0	1,6	2,5	2,0	1,3	1,6	2,0
<i>E.faecium</i>	ATCC 19434	>64	>64	>64	40,3	>64	40,3	16,0
<i>S.aureus</i>	ATCC 25923	20,2	16,0	25,4	10,1	40,3	20,2	8,0
<i>K.pneumoniae</i>	ATCC 700603	5,0	8,0	8,0	3,2	6,3	3,2	3,2
<i>A.baumannii</i>	ATCC 19606	5,0	2,0	2,0	1,6	2,0	1,6	2,0
<i>P.aeruginosa</i>	ATCC 27853	25,4	16,0	25,4	8,0	20,2	20,2	8,0
<i>E.cloacae</i>	ATCC 13047	12,7	4,0	4,0	2,5	4,0	4,0	2,5

[#] The suffix B7- was omitted from all compound names for clarity.

[§] MIC and MBC were recorded after 18h of incubation at 37°C.

Results are the geometric mean of at least three independent experiments (n = 3).

5. Testing antimicrobial peptides inhibiting protein synthesis in living *E. coli* and *K. pneumoniae* using bio-orthogonal non-canonic amino-acid tagging (BONCAT)

This chapter introduces a research article (and its supplementary materials) published on Frontiers in Microbiology in 2025:

Luigi de Pascale, Agnese D'Amore, Adriana Di Stasi, Martino Morici, Sabrina Pacor, Alessandro Tossi, Thuy Duong Pham, Attilio Fabbretti, Daniel N. Wilson, Mario Mardirossian, Marco Scocchi. **Testing antimicrobial peptides inhibiting protein synthesis in living *E. coli* and *K. pneumoniae* using bio-orthogonal non-canonic amino-acid tagging (BONCAT).** *Front. Microbiol.* 16:1713216. <https://doi.org/10.3389/fmicb.2025.1713216>

The third study tackled the challenges associated with conventional approaches for investigating the intracellular activity of antimicrobial peptides (AMPs), including PrAMPs, which are typically studied through *in vitro* and structural studies or complex and often radioactive *in vivo* methods only in *E. coli*. This research aimed to assess the potential of BONCAT (bioorthogonal noncanonical amino acid tagging) to study PrAMPs and potentially other AMPs in inhibiting the protein synthesis, as a rapid, and fluorescence-based alternative that relies on molecular analogues instead of radioactivity. BONCAT works by enabling the specific incorporation of alkyne-functionalized amino acid analogues into newly synthesized proteins (and potentially other macromolecules) within living cells, which can then be visualized through a click-chemistry reaction involving an azide-conjugated fluorophore. The goal was to study the mode of action of AMPs that inhibit protein synthesis *in vivo*, extending the investigation beyond *E. coli* to include also *K. pneumoniae*, overcoming the limitation to test the protocol on a sole bacterial specie as a model.

As the first author of this study, I contributed to the conceptual and methodological design of the work and was actively involved in data curation, analysis, and interpretation. Furthermore, I was primarily responsible for conducting almost all experimental work and contributed to drafting, critically reviewing, revising, and editing the manuscript.



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Testing antimicrobial peptides inhibiting protein synthesis in living *E. coli* and *K. pneumoniae* using bio-orthogonal non-canonical amino-acid tagging

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Introduction: Antimicrobial peptides (AMPs) inhibit bacteria through diverse mechanisms of action. While many AMPs exert their effects by interacting with and damaging bacterial membranes, a growing subset has been shown to target intracellular processes, such as protein synthesis. In this study, we assessed the suitability of the bio-orthogonal non-canonical amino acid tagging (BONCAT) technique to investigate the mechanism of action on protein synthesis of proline-rich antimicrobial peptides (PrAMPs) in living Gram-negative bacteria.

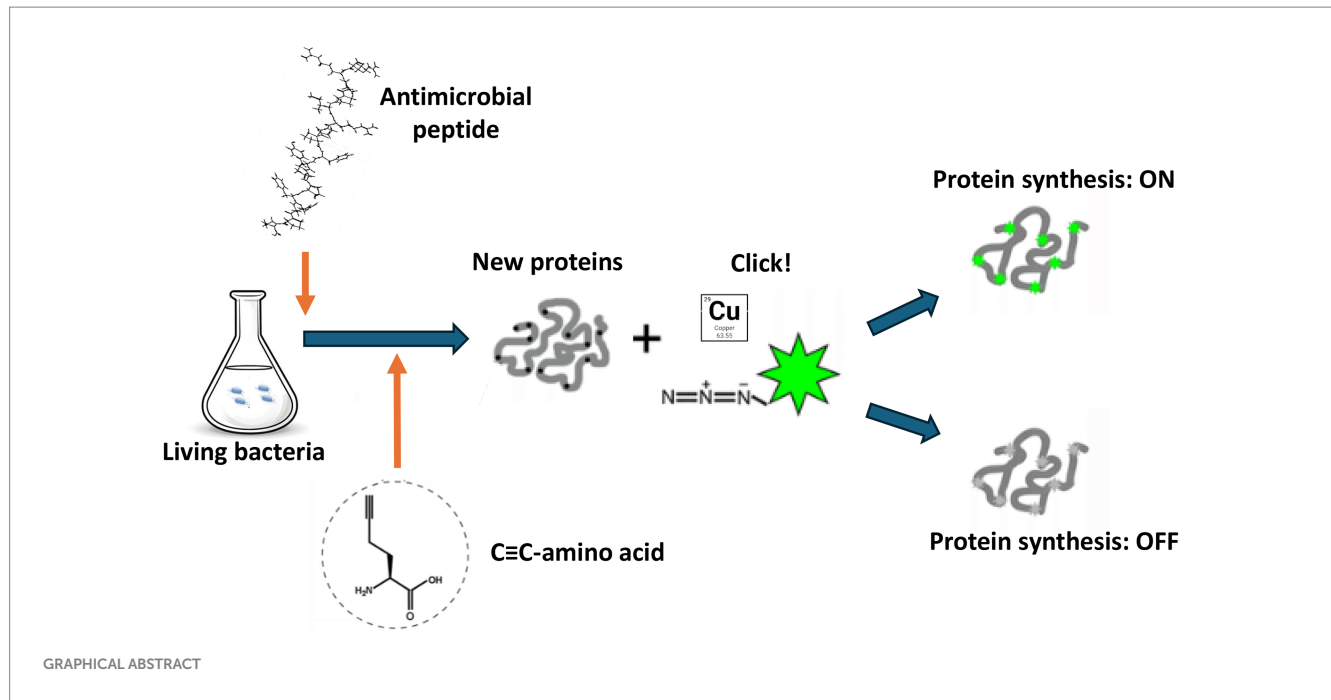
Methods: By combining BONCAT with flow cytometry to evaluate membrane integrity using propidium iodide assay we first validated the effective inhibitory activity of PrAMPs on protein synthesis leaving intact the bacterial inner membrane in both *Escherichia coli* and *Klebsiella pneumoniae*, thus extending beyond *E. coli* as the sole model organism.

Results: We demonstrated that this approach can discriminate between AMP affecting protein synthesis from those with membranolytic activity. We showed that different PrAMPs, significantly reduced the protein synthesis in 10 min, suggesting a very rapid inhibition kinetics. Furthermore, unlike chloramphenicol, PrAMPs demonstrated a prolonged inhibitory effect on protein synthesis even after the peptides were removed from the medium, suggesting a long-lasting post-antibiotic effect.

Discussion: We therefore demonstrated the validity of BONCAT as a tool for studying the molecular mechanisms of PrAMPs and we suggest that, in principle, this method may be extended also to other types of antimicrobial compounds to provide new insights into their mode of action on living bacteria.

KEYWORDS

antimicrobial peptide, protein synthesis, click-chemistry, drug discovery, proline-rich, amino acid analogue



Introduction

Antimicrobial peptides (AMPs), also referred to as host defence peptides, have gained significant attention as potential therapeutic agents for combating infectious diseases (Gera et al., 2022; Magana et al., 2020; Yang et al., 2025) and as innovative immunomodulatory treatments (Mahlpuu et al., 2020). They exhibit strong antibacterial, antiviral, and antifungal activities (Wang, 2014) and are prevalent across multicellular eukaryotes. In fact, most plants and animals express a wide array of AMP genes, particularly in epithelial tissues, and in response to infections (Lazzaro et al., 2020).

While it was once customarily held that AMPs primarily exerted their effects through membrane disruption and permeabilization (Benfield and Henriques, 2020; Travkova et al., 2017), subsequent research has revealed multiple additional mechanisms of action other than disruption of the bacterial envelope (Le et al., 2017; Scocchi et al., 2016; Zhang et al., 2021). Proline-rich AMPs (PrAMPs) are a notable subclass whose mode of action primarily relies on intracellular action with no morphological changes (Anderson et al., 2004; Shi et al., 1996). They can in fact cross cell membranes without damaging them (Podda et al., 2006) and inhibit protein synthesis, resulting in bactericidal activity (Graf and Wilson, 2019; Li et al., 2014; Welch et al., 2020). Other types of AMPs also act internally, for instance indolicidin, has been shown to significantly impact on bacterial DNA synthesis (Hsu et al., 2005), whereas microcin J25 (MccJ25) primarily inhibits RNA polymerase (Mukhopadhyay et al., 2004). Furthermore, other AMPs are suggested to drive the compaction of nucleic acids and modulate phase transitions (Sneideris et al., 2023).

PrAMPs are widespread, having independently evolved in many arthropods as well as in all families of the *Artiodactyla* order of mammals (Tossi et al., 2024), where they function as the active domains of cathelicidins (Scocchi et al., 2011; Welch et al., 2020). PrAMPs exhibit a narrower spectrum of activity compared to membranolytic AMPs, both *in vitro* and *in vivo*, being mainly directed at Gram-negative bacteria (Benincasa et al., 2010; Berthold et al., 2013;

Runti et al., 2017) and without damaging host eukaryotic cells (Armas et al., 2021; Hansen et al., 2012; Li et al., 2014). Structural and biochemical studies on a representative number of insect and mammalian PrAMPs have shown that they enter the cytoplasm of *E. coli* by exploiting bacterial transporters (Krizsan et al., 2015; Mattiuzzo et al., 2007) without perturbation of cell membranes and then bind to the 50S subunit of bacterial ribosomes, thereby inhibiting protein synthesis (Graf et al., 2017). The binding site of all so far investigated PrAMPs is situated within the nascent polypeptide exit tunnel, although distinct inhibitory mechanisms may divide PrAMPs into two classes (I and II), according to the step of the protein synthesis that is impaired (Roy et al., 2015; Seefeldt et al., 2016, 2015).

Our understanding of the intracellular mechanism of action of AMPs, and specifically PrAMPs, has primarily been derived from functional studies (Krizsan et al., 2014; Mardirossian et al., 2020, 2014), structural studies performed using *in vitro* systems (Florin et al., 2017; Gagnon et al., 2016; Raulf et al., 2025; Seefeldt et al., 2016, 2015), and studies using radioactive tracers to treat bacteria (Mardirossian et al., 2018, 2014; Subbalakshmi and Sitaram, 1998). However, all of these studies have been conducted on, and are limited to, *E. coli* (Florin et al., 2017; Mardirossian et al., 2018; Taniguchi et al., 2016), while evidence has emerged suggesting that the mechanism of action of AMPs, including PrAMPs, can vary significantly across different bacterial species (Di Stasi et al., 2025; Oliveira Júnior et al., 2025). The complexity of these techniques and their inherent limitations hinder the assessment of protein synthesis inhibition in the development of peptide analogues with improved properties, discouraging evaluation studies on a large number of compounds and/or testing on a broad range of bacterial species.

Amino acid analogues were successfully employed about a decade ago to detect newly synthesized proteins within bacteria through a click chemistry technique known as Bioorthogonal Non-canonical Amino Acid Tagging (BONCAT) (Hatzenpichler et al., 2014). In this approach, bacterial cells are incubated with methionine analogues, such as L-homopropargylglycine (HPG) or L-azidohomoalanine (AHA), which

carry azide or alkyne groups. Once these analogues are incorporated into newly synthesized proteins, they can be fluorescently labelled via a copper(I)-catalysed azide-alkyne cycloaddition (click chemistry) reaction with corresponding alkyne- or azide-labelled fluorophores. This allows for the visualization of cells actively engaged in protein synthesis through fluorescence (Lindivat et al., 2020) and, by extension, also highlights cells where protein synthesis is inhibited. However, despite BONCAT's broad suitability for studying protein synthesis inhibitors, its application to Proline-Rich PrAMPs remained unexplored.

