Biological Properties of *de novo* Generated Linear Cationic Antimicrobial Peptides

by

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I am here because of penicillin…

Sir Alexander Fleming

Declaration

I, Margarita Karapetian, declare hereby that I am the sole author of this thesis. Any previously published or accepted for publication materials by other authors have been used in accordance with proper citation rules.

This thesis contains no material which has been accepted as part of the requirements of any other academic degree or non-degree program.

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აბსტრაქტი

ანტიმიკრობული პეპტიდები (ამპ) პერსპექტიულ კანდიდატებად მოიაზრებიან ანტიბიოტიკებისადმი რეზისტენტობის წინააღმდეგ საბრძოლველად, რომელიც 21-ე საუკუნის ერთ-ერთ უდიდეს გამოწვევას წარმოადგენს. ტრადიციული ანტიბიოტიკებისგან განსხვავებით, ამპ-ები კლავენ ბაქტერიებს ერთდროულად მრავალი ბაქტერიული სამიზნესთან ურთიერთქმედებით, რაც ბაქტერიებისთვის რეზისტენტობის განვითარებას ართულებს. ამპ-ებს ასევე შეუძლიათ გამოავლინონ ანტისიმსივნური, ანტივირუსული, სოკოს საწინააღმდეგო, პარაზიტების საწინააღმდეგო, იმუნომოდულატორული და ბიოფირის საწინააღმდეგო აქტივობები.

თუმცა, მიუხედავად ამგვარი ღირებული ბიოლოგიური პოტენციალისა, არსებული ამპ-ების ფართო გამოყენება ანტიბიოტიკებისადმი მდგრადი ინფექციების სამკურნალოდ შეზღუდულია მათი ტოქსიკურობისა და ფიზიოლოგიურ პირობებში არასტაბილურობის გამო. სხვადასხვა მიდგომას შორის, რომელიც გამოიყენება ზემოთ აღნიშნული შეზღუდვების დასაძლევად და სასურველი ბიოლოგიური თვისებების მქონე ამპ-ის შესაქმნელად, პრედიქციის ალგორითმებზე დაფუძნებული, მიზან-მიმართული de novo დიზაინი წარმოადგენს ერთ-ერთ ყველაზე სწრაფ და ეკონომიურ მეთოდს.

In silico შექმნილი ამპ-ების ბიოლოგიური აქტივობები და მოქმედების მექანიზმები ნაკლებად არის შესწავლილი. მოცემულ ნაშრომში ჩვენ გამოვიკვლიეთ *in silico* დიზაინით შექმნილი 13 ხაზოვანი კათიონური ანტიმიკრობული პეპტიდი, კერძოდ, მათი ანტიბაქტერიული აქტივობა (დამოუკიდებლად ან კომერციულ ანტიბიოტიკებთან კომბინაციაში), ტოქსიკურობა და პროტეაზების მიმართ სტაბილურობა.

შერჩეულ იქნა 4 პეპტიდი, რომელმაც გამოავლინა ყველაზე პერსპექტიული თერაპიული თვისებები, როგორიცაა მაღალი ანტიბაქტერიული აქტივობა, დაბალი ტოქსიკურობა, სტაბილურობა პროტეაზების მიმართ. შერჩეული ოთხი კამპ-დან სამმა (24L, L1L და L1D) აჩვენა სინერგია კომერციულ ანტიბიოტიკებთან. ამავდროულად, იწვევდნენ ბაქტერიული მებრანის დესტაბილიზაციას/დაზიანებას რაც გამოიხატებოდა ფოსფოლიპიდების ინტენსიურ გადანაწილებაში და ვეზიკულიზაციაში. ხოლო 24D-პეპტიდი (რომელიც ავლენდა უმაღალეს ანტიბაქტერიულ აქტივობას, მაგრამ არ აჩვენებდა სინერგიას არც ერთ შესწავლილ ანტიბიოტიკთან) აფერხებდა ბაქტერიული უჯრედის დაყოფის პროცესს.

ჯამში, ეს ნაშრომი წარმოადგენს მრავალმხვრივ კვლევას, რომელიც აღრმავებს ცოდნას de novo შექმნილ კათიონური ანტიმიკრობული პეპტიდების უნიკალურ თვისებებზე.