In this study, we evaluated BONCAT as a versatile tool for studying the inhibitory effects of PrAMPs on translation in Gram-negative bacteria. Using BONCAT coupled with flow cytometry, we monitored the reduced incorporation of fluorophore-labelled L-homopropargylglycine into newly synthesized proteins in *E. coli* and *K. pneumoniae* cells with non-compromised membranes. We analyzed three different PrAMPs in parallel with reference antibiotics possessing well-established molecular targets to validate the application utility in detecting protein synthesis inhibition by PrAMPs. Furthermore, we assessed the timing of inhibition and the post-antibiotic effects of the PrAMPs in *E. coli*. To demonstrate the method's broad utility in mechanistic screening, we combined BONCAT with the PI-assay (Propidium Iodide assay), including colistin and a lytic AMP as controls, thereby establishing a tool for discriminating protein synthesis inhibition from membranolytic activity. Our results indicate that BONCAT is highly effective in investigating the mode of action of PrAMPs and could, in principle, be applied to other types of AMPs or antimicrobial compounds to assess their impact on protein synthesis in living bacteria.

Materials and methods

Peptides and antibiotics

All solid-phase synthesised peptides were purchased from NovoPro Bioscience (Shanghai, China) with a purity of > 95%. PMAP (12–31) was a generous gift from University of Padoa (Biondi et al., 2023). All the peptides were resuspended in 500 μ L of 10 mM HCl and lyophilised three times to remove trifluoroacetic acid (TFA) as a counterion. They were then resuspended in sterile Milli-Q water and quantified spectrophotometrically at 214 nm and 280 nm (Ultraspec 2,100 pro, Amersham Bioscience). The molar extinction coefficients were calculated in accordance with Kuipers and Gruppen (2007). Colistin, chloramphenicol, rifampin and nalidixic acid were purchased from Sigma-Aldrich (USA). Chloramphenicol (CHL) and tetracycline (TET) were dissolved in ethanol (95% v/v) at concentrations of 3.5 mg/mL and 7.5 mg/mL, respectively. Rifampicin (RIF) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at the concentration of 10 mg/mL. Nalidixic Acid (NDX) was dissolved in DMSO at a concentration of 2.5 mg/mL. Colistin (COL) was dissolved in sterile Milli-Q water at 2.5 mg/mL.

Chemicals and solutions

The amino acid analogue homopropargylglycine (HPG) (Kiick et al., 2002) was purchased from Thermofisher Scientific: concentrated solution of 500 μ M was prepared in Milli-Q water from

a stock solution at 100 mM in DMSO. CuSO₄ was purchased from Sigma-Aldrich (USA) and solved in Milli-Q water at 20 mM before being filtered (0.2 μ m filter). Tris(3-hydroxypropyltriazolylmethyl) amine (THPTA) was purchased from Sigma-Aldrich (USA) and dissolved in 0.2 μ m filtered Milli-Q water at 50 mM. Alexa Fluor azide 488 was purchased from Thermofisher Scientific and solved in DMSO at 10 mM. Sodium ascorbate and Aminoguanidine were both purchased from Sigma-Aldrich (USA) and dissolved in Milli-Q water at 100 mM before being filtered (0.2 μ m filter). All the mentioned reagents were stored at -20°C , except for HPG and copper sulfate, both of which were stored at 4°C (the former screened from light exposure).

A premix solution was prepared by mixing 20 mM copper sulfate, 50 mM THPTA and 10 mM Alexa Fluor azide 488 in the ratio 20:40:1. The premix solution was always freshly prepared and incubated for 3 min in the dark at 25°C before use. Propidium iodide, purchased from MERCK LIFE SCIENCE S.R.L, was dissolved in PBS at a concentration of 0.1 mg/mL, and stored in the dark at 4°C .

Bacteria strains and growth conditions

E. coli ATCC 25922, *K. pneumoniae* ATCC 700603 and ATCC 13883, *A. baumannii* ATCC 19606, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, *E. cloacae* ATCC 13047 and *E. faecium* ATCC 19434 were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and from the Leibniz institute DSMZ (Braunschweig, Germany) and were stored at -80°C as glycerol stock until use. M9 Minimal Salts Base 1x (M9 medium base) was prepared diluting sterilized BD Difco™ (New Jersey, U.S.) Dehydrated Culture Media M9 medium base 5x were prepared and stored at 4°C for up to a month. All assays with bacteria were performed in 0.2 μ m filtered M9 minimal salts supplemented with 0.2 μ m filtered 2 mM MgSO₄ and 0.2 μ m filtered 5 mM glucose (supplemented M9 medium).

All bacterial cultures were grown aerobically overnight in a shaker at 130 rpm and 37°C in supplemented M9 minimal salt medium with the addition of 1% (v/v) Mueller Hinton broth (MHB, Difco). The next day, the cultures were washed and resuspended in fresh supplemented M9 medium.

Minimum inhibitory concentration (MIC) assay

Minimum inhibitory concentration (MIC) assay was performed as reported previously (Sola et al., 2020). An overnight culture of *E. coli* ATCC 25922 or *K. pneumoniae* ATCC 700603, prepared as described above was incubated at 37°C until reaching an optical density of approximately 0.5–0.6 at 600 nm. The culture was then diluted in supplemented M9 medium at the concentration of 1×10^7 CFU/mL. Each peptide, diluted at a starting concentration of 128 μ M in supplemented M9 medium was added to the first column of wells of a 96-well round-bottom microplate (Sarstedt, Milan, Italy). Serially two-fold dilution (1:1) were performed across subsequent wells to a final volume of 50 μ L. 50 μ L of bacterial suspension (1×10^7 CFU/mL) were then added to each well, to a final bacterial concentration in each well to 5×10^6 CFU/mL per well. Untreated bacterial culture was used

as growth control, while 100 μ L of uninoculated supplemented M9 medium was used to verify the sterility of the medium. The plates were sealed with parafilm to minimize evaporation and incubated at 37 °C for 18 h. The minimum inhibitory concentration (MIC) was visually determined as the lowest peptide concentration in a well with no visible bacterial growth. Data are reported as the mode of at least three independent experiments.

Bacteria grow rate assays

An overnight culture of *E. coli* ATCC 25922, prepared as described above, was diluted to a final concentration of 5×10^6 CFU/mL and then incubated at 37 °C for 30 min in the presence or absence of each peptide or antibiotic at various concentrations, with a final volume of 1 mL. To measure the post-antibiotic effect (PAE) of antimicrobials, the compounds were removed from the supernatant by centrifugation (16,000 $\times$ g for 5 min) and bacteria were washed and resuspended in 1 mL of fresh supplemented MHB medium, to promote faster growth visible as increased absorbance. 200 μ L of each sample was then transferred to a 96-well round-bottom microplate (Sarstedt, Milan, Italy), which was then placed in a Nanoquant Infinite-M200Pro plate reader with stable temperature at 37 °C. Each sample was surrounded by water-filled wells to reduce its own evaporation. Optical density at 600 nm was then recorded for each sample every hour for up to 18 h. PAE was calculated according to [Holfeld et al. \(2018\)](#) as the difference between the time needed for a cell culture incubated with an antibiotic substance to reach half-maximum absorbance of the control culture and the time that the control culture needed to reach half-maximum absorbance of the stationary phase. According to this definition, the PAE of each compound (expressed as time) was calculated based on the OD₆₀₀ value of its respective replicate, which corresponded to half of the maximum absorbance reached by the control culture in the stationary phase (at 18 h). The mean PAE values (PAE in hours) of each compound, were obtained by averaging the times from the three independent experiments.

Homopropargylglycine incorporation into bacteria

We used a modified protocol from [Lindivat et al. \(2020\)](#). Briefly, overnight cultures of *E. coli* ATCC 25922 or *K. pneumoniae* ATCC 700603, prepared as described above, were diluted to a final concentration of 5×10^6 CFU/mL and allowed to adjust for 30 min at 37 °C. The culture was then incubated at 37 °C in the dark for 10 or 30 min in the presence or absence of each peptide or antibiotic at various concentrations, with a final volume of 1 mL. At this point, only in certain specified experimental assays, the compounds were removed from the supernatant by centrifugation (16,000 $\times$ g for 5 min) and bacteria were washed and resuspended in fresh supplemented M9 medium. Then, 32 μ L of a 500 μ M homopropargylglycine (HPG) solution were added to each sample, resulting in a final concentration of 16 μ M in a final volume of 1 mL. Also included in each assay were two untreated controls containing only bacteria and bacteria treated with 16 μ M HPG alone, respectively. All samples were then incubated at 37 °C in the dark for

30 min. After incubation, samples were washed once with 1X filtered Phosphate Buffered Saline (PBS) (centrifuged at 16,000 $\times$ g for 5 min) and resuspended in PBS. After washing, samples were fixed with formaldehyde (Sigma Aldrich, USA) to a final concentration of 3% in 1 mL for 1 h at 25 °C. Subsequently, the samples were centrifuged at 16,000 $\times$ g for 5 min and resuspended in 1 mL of PBS. If not analysed immediately, the fixed samples were stored at 4 °C prior to performing the click chemistry reaction. Samples were centrifuged at 16,000 $\times$ g for 5 min and bacteria cells were dehydrated by ethanol that was added sequentially to achieve a final concentration of 50% (v/v) (3 min of incubation), 80% (v/v) (3 min of incubation), and 95% (v/v) (3 min of incubation). After dehydration, the samples were centrifuged (16,000 $\times$ g, 5 min) and resuspended in PBS. For cell analysis by SDS-PAGE, the following variations were implemented: bacterial cells were diluted in supplemented M9 medium to a final density of $\sim 1 \times 10^9$ CFU/mL, and HPG was added to the samples at a final concentration of 160 μ M. Bacteria were incubated with HPG for 0, 15, 30, 60, or 120 min at 37 °C in the dark.

Cu(I)-catalysed alkyne- azide click chemistry reaction

The click reaction was carried out according to [Rostovtsev et al. \(2002\)](#). 15 μ L of a pre-mix solution (prepared as described above) were added to each sample containing fixed bacteria that had incorporated HPG (30 μ L of pre-mix solution in case of samples prepared for SDS-PAGE analysis), resulting in the following final concentrations in each sample: 0.1 mM copper sulfate, 0.5 mM Tris (3-hydroxypropyltriazolylmethyl)amine (THPTA), and 2.5 μ M Alexa Fluor azide 488. Additionally, 50 μ L of freshly prepared 100 mM sodium ascorbate and 50 μ L of 100 mM aminoguanidine were added (100 μ L in case of samples prepared for SDS-PAGE analysis), bringing the final volume to 1 mL. The samples were gently inverted once to mix and incubated for 30 min in the dark at 25 °C. Finally, each sample was washed with PBS once (centrifuged at 16,000 $\times$ g for 5 min) and resuspended in 1 mL PBS.

Bacteria analyses by flow cytometry

An overnight culture of *E. coli* ATCC 25922, or of *K. pneumoniae* ATCC 700603, prepared as described above, was diluted to a final concentration of 5×10^6 CFU/mL and allowed to adjust for 30 min at 37 °C under shaking. Membrane permeabilization of bacteria was then assessed by incubating bacteria at 37 °C, in a final volume of 1 mL, with varying concentrations of each peptide or antibiotic used for the BONCAT assay. 5 min before the ending of the selected incubation time (40 min), propidium iodide probe was added to the samples to a final concentration of 10 μ g/mL before analysis using a flow cytometer. A positive control consisted of *E. coli* cells dehydrated with ethanol (as described above), while untreated cells were used as negative control. The fluorescence of untreated cells was used to establish the gating for positive PI-stained cells. Fixed bacterial cells, subjected to the click chemistry reaction to link the Alexa Fluor azide 488 probe, were analysed by flow cytometry and, among the gates-selected ones, only those that tested positive for Propidium Iodide, and thus permeabilized, were considered for the

calculation of the mean fluorescence intensity (MFI) of the Alexa Fluor azide 488.

Samples were analyzed by an Attune NxT® (ThermoFisher) equipped with a blue laser (488 nm) and photomultiplier tubes (PMTs) BL1 (525/50) and BL3 (695/40) to collect fluorescence. All parameters were set in logarithmic scale and the signals were recorded as Area. Forward scattering (FSC) was set to 300 mV, side scattering (SSC) to 350 mV and thresholds to 0.2 for FSC and 0.5 for SSC. The compensation matrix was performed according to the tool software. Hierarchical gates and acquisition flow rate (200 μ L/min) were set to avoid coincident events. The files were saved as FCS 3.1 and analysed by FCS Express *De Novo* software v7. Flow cytometry data are presented as means $\pm$ SD from three independent experiments ($n = 3$).

Analysis of HPG incorporation into bacteria by SDS PAGE

At the end of the click-chemistry incubation, each sample was washed with PBS once (centrifuged at $16,000 \times g$ for 5 min) and resuspended in 50 μ L volume, consisting of 37.5 μ L of Milli-Q water and 12.5 μ L of loading buffer 4 $\times$ (0.125 M Tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8), and stored at -20°C until use. Prior to analysis, samples were thawed, incubated on a thermoblock at 90°C for 10 min and then centrifuged. 15 μ L of the supernatant were loaded into the wells of a 4% polyacrylamide stacking gel. Protein samples were analysed by SDS-PAGE using a standard Laemmli Tris-glycine system. Electrophoresis was conducted at 200 V in Tris-glycine-SDS running buffer until the bromophenol blue band reached the gel bottom. After separation, the gel was scanned using the ChemiDoc system (BIO-RAD) selecting the appropriate channel for Alexa Fluor azide 488 to detect the fluorescence signal of the probe. Subsequently, the gel was stained overnight with Coomassie Brilliant Blue R-250 and destained using 10% (v/v) acetic acid in deionized water. The signal intensity of Coomassie Brilliant Blue R-250 was detected using ChemiDoc system at the appropriate channel for the dye. The ImageJ processing program was used to quantify and normalize the Alexa Fluor azide 488 fluorescence signals of each sample with the respective signal relative to Coomassie Brilliant Blue R-250. The results obtained were then reported in this paper in the form of a percentage compared with a control sample treated with HPG for the maximum time we tested (120 min), considered as 100%.

ESKAPEs ribosome preparation

The ribosomes used in this study were purified as described previously (Di Stasi et al., 2025). Briefly, to purify the 70S ribosomes of ESKAPE pathogens, the bacterial strains *E. faecium* ATCC 19434, *S. aureus* ATCC 25923, *K. pneumoniae* ATCC 13883, *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853 and *E. cloacae* ATCC 13047 were grown in 2 L of Luria-Bertani (LB) medium, at 37°C , until the mid-log phase was reached. After centrifugation at $7,000 \text{ rpm}$ ($12,000 \times g$) for 15 min at 20°C (JLA rotor 8.1000), the bacterial pellet was resuspended in 50 mL of saline (0.9% w/v NaCl) and re-centrifuged at $15,000 \text{ rpm}$ ($27,000 \times g$) for 10 min at 20°C (JA

rotor 25.50). The bacterial pellet was resuspended in 10 mL of Nirenberg Buffer (NB) (10 mM Tris-HCl pH7.7, 60 mM NH_4Cl , 10 mM Mg acetate). The cells were then stored in liquid nitrogen at -80°C and then thawed at the time of use. After thawing, the cells were subjected to sonication (Misonix 3,000) to induce lysis and dithiothreitol (DTT) was added at a final concentration of 1 mM with subsequent centrifugation at $15,000 \text{ rpm}$ ($18,000 \times g$) for 30 min at 4°C to purify the cell extract. The supernatant was subsequently ultracentrifuged at $80,000 \text{ rpm}$ ($320,000 \times g$) for 2 h at 4°C (S80-AT3 rotor) to resuspend the ribosomal pellet in NB with 1 mM DTT. Finally, the solution was cleared of the last remaining impurities by centrifugation at $15,000 \text{ rpm}$ ($18,000 \times g$) for 15 min at 4°C , and the ribosomes subjected to quantification by Nanodrop (A_{260}). All buffers mentioned in this paragraph were used after being filtered (MWCO = $0.2 \mu\text{m}$).

In vitro translation assay

The *in vitro* translation assay was performed by using the Δ ribosome from the *E. coli* PURExpress system New England Biolabs (NEB), as reported previously (Di Stasi et al., 2025; Raulf et al., 2025). Purified ESKAPE ribosomes, obtained as described in the previous paragraph, were used to replace *E. coli* ribosomes in the PURExpress kit by inserting 1 μ L of their suspensions (about 8 pmol of 70S subunit) into 5 μ L of PURExpress reaction mixture with the addition of 1 μ L of antibiotics. Each sample contained 16 ng/ μ L of mRNA for firefly luciferase (Fluc) transcribed *in vitro* by T7 polymerase (Thermo Fisher Scientific) from a pIVEX-2.3MCS vector containing the Fluc gene. The reaction mixture was incubated 2 min at 32°C (with stirring at 600 rpm) before mRNA addition, then incubated at 32°C for 30 min (600 rpm). Each reaction was stopped by the addition of 5 μ L of kanamycin (50 mg/mL) and transferred to the wells of a 96 microplate (Greiner Lumitrac, non-binding, white, chimney plates). Finally, 40 μ L of luciferase assay substrate solution were added to the wells (Promega, E1501), and the luminescence measured using a plate reader (Tecan Infinite 200 PRO). The negative control of the test was nuclease-free water in place of the antibiotic, whose values were used to normalize the absolute luminescence values of the samples. This experimental assay was conducted in triplicate, using individually prepared reaction mixtures.

Statistical analysis

The results of histograms presented in this work are shown as a percentage compared with the fluorescence of the untreated positive control (incubated with HPG and Alexa Fluor azide 488). Data are the mean $\pm$ SD of at least three experiments ($n \geq 3$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the untreated sample (one-way ANOVA test).

Results

To evaluate the potential inhibitory effects of antibacterial peptides on protein synthesis, we established a protocol based on the BONCAT method (Hatzepichler et al., 2014; Lindivat et al., 2020). First, we validated the efficient incorporation of the

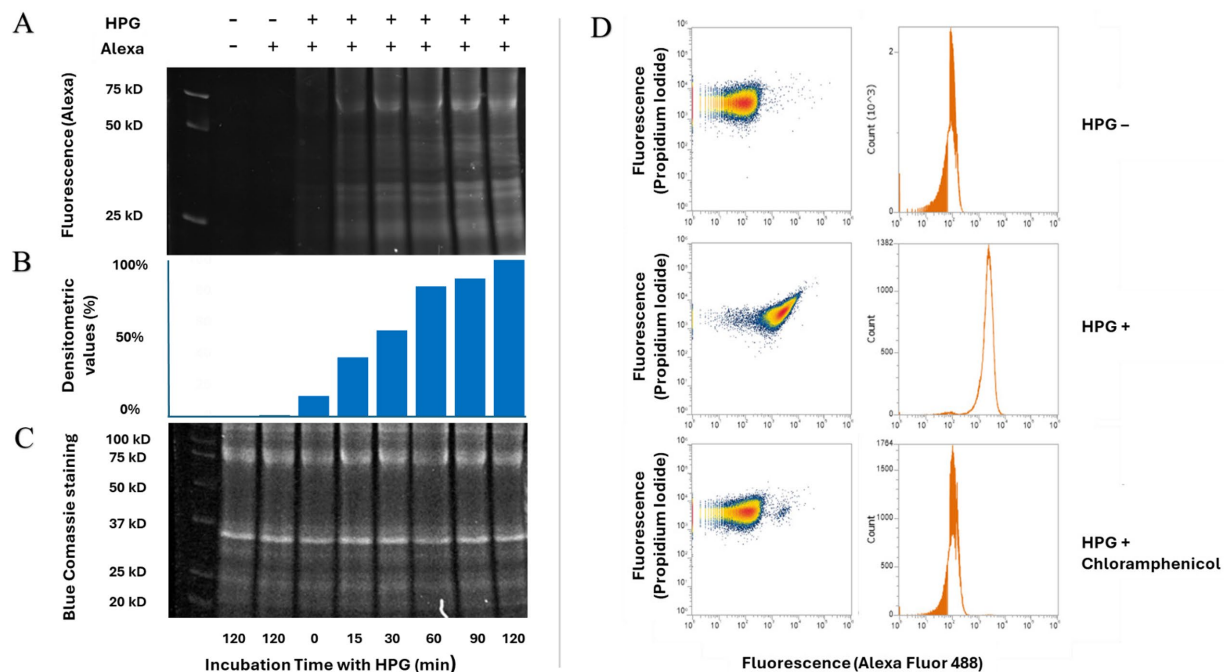


FIGURE 1

Incorporation of HPG in growing *E. coli* ATCC 25922. (A–C) SDS page of proteins extract from *E. coli* ATCC 25922 treated with homopropargylglycine (HPG) from 0 min up to 120 min. Proteins detected by fluorescence given by the linked Alexa 488 fluor probe (A); densitometric analysis of fluorescent protein bands (B); and proteins visualized by Coomassie blue staining (C). (D) Flow cytometric analysis of *E. coli* cells treated with Alexa Fluor azide, HPG or HPG in the presence of chloramphenicol. Cells were pre-incubated for 10 min with chloramphenicol, followed by a 30-min incubation with the amino acid analogue HPG. After fixation, samples underwent a click chemistry reaction with the fluorescent probe Alexa 488 to label newly synthesized proteins. The data are representative of three replicate experiments with highly similar results.

methionine analogue, homopropargylglycine (HPG), into newly synthesized proteins under the selected bacterial growth conditions. *E. coli* ATCC 25922 cells were incubated for up to 120 min in M9 salts medium supplemented with 2 mM MgSO₄ and 5 mM glucose, containing HPG but lacking any external amino acid source. Growth was monitored during this period (see [Supplementary Figure S1](#)). *E. coli* cells were then fixed and underwent a click chemistry reaction to specifically label HPG-containing proteins with an Alexa Fluor-azide probe. Subsequent SDS-PAGE analysis confirmed that bacterial proteins became fluorescent upon treatment, with a detectable signal appearing after only 15 min of incubation with HPG and increasing up to 120 min ([Figures 1A–C](#)). No fluorescence was observed in samples not treated with HPG ([Figures 1A–C](#)). We also analysed the HPG incorporation in proteins using flow cytometry ([Figure 1D](#)). To ensure optimal penetration of the fluorescent probe in bacteria, enabling therefore its binding to newly synthesised proteins, we confirmed that the cell fixation protocol enabled a high level of cell permeabilization to PI ([Supplementary Figure S2](#)). Additionally, we verified that the HPG concentration used did not affect bacterial growth ([Supplementary Figure S3](#)).

Next, we investigated the effects of PrAMPs on protein synthesis, monitoring HPG incorporation in cells treated with a panel of antimicrobials. This panel included three different proline-rich peptides (PrAMPs): Bac7(1–35), Bac7(1–16), and B7-005, all previously shown to inhibit *E. coli* protein synthesis ([Mardirossian et al., 2020, 2014; Seefeldt et al., 2016](#)). For comparison, we also tested an antibiotic known to inhibit protein

synthesis (e.g., chloramphenicol, CHL), and another two with different cellular targets (e.g., rifampin, RIF, and nalidixic acid, NDX).

Before running these assays, we determined the MICs of all these compounds under the conditions to be used for BONCAT experiments, i.e., in the supplemented M9 salts medium and using 5×10^6 CFU/mL *E. coli* cells ([Table 1](#)). Subsequently we confirmed the PrAMPs have not membrane-permeabilizing activity at their respective MICs, under the same specific experimental conditions, using flow cytometric analysis after incubating with PI ([Table 1](#)).

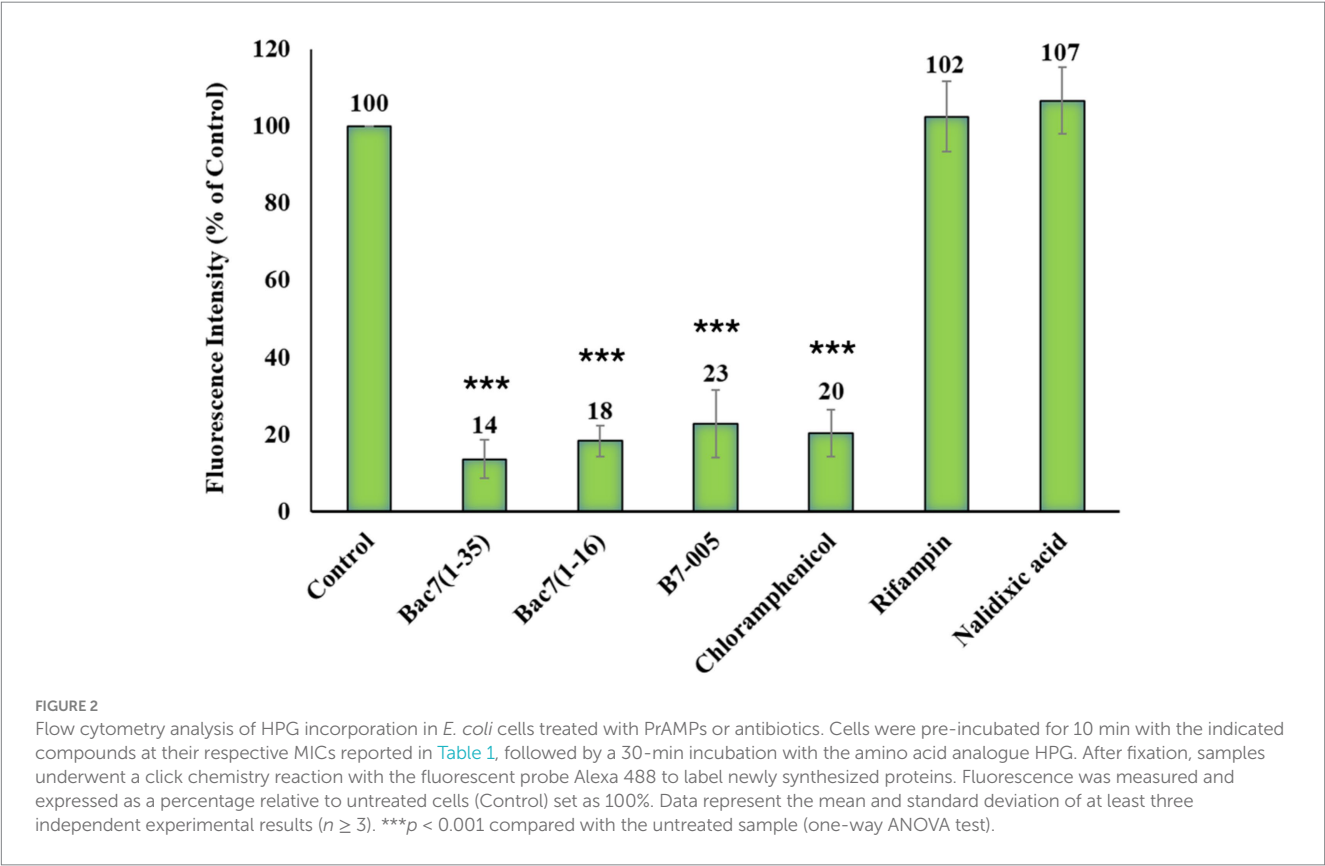
Subsequently, log-phase *E. coli* ATCC 25922 cells (5×10^6 CFU/mL) were incubated with each antimicrobial at its MIC value for 10 min. The amino acid analogue HPG was then added to the bacterial suspension and the incubation was prolonged for further 30 min. Subsequently bacteria were fixed, subjected to a click chemistry reaction, and analysed by flow cytometry to estimate the mean fluorescence intensity (MFI) values of the population ([Figure 2, Supplementary Table S3](#)).