Abstract

Antimicrobial peptides (AMPs) have emerged as promising candidates in combating antibiotic resistance – a growing issue in healthcare. In contrast to conventional antibiotics, AMPs kill bacteria through simultaneous interaction with multiple bacterial targets thus, making it harder for bacteria to develop resistance. Together with this attractive feature, AMPs can also exhibit anti-cancer, anti-viral, anti-fungal, anti-parasite, immunomodulatory, and anti-biofilm activities. However, despite such valuable biological potential, a wide-range application of existing AMPs for the treatment of antibiotic-resistant infections is limited due to their toxicity and instability under physiological conditions. Among various approaches that have been employed to overcome the above-mentioned limitations and generate AMPs with desired biological properties, the *de novo* design, based on target-specific prediction tools, represents one of the fastest and most cost-effective methods. Yet, biological activities and mechanisms of action of *in silico* generated AMPs are not being intensively studied. In the present work, we investigated 13 *in silico* designed linear cationic antimicrobial peptides (LCAMPs) for their antibacterial activities (alone or in combination with commercial antibiotics), toxicity, and stability toward proteases. 4 LCAMPs showing the most promising therapeutic properties, such as high antibacterial activity, low cytotoxicity, and stability towards proteases, were selected and further studied for their modes of action on bacterial outer and inner targets.

Three out of these four LCAMPs revealed synergy with commercial antibiotics and appear to be membrane-active, inducing intensive phospholipid redistribution, blebbing and disruption of cytoplasmic bacterial membrane, while one LCAMP (showing the highest antibacterial activity but not showing synergy with any of the tested antibiotics) impairs bacterial cell division.

Overall, this work represents a comprehensive study providing insights into the unique characteristics of cationic AMPs.

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List of Abbreviations

AAMP	Anionic antimicrobial peptides
ABC	ATP binding cassette
ABR	Antibiotic resistant
AMP	Antimicrobial Peptides
AMR	Antimicrobial Resistance
APP	Antibacterial Peptide Prediction
ARG	Antibiotic Resistance Genes
Arg	Arginine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BC	Before Christ
BPPL	Bacterial Priority Pathogens List
BSA	Bovine Serum Albumin
CAP	Covalently attached protein
CAS	CRISPR-associated proteins
CBD	Cell wall-binding domains
CCD	Charge-coupled device
CD	Circular dichroism
CDC	Centers for Desease Control and Prevention (CDC)
CFU	Colony Forming Units
CIP	Ciprofloxacin
CL	Cardiolipin
CLSI	Clinical and Laboratory Standards Institute
COVID	Coronavirus disease
CRAMP	Cathelin-related antimicrobial peptide
CRE	Carbapenem-Resistant Enterobacterales
CRISPR-CAS	Clustered, regularly interspaced, short palindromic repeats
CSA	Cationic steroid antimicrobials
DBAASP	Database of Antimicrobial Activity and Structure of Peptides
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double-strand brakes
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electro mobility shift assay
ERY	Erythromycin
ESBL	Extended-Spectrum Beta-Lactamase
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FICi	Fractional Inhibitory Concentration index
FITC	Fluorescein isothiocyanate
FM 4-64	Fei-Mao styryl, N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide

GDP	Guanosine diphosphate
GlcNAc	N-acetylglucosamine
GTP	Guanosine-5'-triphosphate
HD	Human defensin
HDP	Host defense peptides
HPLC	High-performance liquid chromatography
HU	Histon-like protein
IB	Inclusion body
IE	Inoculum Effect
IM	Inner membrane
IMP	Integral membrane protein
LB	Lisogeny- Broth
LCAMP	Linear Cationic Antimicrobial Peptides
LMA	Low melting point agarose
LP, Lpp	Lipoprotein
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MAG	Magainin
MALDI	Matrix-assisted laser desorption/ionization
MATE	Multidrug and toxin extrusion
mDap	diaminopimelic acid
MDR	Multidrug-resistant
MFS	Major Facilitator Superfamily
MIC	Minimum Inhibitory Concentration
ML	Machine learning
mRNA	Messenger RNA
MRSA	Methicillin-Resistant Staphylococcus aureus
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide
MurNAc	N-acetylmuramic acid
MV	Memebrane vesicles
NAO	3,6-bis(dimethylamino)-10-nonyl-acridinium bromide
NAP	Nucleoid - associated proteins
NC	Negative control
ND	Not determined
NT	Not digested
O/N	Overnight
OD	Optical density
OIMV	Outer Inner Membrane Vesicle
OM	Outer membrane
OMP	Outer membrane protein
OmpA	Outer membrane protein A
OMV	Outermembrane vesicles
PAGE	Polyacrylamide gel electrophoresis
Pal	peptidoglycan-asscociated lipoprotein
PBP	Penicillin-binding proteins
PBS	Phosphate buffered saline solution
PD	Partially digested