We observed a significant fluorescence intensity decrease (75–90%) in bacterial samples treated with each of the three PrAMPs compared to the untreated bacteria (set as 100%). All three PrAMPs similarly inhibited HPG incorporation after 40 min of total incubation ([Figure 2](#)). As expected, chloramphenicol (CHL) caused an 80% reduction in fluorescence at its MIC. In contrast, rifampin (RIF) and nalidixic acid (NDX) did not affect fluorescence intensity, consistent with their distinct mechanisms of action targeting transcription and DNA replication, respectively ([Figure 2](#)).

TABLE 1 Antimicrobial activity (MIC) and cell membrane permeabilization assay of different antimicrobial peptides and antibiotics against 5×10^6 CFU/mL *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 in supplemented M9 medium.

Compound	<i>E. coli</i> ATCC 25922		<i>K. pneumoniae</i> ATCC 700603	
	MIC (μ M) *	Membrane permeabilization (% PI +cells) **	MIC (μ M)	Membrane permeabilization (% PI +cells)
Bac7(1–35)	1	<1%	4	<1%
Bac7(1–16)	1	<1%	4	<1%
B7-005	2–8	<1%	4	<1%
PMAP(12–31)	128	38%	n.d	n.d
COL	16	1%	16	n.d
RIF	2	<1%	16	<1%
NDX	16	<1%	n.d.	n.d.
CHL	32	<1%	n.d.	n.d.
TET	n.d.	n.d.	32	<1%
Ethanol	n.d.	78%	n.d.	96%

* Minimum Inhibitory Concentrations (MICs). MIC assays were performed using bacteria at an initial inoculum of 5×10^6 CFU/mL. The MIC was defined as the lowest antimicrobial concentration showing no visible bacterial growth after 18 h of incubation at 37 °C. Values represent the mode MIC from at least three independent experiments ($n \geq 3$). ** Cell permeabilization was assessed by incubating bacteria (5×10^6 CFU/mL) with each compound at its respective MIC for 40 min. Results are expressed as the percentage of PI-positive cells relative to the total bacterial count. Values represent the mean of three independent experiments ($n = 3$). CHL: chloramphenicol; RIF: rifampin; NDX: nalidixic acid; TET: tetracycline; COL: colistin. N.d.: Not determined.



The BONCAT protocol therefore effectively reported the inhibitory activity of PrAMPs on protein synthesis in living *E. coli* cells. We then verified whether this method could be applied also to other AMPs with unknown target with to define whether they are inhibitors of protein synthesis. Since most AMPs act on bacterial membranes, we investigated whether a combination of BONCAT

and permeabilization assay was a valid approach to discriminate between genuine intracellular inhibitors and membrane-acting compounds.

For this, we utilized two distinct membranolytic peptides: the alpha-helical PMAP (12–31), an active fragment derived from the known lytic AMP PMAP-36 (Biondi et al., 2023; Scheenstra et al., 2019) and the peptide antibiotic colistin, COL (Taglialegna, 2023). Analogous to the PrAMPs previously used, we determined MIC values and membrane permeabilization activity using the same medium and bacterial density described above (Table 1). Both colistin and PMAP(12–31) displayed a significant increase in MIC values (ranging from 8 to 32-fold) at higher bacterial concentrations under the conditions used (Supplementary Table S1). Quite surprisingly, colistin showed no membrane permeabilization at its MIC after 40 min of incubation. In contrast, PMAP(12–31), at its MIC, permeabilized 38% of the bacterial population. We then applied both colistin and PMAP(12–31) to the same BONCAT protocol used for PrAMPs, and for comparison, we used also chloramphenicol and rifampin as positive controls for direct protein synthesis and transcription inhibition, respectively. We observed that the AMP PMAP(12–31) determined a decrease in cell fluorescence (–49%) after 40 min of incubation concurrent to an increase of bacterial cell permeabilization (+38%) compared to the untreated control (Figure 3). In contrast, colistin, affected neither the integrity of bacterial membranes within the timeframe we applied nor decreased bacterial fluorescence, thus indicating even no indirect inhibition of protein synthesis (Figure 3). Both these cases suggest a positive correlation between the extent of cell permeabilization and a decrease in fluorescence due to indirect

inhibition of protein synthesis. Therefore, a combined assay that includes both cell membrane permeabilization and BONCAT can distinguish a specific, primary inhibition of protein synthesis from the indirect effects of a generic metabolic failure, which occurs in fatally permeabilized cells.

We then evaluated the applicability of this assay to bacterial species other than *E. coli*. To assess which of the ESKAPE pathogens could be most efficiently targeted by Bac7(1–16) in terms of their ability to block protein synthesis. We therefore performed an *in vitro* protein synthesis inhibition assay. Functional ribosomes extracted and purified from each of the ESKAPE pathogens were combined with the commercial purified *E. coli* translational machinery, which was deprived of its own ribosomes. This reconstituted a complete, functional translational machinery including the ribosomes derived from all the ESKAPE pathogens. *E. coli* ribosomes were also used for comparison. *In vitro* protein synthesis reactions were performed in the presence and in absence of Bac7(1–16), evaluating the productions (and activity) of firefly luciferase as reporter. The peptide impaired protein synthesis in all bacterial strains, albeit with different efficiency (Figure 4). We found that Bac7(1–16) at 5 μ M inhibited 90% *K. pneumoniae* ribosomes, a value comparable to, of even exceeding, the inhibition observed for *E. coli* ribosomes in the same assay (Figure 4).

We repeated the BONCAT protocol with *K. pneumoniae* cells after confirming the strain's ability to grow in supplemented M9 medium base devoid of amino acids (see Supplementary Figure S1). We also determined the MIC values for the same panel of PrAMPs against 5×10^6 cells/mL *K. pneumoniae* ATCC 700603 cells in supplemented M9 medium. Overall, *K. pneumoniae* was susceptible to all PrAMPs,

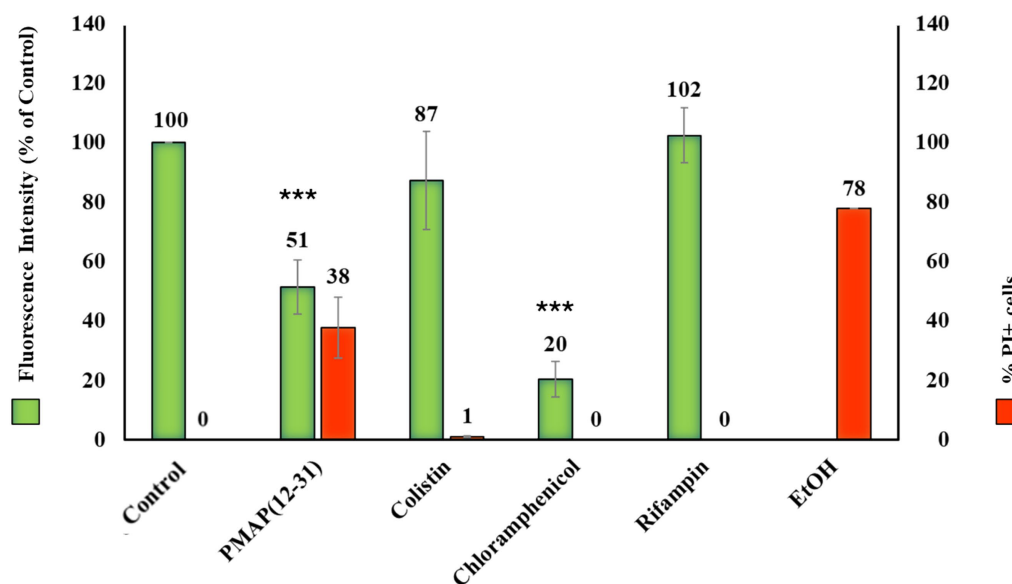


FIGURE 3

Flow cytometry analysis of *E. coli* cells treated with membranolytic peptides or antibiotics. Fluorescence (in green) is due to cells incubated 30 min with HPG, then fixed and subjected to click-chemistry with the fluorescent probe Alexa azide 488. Fluorescence percentages were recorded for cells treated with or without the indicated compounds for 10 min, followed by incubation with HPG for an additional 30 min. Each peptide or antibiotic was tested at its MIC concentration reported in Table 1. Cell permeabilization (in red) was assessed by incubating the bacteria with each compound at their MICs for 40 min. Ethanol was used as a positive control for membrane permeabilization. The results are expressed as the percentage of PI-positive cells compared to the total number of bacteria. Data represent the mean and standard deviation of at least three independent experiments ($n \geq 3$).

*** $p < 0.001$ compared with the untreated sample (one-way ANOVA test).

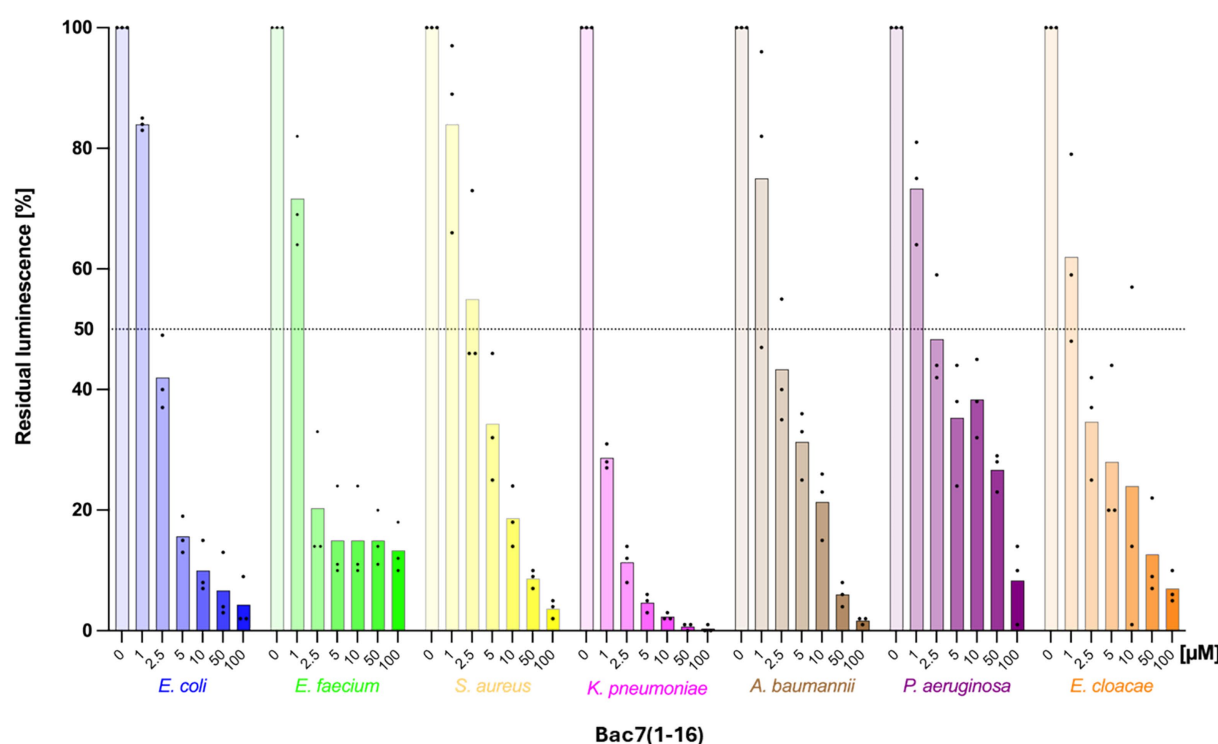


FIGURE 4

Inhibition of *in vitro* protein synthesis in different bacteria by Bac7(1–16). The assay was performed using the PURExpress system (NEB), supplemented with extracted and purified *E. coli* or distinct ESKAPE functional ribosomes. Samples treated with water (instead of the peptide) served as positive controls, with their efficacy set at 100%. Histograms represent the average of at least three independent experiments ($n \geq 3$), with individual results indicated by black dots.

even though the MIC values of Bac7(1–16) and Bac7(1–35) were 4-fold higher than those for *E. coli*, while B7-005 displayed an overall comparable activity on both the bacterial species (Table 1). We also excluded any potential damaging effects on membranes at the MIC, to ensure any variation of the protein synthesis evaluated by BONCAT was a direct effect on the translation and not a downstream of membrane disruption (Table 1).

PrAMPs significantly inhibited incorporation in HPG in *K. pneumoniae* (Figure 5), with inhibition percentages similar to those observed in *E. coli* (Figure 2). As expected, tetracycline, the antibiotic used as a control for protein synthesis inhibition, caused a relevant reduction (–65%) in fluorescence at its MIC. These results indicate that PrAMPs are at least as effective as tetracycline at inhibiting protein synthesis in *K. pneumoniae*. In contrast, rifampin did not affect fluorescence (Figure 5), consistent with its mechanism of action targeting RNA transcription while not also affecting downstream protein synthesis within a short timeframe. Therefore, we confirmed the effectiveness of BONCAT in verifying the impact of PrAMPs on protein synthesis in another clinically relevant pathogen besides the bacterial model *E. coli*. Furthermore, this study provides the first evidence that the tested mammalian PrAMPs inhibit protein synthesis in living *K. pneumoniae* cells.

Subsequently, we utilized the BONCAT protocol to further investigate the mechanism of protein synthesis inhibition by PrAMPs. Specifically, we examined the kinetics of translation inhibition and whether the inhibitory effects of the peptides persisted after the direct exposure of the bacterial culture to the

drugs. To this end, the assay was limited to PrAMPs Bac7(1–16) and Bac7(1–35) only, as their mode of action relies purely on the inhibition of protein synthesis, unlikely B7-005 (Mardirossian et al., 2020). *E. coli* cells were incubated with Bac7(1–35) and Bac7(1–16) for 10 or 30 min, as described above, and antibiotics were used as a comparison. Following the incubation with the antimicrobials, bacteria were pelleted to remove the drugs from the medium and then re-incubated for 30-min with HPG.

The bacterial protein synthesis in cells treated with Bac7(1–35) and Bac7(1–16) remained significantly affected even after the washout (Figure 6). Under our experimental setting, Bac7(1–35) demonstrated very rapid activity, reducing almost completely protein synthesis, i.e. –88% after just 10 min and by –98% after 30 min of incubation. In contrast, chloramphenicol lost most of its inhibitory effects once removed from the medium (Figure 6), which implies a fundamental difference in its mechanism of action compared to the PrAMPs. An unexpected reduction in fluorescence of over 20% was however observed with rifampicin (Figure 6).

Unlike the antibiotic CHL, PrAMPs therefore maintained its inhibitory effect even after the peptide in the medium was washed out of the bacterial cell cultures. This led us to investigate whether this persistent inhibition by PrAMPs might also affects the bacterial growth rate.

To this end, *E. coli* cultures were incubated for 30 min with each of the PrAMPs as well as with control antibiotics at their respective MICs. The antimicrobial compounds were then washed out and bacterial growth was monitored by measuring optical density at 600 nm for 18 h.

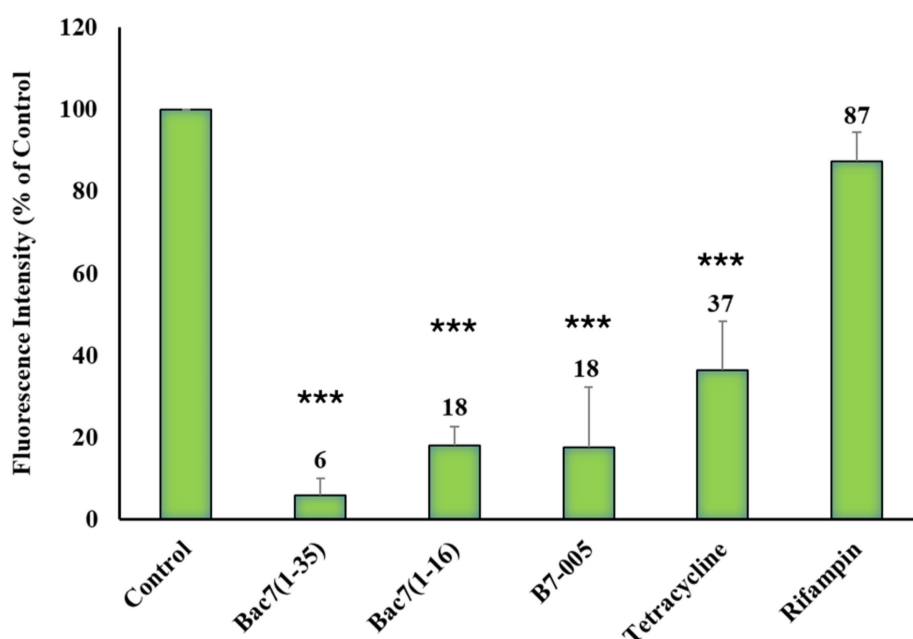


FIGURE 5

Flow cytometry analysis of HPG incorporation in *K. pneumoniae* cells treated with PrAMPs or antibiotics. Cells were pre-incubated for 10 min with the indicated compounds at their respective MICs (reported in the Table 1), followed by a 30-min incubation with the amino acid analogue HPG. After fixation, bacterial samples underwent a click chemistry reaction with the fluorescent probe Alexa Fluor azide 488 to label newly synthesized proteins. Fluorescence was measured and expressed as a percentage relative to untreated cells (Control) set as 100%. Data represent the mean and standard deviation of at least three independent experimental results ($n \geq 3$). *** $p < 0.001$ compared with the untreated sample (one-way ANOVA test).

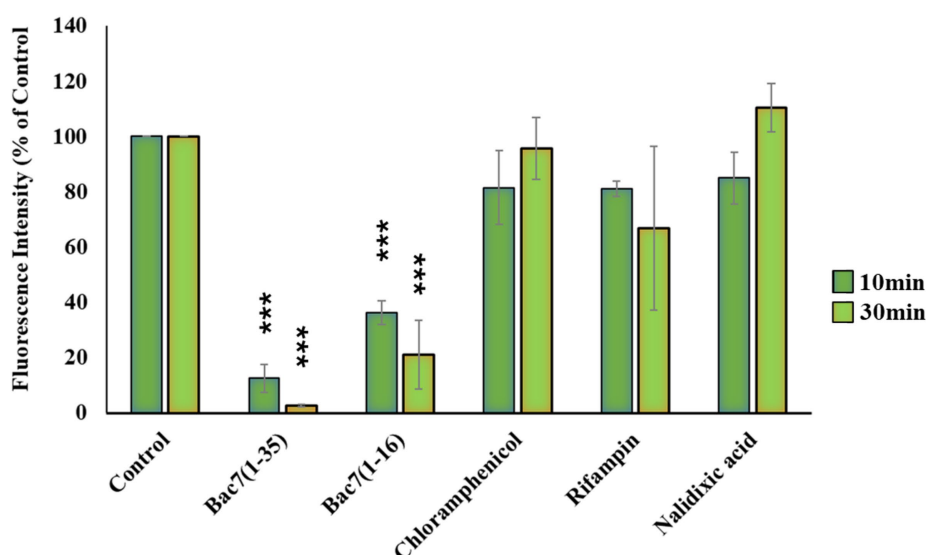
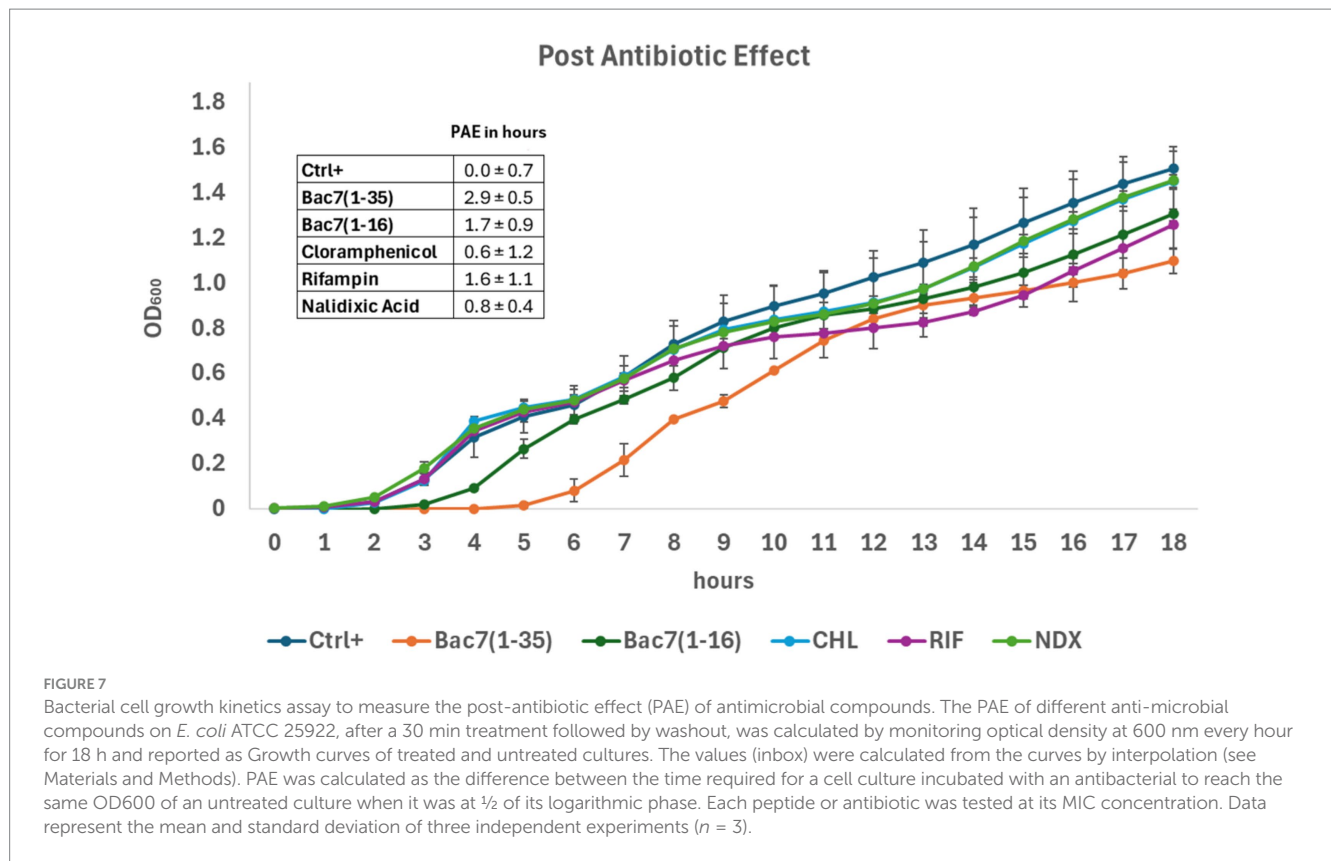


FIGURE 6

Flow cytometry analysis of *E. coli* protein synthesis inhibition after antimicrobial washout. The figure displays the fluorescence of newly synthesized proteins in *E. coli* after treatment with PrAMPs or control antibiotics. Cells were initially treated for 10 or 30 min at their minimum inhibitory concentration (MIC) before a complete washout of the compound. Protein synthesis was then assessed by incubating the cells with homopropargylglycine (HPG) for 30 min, followed by fixation and click chemistry with Alexa Fluor azide 488. Fluorescence was measured and expressed as a percentage relative to untreated cells (Control) set as 100%. Data, reflect the mean and standard deviation of fluorescence levels from a minimum of three independent experiments ($n \geq 3$). *** $p < 0.001$ compared with the untreated sample (one-way ANOVA test).

The results indicate a temporary suppression of bacterial growth, manifesting as a delay in the growth of treated cells compared to untreated cells (Figure 7). This phenomenon is precisely defined as the

Post-Antibiotic Effect (PAE) (Craig, 1993; Spangler et al., 1998), and PAE values calculated for different antimicrobial agents are shown in Figure 7. Specifically, Bac7(1–35) exhibited the longest-lasting PAE,



preventing for 3 h the growth of treated bacteria compared to the untreated sample. Similarly, Bac7(1–16) showed a PAE, albeit shorter than Bac7(1–35) but comparable to other antibiotics such as rifampicin. In contrast, chloramphenicol demonstrated a PAE of about half an hour. This is in agreement with our protein synthesis inhibition assays, where the inhibitory effect of CHL was largely lost once removed from the medium. The PAE is also qualitatively reflected in the lag times observed for bacterial growth.