PDB	Protein Data Bank
PE	phosphatidylethanolamine
PFA	Paraformaldehyde
PFGE	Pulsed-field gel electrophoresis
PG	phosphatidylglycerol
PGN	Peptidoglycan
PrAMPs	proline-rich antimicrobial peptides
PTM	Posttranslationally modified
QSAR	Quantitative structure - activity relationship
RBC	Red blood cells
RFP	Red fluorescent protein
RNA	Ribonucleic Acid
RNAP	RNA polymerase
RNase	Ribonuclease
RND	Resistance Nodulation Division
ROS	Reactive oxygen species
RPF	Red Fluorescent Protein
RR	Rifampicin-resistant
rRNA	Ribosomal RNA
RT	Room temperature
S	Svedberg's unit
SDS	Sodium Dodecyl Sulfate
SMR	Small multidrug resistance
TB	Tuberculosis
TET	Tetracyclines
topA	Topoisomerase A
TP	Tachyplesin
TRIC	Tetramethylrhodamine-isothiocyanate
tRNA	Transfer RNA
TU	Thiourea
UDP-GlcNAc	Uridine-diphosphate-N-acetylglucosamine
US	United States
VAPGH	Virion-associated peptidoglycan hydrolases
VRE	Vancomycin-Resistant Enterococcus
WHO	World Health Organization
WTA	Wall teichoic acid

Chapter 1 : Introduction.

1.1. Antibiotic resistance as a global threat to human health.

Bacterial antimicrobial resistance (AMR) is a global phenomenon associated with the resistance of bacteria towards the action of antibiotics, caused by excessive and/or uncontrolled usage of broad-spectrum antimicrobials in medicine, animal-related practices, and the release of antibiotics into the environment [1–4]. AMR has emerged as a public health problem of the 21st century.

The increasing spread of AMR among microbe populations is largely attributed to the spread of antibiotic resistance genes (ARGs), which bacteria can acquire to develop resistance towards antibiotics [3]. ARGs (conferring resistance to most commercially available antibiotics such as aminoglycosides, macrolides, quinolones, and others) are present in the environment and can be horizontally and vertically transferred within the microbial community, thus facilitating the spread of AMR [5–8].

According to predictive statistical models, an estimated 4.95 million deaths were linked to bacterial AMR in 2019, with 1.27 million directly attributable to bacterial AMR [9]. Six major pathogens have been listed to be responsible for 70% of AMR attributable death cases half of which were caused by *Escherichia coli* (25%) and *Staphylococcus aureus* (26%), the rest 4 pathogens include *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Fig. 1). Resistance to fluoroquinolones and β-lactam antibiotics (ie, carbapenems, cephalosporins, and penicillins) accounted to 70% of AMR death cases.

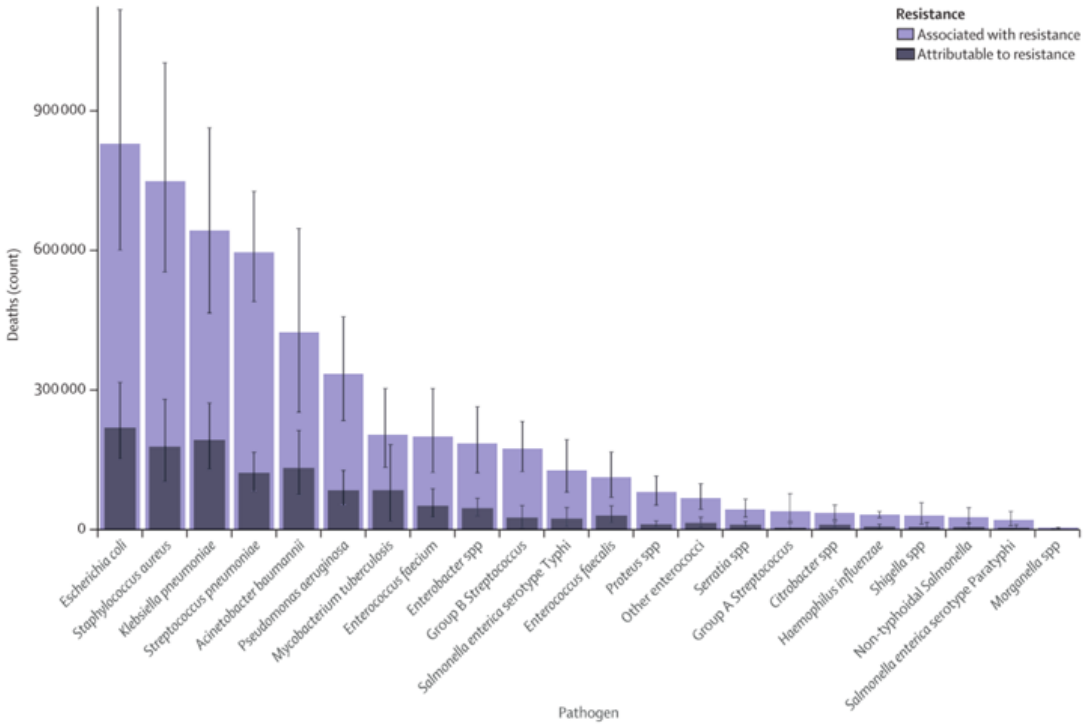


Figure 1.Global deaths (counts) attributable to and associated with bacterial antimicrobial resistance by pathogens in 2019. Estimates were aggregated across drugs, accounting for the co-occurrence of resistance to multiple drugs. Error bars show 95% uncertainty intervals. (From Lancet, 2022).