Discussion

AMPs are a promising alternative to classical antibiotics. However, to consider them as therapeutic agents, it is crucial to understand their mechanisms of action (MoA). Over recent years, in fact, numerous methods have been developed, adapted, and refined to investigate the MoA of AMPs. Benfield and Henriques (2020), Schäfer and Wenzel (2020), Zhang et al. (2021). Nonetheless, it is important to acquire information on their MoA on living bacteria, which can be fundamentally different from their effect in simplified artificial models (Schäfer and Wenzel, 2020). Additionally, the MoA of an AMP can differ significantly among bacterial species (Di Stasi et al., 2025), underlining the need for specific characterization of each AMP/pathogen combination.

The primary objective of this work was to optimize and apply the BONCAT (Bioorthogonal Non-Canonical Amino Acid Tagging) method to investigate the *in vivo* intracellular mechanism of PrAMPs. This approach has been widely used in various fields, including eukaryotic cells (Dieterich et al., 2006) and bacterial studies to analyse mechanisms such as pathogenesis and virulence (Ignacio et al., 2021),

quorum sensing (Bagert et al., 2016) and response to antibiotics (Babin et al., 2017). Our findings demonstrate that the BONCAT method is also an effective and reproducible tool for studying the intracellular mechanisms of AMPs that affect protein synthesis in living bacteria.

The protocol was initially validated using three well-characterized PrAMPs: Bac7(1–35), Bac7(1–16), and B7-005. These peptides have previously been shown to inhibit protein synthesis using complementary techniques, such as structural studies (Graf et al., 2017; Mardirossian et al., 2020; Seefeldt et al., 2016) or the incorporation of radioactive amino acids (Mardirossian et al., 2014). All the tested PrAMPs almost completely inhibited the synthesis of new proteins in living *E. coli*, which demonstrates that BONCAT is a suitable and specific tool for studying AMPs acting as protein synthesis inhibitors in bacteria.

We then demonstrated that this method can also be applied to bacterial species other than the model organism, *E. coli*. After testing the effect of PrAMPs on a cell-free ribosomal system, we selected *K. pneumoniae* due both to its clinical importance and because we established that its ribosomes are highly sensitive to PrAMP inhibition. The BONCAT results for *K. pneumoniae* were very similar to those observed in *E. coli*, suggesting that this method may be further extended to other susceptible bacteria able to grow in minimum media, thereby providing a versatile tool for MoA studies. Moreover, it represents the first observation of the MoA of PrAMPs on a living bacterial pathogen other than *E. coli*. Further investigation is warranted, especially in light of recent evidence indicating that molecular mechanism studies of antibacterial compounds on *E. coli* are not necessarily predictive of their action in other bacteria (Di Stasi et al., 2025).

A significant challenge in evaluating the mode of action of any antibacterial agent is distinguishing a primary, direct effect on a specific pathway or target, from the secondary consequences of cell death. For instance, an antibacterial agent acting primarily by compromising the cell membrane will eventually lead to a decline in protein synthesis as a result of ion influx, cellular content leakage, and cessation of energy production.

Our results with the lytic antimicrobial peptide PMAP(12–31) illustrate this point: it simultaneously decreased amino acid incorporation and caused membrane damage. In contrast, the normally membranolytic antibiotic colistin, which was unable to kill bacteria by lysis under the assay conditions we used, also did not affect protein synthesis. Crucially, the tested PrAMPs did not alter membrane permeability at protein synthesis-inhibitory concentrations. We showed this by combining the BONCAT protocol with a classic Propidium Iodide (PI) cytofluorimetric assay (Rosenberg et al., 2019), and this combination of techniques easily distinguishes a genuine ribosomal effect from an unspecific consequence of membrane disruption.

Overall, this combined method is well-suited for a variety of applications. It could be used to confirm the mode of action of a putative protein synthesis inhibitor or to investigate new antibacterial agents with unknown mechanisms. It therefore provides a robust platform to study the numerous AMPs for which an intracellular mode of action has been postulated but currently still lacks consolidated *in vivo* data (Le et al., 2017; Schäfer and Wenzel, 2020). Moreover, the BONCAT protocol could be applied for evaluating large numbers of potential antimicrobial agents and/or a broad range of target bacterial species, overcoming the complexity and limitations often associated with other techniques. A further optimization of BONCAT toward a high-throughput assay may foster the development of drugs targeting the bacterial protein synthesis.

Furthermore, BONCAT enabled us to precisely study the kinetics of PrAMPs inhibition of protein synthesis, an aspect not as thoroughly investigated using previous techniques. For instance, the killing kinetics of Bac7(1–35), Bac7(1–16) and B7-005 has previously been investigated using the viable colony count method. Results indicated that they determine a 10- to 100-fold reduction in viable *E. coli* cells within 1 h, at their minimum inhibitory concentrations (MICs), with Bac7(1–16) being significantly less rapid (Di Stasi et al., 2025; Podda et al., 2006), while our study reveals a more rapid action on their molecular target than on the whole microorganism. These peptides almost completely inhibited the incorporation of the amino acid analogue HPG into proteins after just 10 min of exposure. This demonstrates PrAMPs reach their molecular target on a timescale that is not very different from membranolytic peptides (Fantner et al., 2010) and comparable to, or shorter than that of traditional antibiotics (Harvey and Koch, 1980; Zhang et al., 2023). This finding shows that PrAMPs bind to ribosomes within minutes, a timeframe shorter than previously hypothesized for this class of molecules.

This work also highlighted a notable post-antibiotic effect (PAE), a phenomenon defined as the persistent suppression of bacterial growth after a brief exposure to an antimicrobial agent (Craig, 1993; Spangler et al., 1998). In the current study this effect was observed for the PrAMPs Bac7(1–35) and Bac7(1–16). The duration of the PAE *in vitro* is known to correlate with the antibiotic concentration, the duration of the exposure to the drug, and the bacterial species studied (Li et al., 1997). Our results showed a greater PAE for these PrAMPs compared to rifampicin, nalidixic

acid and chloramphenicol, all used at their MICs. While rifampicin is known to induce a significant PAE (Chan et al., 2001) and nalidixic acid and chloramphenicol on the contrary generate only a moderate PAE (Athamna et al., 2004), our PrAMPs demonstrated a pronounced and sustained effect (see Figure 7). This finding is consistent with a previous study on ten different PrAMPs, including the full-length Bac7(1–60), used at their MIC for a one-hour exposure against *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Holfeld et al., 2018). In the current study, we also achieved a comparable growth arrest, lasting for several hours with 30 min exposure time. Our analysis revealed that after the removal of the peptides, protein synthesis remained inhibited for at least 30 min. It is plausible that a fraction of the peptides remains bound to their ribosomal targets even after the extracellular peptide concentration are reduced. A previous study demonstrated that Bac7(1–35) can accumulate at high concentrations within the *E. coli* cytoplasm (Mardirossian et al., 2014), making it highly likely that residual intracellular levels are sufficient to sustain inhibition of protein synthesis, thus prolonging the PAE. These observations have important pharmacodynamic implications, suggesting that PrAMPs can continue to inhibit bacterial protein synthesis even when their extracellular concentration falls below the effective threshold due to pharmacokinetic events (e.g., renal or hepatic clearance) upon a potential *in vivo* administration.

In addition, we observed that the efficacy of the PrAMP antibiotics used in this study, as measured by their MIC, did not decline when the bacterial density increased by 20-fold with respect to standard MIC procedures. In contrast, colistin and PMAP(12–31) exhibited an 8- to 64-fold increase in their MICs under the same conditions (see Supplementary Tables S1, S2). It is known that the efficacy of several antibacterial compounds decreases with increasing bacterial density (inoculum effect) (Loffredo et al., 2021), often due to reduced compound availability, due to effects such as sequestration by bacterial membranes. The increased total surface area of cell membranes, which are the principal targets of these compounds, results in more molecules binding, thereby requiring higher concentrations to achieve the threshold for the same antimicrobial effect (Loffredo et al., 2021). However, the PrAMPs tested in this study appear to bypass the limitations associated with the inoculum effect, confirming previous findings for Bac7(1–16) (Loffredo et al., 2021). The consistent MICs, regardless of the inoculum size, suggest that the mode of action of PrAMPs is not primarily influenced by membrane sequestration. It is plausible that at the MIC values, these PrAMPs are sufficient to saturate their intracellular binding sites, and that an increase in cell number does not significantly reduce the active peptide concentration required to inhibit protein synthesis, thus maintaining efficacy above the necessary threshold.

The combination of a long-lasting post-antibiotic effect (PAE) and the absence of an inoculum effect makes PrAMPs potentially advantageous in complex clinical contexts. For example, these characteristics could lead to reduced frequency of administration compared to traditional antibiotics and enable their effective use under conditions of high bacterial load.

In conclusion, this work contributes significantly to the functional characterization of PrAMPs. Moreover, our application, which combines permeabilization assays with BONCAT, establishes a suitable and powerful tool to study the mode of action of new antimicrobial compounds, including both AMPs and non-peptidic agents, even in the absence of preliminary information on their mechanism of action. The

utility of this approach lies in its ability to foster the advancement of screening strategies for new antimicrobial agents, a necessity now more than ever in response to the global emergency of bacterial resistance.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary material](#), further inquiries can be directed to the corresponding author.

Author contributions

LP: Data curation, Investigation, Methodology, Validation, Writing – original draft. AD'A: Investigation, Methodology, Writing – original draft. AS: Data curation, Writing – original draft, Investigation, Methodology. MMo: Investigation, Writing – review & editing. SP: Methodology, Supervision, Writing – original draft. AT: Conceptualization, Funding acquisition, Writing – review & editing. TP: Investigation, Methodology, Writing – original draft. AF: Data curation, Writing – review & editing, Formal analysis. DW: Writing – review & editing, Supervision. MMA: Writing – review & editing, Conceptualization, Funding acquisition, Writing – original draft. MS: Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing, Data curation, Supervision.

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Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2025.1713216/full#supplementary-material>

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Supplementary materials:

1- Supplementary figure S1

Growth curves of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 in supplemented M9 medium

2- Supplementary figure S2

Representative flow cytometric image of fixed *E. coli* cells stained with propidium iodide (PI)

3- Supplementary figure S3

Colony growth assay in the presence of 16 μ M HPG

4- Supplementary table S1

Comparison of MIC values for peptides and antibiotics against *E. coli* ATCC 25922 in different media at varying bacterial concentrations

5- Supplementary table S2

Comparison of MIC values for peptides and antibiotics against *K. pneumoniae* ATCC 700603 under different media and bacterial concentrations

6- Supplementary table S3

Raw data for mean fluorescence intensity (MFI) of HPG incorporation in *E. coli* cells treated with PrAMPs or antibiotics

1. Supplementary figure S1

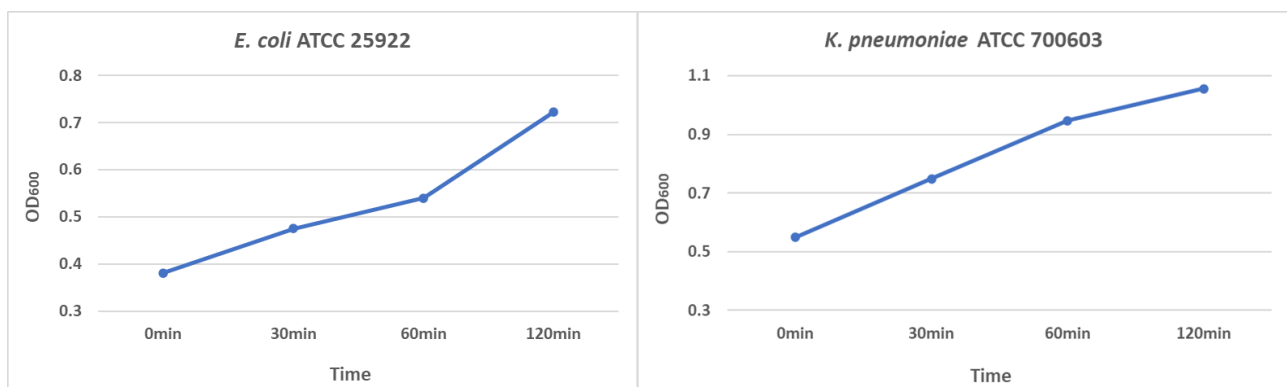


Figure S1: growth curves of *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 in supplemented M9 medium. Overnight cultures of *E. coli* ATCC 25922 (left panel) and *K. pneumoniae* (right panel), prepared as described in the Materials and Methods section, were resuspended in fresh supplemented M9 medium. Optical density at 600 nm (OD₆₀₀) was measured at time zero and after 30 minutes, 1 hour, and 2 hours of incubation at 37 °C with shaking at 130 rpm. The supplemented M9 medium supported the growth of both bacterial strains, with OD₆₀₀ values approximately doubling within the first two hours, indicating active proliferation under the tested conditions.

2. Supplementary figure S2

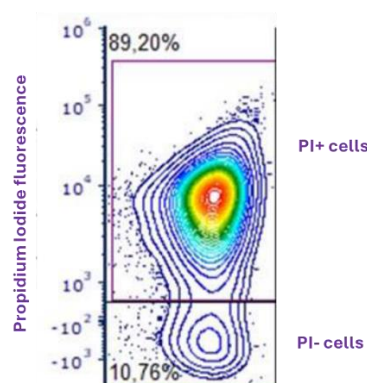


Figure S2: Representative flow cytometric image of fixed *E. coli* cells stained with propidium iodide (PI). *E. coli* ATCC 25922 cells were permeabilized with EtOH, fixed with formaldehyde, and analysed by flow cytometry using PI (Rosemberg et al., 2019 as described in the Materials and Methods section. The gating strategy shown in the figure was established by analysing a control population of *E. coli* cells that were not subjected to fixation or permeabilization but incubated for 5 minutes with PI. In the sample shown, the gate for PI-positive (PI+) cells was set taking into account a sample without PI. Data acquisition was performed using Attune Flow Cytometer Software, and analysis was carried out using FCS Express Flow Cytometry software. Fixation and permeabilization resulted in approximately 90% of *E. coli* ATCC 25922 cells being PI+, indicating successful membrane permeabilization. Only PI+ cells were considered for the calculation of Alexa Fluor azide 488 MFI.

3. Supplementary figure S3

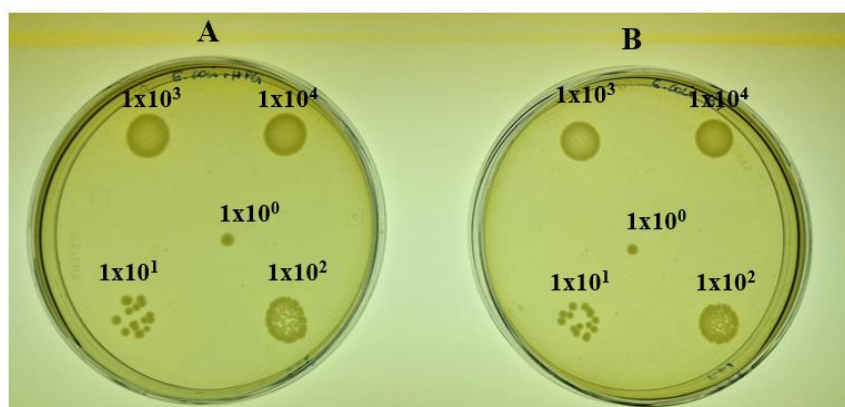


Figure S3: Colony growth assay in the presence of 16 μ M homopropargylglycine (HPG). To assess potential toxicity to bacterial cells, *E. coli* ATCC 25922 (5×10^6 CFU/mL), prepared as described in the Materials and Methods section, was incubated with 16 μ M HPG (kiick et al., 2022) for 30 minutes in supplemented M9 medium, under conditions consistent with those used in BONCAT assays. Following incubation, serial 1:10 dilutions of the sample were performed in supplemented M9 medium, and each dilution was spotted onto MHB-agar plates. Plates were then incubated at 37 °C for 24 hours. Colony counts revealed no observable difference between the HPG-

treated sample (panel A) and the untreated control (panel B), indicating that the methionine analogue HPG does not exert cytotoxic effects on *E. coli* under the tested conditions.

4. Supplementary table S1

Table S1: Comparison of MIC values for peptides and antibiotics against *E. coli* ATCC 25922 in different media at varying bacterial concentrations.

Compounds	MIC (μM)		
	Supplemented M9 5x10 ⁶ CFU/mL	Mueller Hinton	
		5x10 ⁶ CFU/mL	2,5x10 ⁵ CFU/mL
Bac7(1-35)	1	1-2	0,5
Bac7(1-16)	1	2	1
B7-005	2-8	2-8	1
COL	16	4	0,5 *
PMAP(12-31)	128	64	2
CHL	32	64	16

* Colistin (COL) at this bacteria concentration determined around 50% permeabilized cells (PI+) after 40 minutes of incubation

The MIC assays were performed in supplemented M9 medium or in MHB medium, as described in the Materials and Methods section, using final bacterial concentration of 2,5×10⁵ or 5×10⁶ CFU/well. Plates were incubated at 37 °C for 18 hours and sealed with parafilm to minimize evaporation. The values reported in the table represent the mode from at least three independent experiments. As expected (Loffredo et al., 2021), a 20-fold increase in cell density resulted in a dramatic rise in the MIC of PMAP(12-31) (Biondi et al., 2023) (32-fold) and a moderate increase in the MIC of colistin (COL) (Taglialegna et al., 2023) and chloramphenicol (CHL) (4-8-fold). In contrast minor changes (2-fold) were observed for PrAMPs. The medium used for MIC determination also influenced *E. coli* susceptibility, though to a lesser extent. Specifically, a 2-4-fold increase in MIC was observed for the lytic peptides colistin and PMAP(12-31) when tested in M9 medium compared to MHB.

5. Supplementary table S2

Table S2: Comparison of MIC values for peptides and antibiotics against *K. pneumoniae* ATCC 700603 under different media and bacterial concentrations.

Compounds	MIC (μM)	
	Supplemented M9 5x10 ⁶ CFU/mL	Mueller Hinton
		2,5x10 ⁵ CFU/mL
Bac7(1-35)	4	2
Bac7(1-16)	4	4
B7-005	4	2
COL	16	1

The MIC assays were performed as described in the Materials and Methods section, using either the conditions set for the BONCAT assay (supplemented M9 medium and 5×10^6 CFU/well) or standard conditions (MHB medium and 2.5×10^5 CFU/well). Plates were incubated at 37 °C for 18 hours and sealed with parafilm to minimize evaporation. The values reported in the table represent the mode from at least three independent experiments. The 20-fold increase in cell density in the presence of supplemented M9 medium resulted in a 16-fold increase in the MIC of colistin, while only minor changes (1-2-fold) were observed for PrAMPs.

6. Supplementary table S3

Table S3: Mean fluorescence intensity (MFI) of HPG incorporation in *E. coli* cells treated with PrAMPs or antibiotics

Exp. n.		Control	Bac7(1-35)	Bac7(1-16)	B7-005	CHL	RIF	NDX
1°	Mean Fluorescence Intensity	3654		580		953		3257
	% of control	100		16		26		89
2°	Mean Fluorescence Intensity	1699		221		352	1559	1724
	% of control	100		13		21	92	101
3°	Mean Fluorescence Intensity	2496		323		351	2598	2611
	% of control	100		13		14	104	105
4°	Mean Fluorescence Intensity	2547	246				2547	
	% of control	100	10				100	
5°	Mean Fluorescence Intensity	2861	263	468				3135
	% of control	100	9	16				110
6°	Mean Fluorescence Intensity	2540	429		321		2890	
	% of control	100	17		13		114	
7°	Mean Fluorescence Intensity	1957	368	352				2267
	% of control	100	19	18				116
8°	Mean Fluorescence Intensity	1273		236				1402
	% of control	100		19				110
9°	Mean Fluorescence Intensity	1493		350				1720
	% of control	100		23				115
10°	Mean Fluorescence Intensity	1334		262				1424
	% of control	100		20				107
11°	Mean Fluorescence Intensity	1661		417				
	% of control	100		25				
12°	Mean Fluorescence Intensity	2446		488	695			
	% of control	100		20	28			
13°	Mean Fluorescence Intensity	2423			657			
	% of control	100			27			

We performed the BONCAT assay as described in the Materials and Methods section. The accompanying Table shows the raw mean fluorescence intensity (MFI) data obtained by flow cytometry across all experiments used to prepare the Figure 2. The MFI value of each sample (treated with antibiotics or PrAMPs or left untreated), was normalized and expressed as a percentage relative to the respective untreated control (% of control) within the same experimental run. The final data points presented graphically in the Figure 2 histogram of the main article represent the average of these normalized percentage values for each antimicrobial compound.

6. GENERAL DISCUSSION

The mounting threat posed by the ESKAPE pathogens has placed Antimicrobial Peptides (AMPs), particularly cathelicidins, at the forefront as a promising, non-traditional class of antibiotics. While the potential of these compounds is clear, their role in clinical practice remains largely theoretical rather than actual. This potential is hindered by some critical limitations of AMPs that inhibit their transition to a clinical necessity. The three studies comprising this thesis directly address these critical barriers. By focusing on design, characterization, and mechanistic investigation, the work presented here aims to provide novel and necessary elements to determine if these limitations can be overcome.

The **first study** focused on optimizing the porcine α -helical cathelicidin PMAP-36. Rational modifications of its sequence and structure led to the identification of eight new derivatives.

The most important finding from this study is that the cathelicidin PMAP-36 can be shortened from both ends, as its central part maintains a broad-spectrum antimicrobial activity against many different bacteria. In fact, the resulting peptide **a** [PMAP(12-31): Ac-KRLKKIGKVLKWIPPIVGS-NH₂)] showed high antimicrobial activity, with MIC values ranging from 1 to 4 μ M, against a broad range of bacteria such as *E. coli*, *P. aeruginosa* and *S. epidermidis*. In addition, this peptide was also effective against pathogens like *A. baumannii* and *K. pneumoniae*, on which PMAP-36 derivatives had never been tested before, while it was not active against the Gram-positive strain *S. aureus*. Supporting this main finding, peptide **g** [PMAP(12-24): Ac-KRLKKIGKVLKWI-NH₂], which is shorter by seven amino acids at the C-terminus, retained strong antimicrobial activity with only a moderate (1- to 8-fold) decrease in potency depending on the pathogen. Importantly, both peptides **a** and **g** remained non-cytotoxic and non-haemolytic at bactericidal concentrations. In terms of antimicrobial activity and cytotoxicity, peptides **a** and **g** emerged as the best candidates among the derivatives studied. Given its superior antimicrobial properties among all the derivatives, and its derivation from the membranolytic peptide PMAP-36 (122), peptide **a** [PMAP(12-31)] was selected for further investigation in the third study of this PhD thesis, which focused specifically on characterizing its mechanism of action and confirmed its expected membranolytic activity on *E. coli* membranes. Importantly, since membranolytic activity can easily be misinterpreted as an inhibition of protein synthesis - because membrane disruption secondarily results in reduced protein production - it is generally crucial to assess both mechanisms in parallel when evaluating antimicrobial peptides. In this context, PMAP(12-31) served as a representative case, confirming that such a combined

analytical approach reliably distinguishes true membranolytic effects from indirect impacts on protein synthesis in *E. coli*.

The substitution of the helix-breaking prolines in peptide **a** with two α -helix-promoting amino acids resulted in a markedly more structured peptide (compound **b**), as CD and NOESY analyses revealed a 94% α -helical conformation in membrane-mimicking environments. The increased hydrophobicity observed for peptide **b** likely reflects an enhanced amphipathic organization of its helical structure, consistent with the rationale of improving peptide–membrane interactions, and may account for its biological activity against *S. aureus* (MIC 4 μ M). These amino acids changes allowed the peptide to acquire higher antimicrobial activity against gram-positive pathogens but at the cost of decreased activity against other bacteria (by 2 to 8-fold) and reduced cell viability. These results agree with previous studies showing that high hydrophobicity and amphipathicity increase haemolytic activity, while less affecting antimicrobial activity (189,190). Similarly, substituting Leu21 in peptide **g** with the helix-promoting residue Aib (peptide **h**) resulted in increased helicity confirmed by CD and NOESY spectra, but without improving the antimicrobial activity. Additionally, the peptide **h**, while remaining non-haemolytic, showed a higher cytotoxicity against HaCat cells than peptide **g**. This outcome was in contrasts with findings on temporin-1DRa, a membranolytic frog skin peptide, where Aib substitutions enhanced both antimicrobial and cytolytic activities (191); further investigation is required to clarify the underlying mechanism behind this phenomenon. Ultimately, except for peptides **a** and **g**, the variants with increased helicity or hydrophobicity showed cytotoxicity levels incompatible with their MIC values for the tested bacteria, often high enough to limit further development. These findings indicate that enhancing helicity or hydrophobicity does not improve activity but increases toxicity, underscoring a key lesson for future design: these parameters must be carefully tuned to reduce cytotoxicity without compromising antimicrobial efficacy.

Furthermore, although N-terminal lipidation was intended to enhance peptide insertion into bacterial membranes and potentially improving membranolytic activity, increasing the lipid chain length corresponded with a reduction in both antimicrobial activity against tested bacteria and helicity under membrane-mimicking conditions (SDS micelles) compared to the starting peptide **b**. Additionally, the elongated peptides also aggregated even at low concentrations and were cytotoxic to HaCat cells and ram red blood cells. The progressive reduction in helicity with increasing lipid tail length indicates that longer alkyl chains may disrupt helix formation, leading to decreased antimicrobial activity in the peptides. This idea is further supported by the behaviour of peptide **f**, where the attachment of a hydrophobic tail at the C-terminus (11-aminoundecanoic acid, Aun) was introduced to assess whether lipid chain position influences biological activity. This modification did not compromise helicity and

had only a moderate impact on antibacterial potency, exhibiting stronger antimicrobial effects and lower cytotoxicity than its same-length analogue **d** (with an N-terminal lauric acid). These variations in activity are likely linked to the significantly higher helical content of compound **f** (82%) compared to peptide **d** (47%). All this suggests that alkyl chains interfere with peptide helicity, consequently reducing antimicrobial activity, emphasizing the importance of α -helix structure as a requirement to maintain antimicrobial activity. Overall, although lipidation and acylation have been shown to enhance or modulate the antibacterial activity of already active AMPs (192–194), including some cathelicidins (195,196), none of the lipid modifications applied in this study to peptide **b** led to derivatives with significant biological improvements, highlighting the importance of helix preservation and careful design in future peptide modifications.

Finally, proteolytic degradation tests in the presence of the serine protease chymotrypsin led most PMAP-36 derivatives to hydrolysis within 10-20 minutes, except for peptides **e** (with an N-terminal palmitic acid) and **h** (with Aib instead of Leu21), whose degradation after 90 minutes remained below 50%. Analysis of proteolytic fragments suggests that the enzyme cleavage sites fall between Leu14 and Lys15 and between Leu21 and Lys22, canonical sites according to literature (197). The presence of the Aib residue in peptide **h** reasonably leads to greater peptide stability, as it replaces the canonical protease cleavage site Leu21, maintained for 180 minutes even in proteolytic degradation tests performed in serum, a more challenging environment for the peptide. Therefore, because proteolytic degradation shortens the *in vivo* lifespan of bioactive peptides and alter their pharmacological profile (198), the introduction of an Aib residue could represent a useful strategy to enhance protease stability in antimicrobial peptides that are both effective and non-cytotoxic.

In **conclusion**, this study successfully identified the central core of PMAP-36 as the key pharmacophore. While the original goal of further reducing cytotoxicity was not fully achieved, both peptide **a** [PMAP(12-31)] and peptide **g** [PMAP(12-24)] retained strong broad-spectrum antimicrobial activity without an increase in cytotoxicity or haemolytic activity at bactericidal concentrations. Importantly, the reduced length of these peptides, combined with maintained efficacy and safety, represents a clear advantage and highlights their potential as promising candidates for further therapeutic development. Furthermore, key findings confirmed that the alpha-helical structure is essential, as confirmed by the failure of the lipidation strategies, all interfering with peptide helicity and consequently reducing antimicrobial properties. On the other hand, an excessive increase in helicity or hydrophobicity was found not affecting particularly the biological activity, while increasing toxicity. Finally, the strategic insertion of the Aib residue (peptide **h**) significantly

enhanced protease stability in serum, offering a path for developing potent and stable drug candidates derived from the core sequence.

In the **second study**, we adopted the opposite strategy, as shortening the peptide chain may affect some desirable properties of AMPs. Specifically, while truncation of cathelicidin-derived PrAMPs preserved activity against the model organism *E. coli*, it also narrowed their antimicrobial spectrum. Therefore, this study focused on enhancing the potency of the 16-amino-acid synthetic PrAMP B7-005 by extending its C-terminus with 6 or 11 amino acids derived from the C-terminal regions of the cetacean PrAMPs TurlA (yielding B7-T6 and B7-T11) and Lip1 (B7-L6 and B7-L11), as well as from the bovine PrAMP Bac7(1-35) (B7-B6 and B7-B11).

The chimeric molecules collectively exhibited enhanced antimicrobial activity against most ESKAPE pathogens, with two- to eight-fold lower MIC values than B7-005. Notably, for each tested strain, at least one chimera was more active than B7-005. Only susceptibility of *E. coli* and *K. pneumoniae* remained unchanged despite the modifications. These extensions of the PrAMP B7-005 thus proved successful in terms of improving antimicrobial activity and spectrum of action. Extending B7-005 to 22 residues restores a length comparable to several natural PrAMPs, typically exceeding 19-20 residues (127), which may be important for effective protein synthesis inhibition (199), and/or for efficient membrane penetration (127). The further increase from 6 to 11 amino acid residues did not generally result in a valuable additional improvement in antimicrobial efficacy.

More specifically, the B7-B and B7-T series show significant improvements in antibacterial activity even if compared to their native peptides Bac7(1-35) and TurlA (188). Notably, B7-L series, containing a Trp18 residue, was also more effective against *P. aeruginosa*, suggesting that the presence of tryptophan in a central or terminal position may be important for targeting this species, as described in previous studies (200,201). Furthermore, B7-T11 and B7-L11 showed the highest activity (MIC = 4 μ M) against *E. faecium*, and were also the most hydrophobic according to the GRAVY index, supporting the idea that hydrophobicity enhances activity against this pathogen, as reported in literature (202,203). Since B7-005 is not effective against *E. faecium* (95), its extension let to target also this pathogen.

Additionally, all chimeras showed little to no reliance on the SbmA transporter, similar to B7-005 (153), indicating that their internalization mechanism in *E. coli* is SbmA-independent, which is advantageous because their antimicrobial spectrum is not restricted to SbmA/BacA-expressing species. Furthermore, regarding biocompatibility, assays on human A549 cells showed that nearly all

chimeric peptides were non-cytotoxic up to 32 μ M. Considering that their MIC values against all tested strains never exceeded 8–16 μ M, this indicates a quite favourable therapeutic window for the chimera series to work on. Notably, peptide B7-B11 showed no cytotoxic effects even at 64 μ M, highlighting its wider therapeutic margin and potential as a safer candidate than B7-005.

Finally, investigation of the chimeras' mechanism of action in *E. coli* revealed that all tested derivatives retain the ability to inhibit bacterial protein synthesis, similar to B7-005. Permeabilization assays further showed that, after 30 minutes of exposure, they cause only modest membrane permeabilization, again comparable to B7-005. These findings suggest that the C-terminal extensions do not alter the mechanism of action of the original peptide at least in *E. coli*. Based with previous evidence that B7-005 can employ different killing strategies across ESKAPE species (95), the improved activity of the chimeras observed in this study against *A. baumannii*, *E. cloacae*, *E. faecium*, and *S. aureus*, as well as of the B7-L series against *P. aeruginosa*, may reflect a shift in their mode of action. In fact, while B7-005 is internalized in *E. coli* and *K. pneumoniae* without major membrane disruption, its activity in the other species appears to involve membrane targeting (95). This suggests that even chimeras may rely more on membrane interactions in these pathogens, benefiting from increased length and/or hydrophobicity, while primarily acting through internalization and protein synthesis inhibition in *E. coli* and *K. pneumoniae*.

In conclusion, this study demonstrated that extending the C-terminus of B7-005 is an effective strategy to enhance its antibacterial performance, leading to new potent PrAMPs with improved activity and a broader spectrum against ESKAPE pathogens. While B7-005 is sufficiently active to kill these bacteria, its 1-16 sequence may be too short for optimal interaction with bacterial targets. In fact, the enhanced potency might reflect a partial shift toward membrane-associated mechanisms in bacteria where envelope targeting plays a more prominent role, possibly requiring longer and/or more hydrophobic sequences. Importantly, the C-terminal extension did not affect the peptides' SbmA independence or increase cytotoxicity, confirming that the structural modifications improved antibacterial efficacy without compromising selectivity.

To validate and better understand the intracellular mechanism of B7-005, this PrAMP was selected for further investigation in the third study of this PhD thesis. Notably, the different structural modifications introduced into B7-005 generated variants with distinct activity profiles, often enhancing potency against certain bacterial species while having minimal or no effect on others. This heterogeneous behaviour highlights how even specific changes in sequence can alter interactions between a peptide and a bacterium in a species-dependent manner, reinforcing the need to investigate

their mechanism of action on a strain-specific basis. In this context, the **third study** aimed to gain deeper insight into the mechanism of action of PrAMPs in living bacteria by applying BONCAT (bioorthogonal noncanonical amino acid tagging), a fluorescence-based method that enables the *in vivo* detection of protein synthesis inhibition across different Gram-negative species (187).

The BONCAT method was validated using three PrAMPs: Bac7(1-35), Bac7(1-16), and B7-005, whose mechanisms of action had already been characterized in previous studies through *in vitro* techniques and structural analyses (130,153,204) or via *in vivo* incorporation of radioactive amino acids (137). The results of this study not only confirm a near-complete inhibition of protein synthesis in *E. coli* by these PrAMPs but also show that the method is extendable to *K. pneumoniae*, a clinically relevant ESKAPE pathogen whose ribosomes proved to be highly sensitive to inhibition by PrAMPs. Using BONCAT, the *in vivo* inhibition of protein synthesis by Bac7(1-35), Bac7(1-16), and B7-005 was confirmed in this bacterial strain as well, providing the first *in vivo* evidence of the mechanism of action of PrAMPs in a bacterial specie other than *E. coli*.

Another challenge was distinguishing between a primary effect of protein synthesis inhibition by antibacterial agents and a similar, but secondary effect due to primary membrane-disruptive activity. Exposure to PMAP(12-31) (peptide *a* in the first study of this PhD thesis) led both to measurable membrane damage and to a reduction in protein synthesis, indicating that its inhibitory effect on translation could be a secondary consequence of membrane disruption. Another well-known lytic peptide, colistin, did not induce membrane permeabilization under the tested conditions, which aligned with the absence of protein synthesis inhibition. In contrast, inhibition of protein synthesis by all PrAMPs was never associated with disruption of the bacterial membrane. These results show that combining a Propidium Iodide (PI)-based membrane permeabilization assay with BONCAT enables discrimination between direct and indirect inhibition of protein synthesis in living bacteria. This combined approach represents a powerful tool to confirm or elucidate the mechanisms of antimicrobial agents

BONCAT also proved to be a valuable tool for precisely studying the kinetics of PrAMP-mediated protein synthesis inhibition, an aspect rarely addressed by previous techniques. The study revealed that Bac7(1-35) and Bac7(1-16) can almost completely inhibit protein synthesis in *E. coli* after just 10 minutes of exposure. For the first time it has been shown that PrAMPs can bind ribosomes in such a short time frame, demonstrating that they inactivate their molecular target on a timescale not so different from that of membrane-lytic peptides (205), and comparable, or even shorter, than that of traditional antibiotics (206,207).

Finally, this study also revealed a post-antibiotic effect (PAE) for Bac7(1-35) and Bac7(1-16). PAE is defined as the persistent suppression of bacterial growth following brief exposure to an antimicrobial agent (208,209). The results show that, after 30 minutes of exposure to the two PrAMPs, Bac7(1-35) in particular exhibited a prolonged PAE on *E. coli* of approximately 3 hours, which is longer than that observed for antibiotics such as rifampicin, nalidixic acid, and chloramphenicol. This finding is consistent with previous studies on various PrAMPs, including full-length Bac7(1-60), in different bacterial strains including *E. coli* (204). A possible explanation for this phenomenon is the prolonged intracellular retention of the peptide after its extracellular levels decline. Indeed, Mardirossian et al. (2014) showed that Bac7(1-35) accumulates at high concentrations in the *E. coli* cytoplasm, suggesting that residual intracellular peptide may sustain translation inhibition and contribute to the PAE (137). Pharmacodynamically, this prolonged PAE suggests that PrAMPs can continue to inhibit bacterial protein synthesis even after their extracellular concentrations decline below the effective threshold, for example due to renal or hepatic clearance. This property could allow them to maintain antibacterial activity for extended periods and potentially enabling less frequent dosing than traditional antibiotics in therapeutic treatment.

In conclusion, the BONCAT method proved to be a fast, cost-effective, and reproducible approach to investigate the mechanism of action of non-lytic antimicrobial compounds that inhibit translation in living bacteria, even without prior mechanistic information. Using three PrAMPs - Bac7(1-35), Bac7(1-16), and B7-005 - the method confirmed a strong and specific inhibition of protein synthesis in both *E. coli* and the pathogen *K. pneumoniae*. BONCAT also effectively distinguished direct translation inhibition from secondary effects related to membrane disruption, and allowed precise kinetic analysis, showing that Bac7-based PrAMPs almost completely blocked translation in *E. coli* within 10 minutes.

7. CONCLUSIONS

This PhD research successfully contributed to overcoming some key barriers limiting the clinical translation of cathelicidins. It demonstrated that rational structural modifications of these peptides can, in different cases, enhance antibacterial activity, expand the antimicrobial spectrum, or improve resistance to proteolytic degradation, while generally maintaining a low cytotoxicity profile. Notably, these studies also highlight that structural changes may make peptides more effective against certain bacterial species than others, underlining the need to clearly define the microbial target for peptide optimization. Overall, these findings provide cathelicidin derivatives with improved activity, broader coverage, and preliminary enhancements in stability and biocompatibility. Importantly, the study generated new mechanistic insights, showing how PrAMPs inhibit bacterial protein synthesis *in vivo* while distinguishing primary intracellular effects from secondary membrane perturbations, thus providing a more comprehensive understanding of their mode of action essential for therapeutic optimization. Overall, these findings advance our knowledge of peptide structure–activity relationships and represent a meaningful step toward the development of safe, stable, effective, and broadly active antimicrobial agents, contributing to bridging the gap toward cathelicidins’ potential therapeutic applications. Furthermore, this thesis introduced a new tool to investigate the mechanisms of action of antimicrobial peptides in living bacteria, representing an important step for future preclinical development in the global fight against antibiotic resistance.

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