Tracking of antimicrobial resistance has significantly slowed during the COVID-19 pandemic. In the US, during the period from 2019 to 2020, according to the Centers for Disease Control and Prevention (CDC), the rate of hospital-onset resistant infections increased by at least 15% [10]. The newest data of July 2024 from the CDC states that in total the rate for six bacterial antimicrobial-resistant infections (Carbapenem-resistant Enterobacterales (CRE), Carbapenem-resistant *Acinetobacter*, *Candida auris*, Methicillin-resistant *S. aureus* (MRSA), Vancomycin-resistant Enterococcus (VRE), Extended-spectrum β-lactamase (ESBL) - producing Enterobacterales, Multidrug-resistant (MDR) *P. aeruginosa*) increased by a combined 20% during the COVID-19 pandemic compared to the pre-pandemic period, peaking in 2021, and remaining above pre-pandemic levels in 2022.

The 2024 WHO Bacterial Priority Pathogens List (BPPL) includes 15 families of antibiotic resistant (ABR) pathogens [11]. The critical priority group is represented by Gram-negative bacteria such as *Acinetobacter baumannii*, various pathogens in the Enterobacterales order that are resistant to last-resort antibiotics, and rifampicin-resistant (RR) *Mycobacterium tuberculosis* [11].

Despite the rise of antibiotic-resistant strains, antibiotics are still an indispensable part of modern medicine. It can't be denied that the use of antibiotics to treat and prevent numerous infections induced by non-resistant strains is still effective. Thus, it is unlikely that humanity will be able to get completely rid of antibiotics in the next few decades. However, understanding the mode of action of antibiotics and the mechanisms underlying the development of antibiotic resistance will substantially contribute to the development of new antimicrobial agents and help to reduce the use of commercial antibiotics.

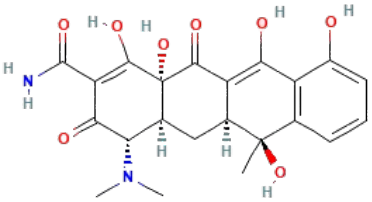
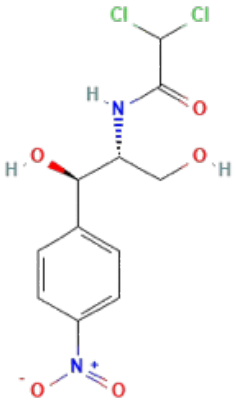
Chapter 2 : Literature overview.

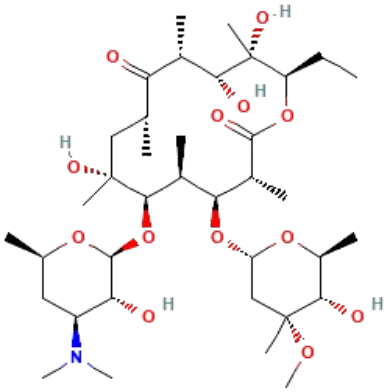
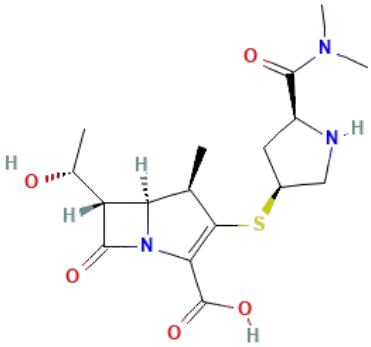
2.1. Historical overview of antibiotics

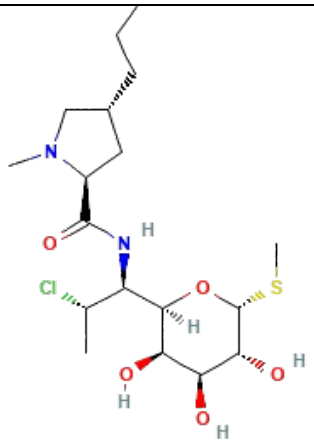
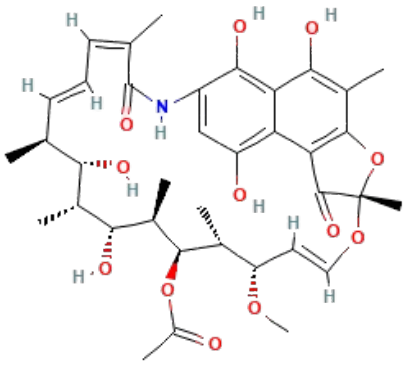
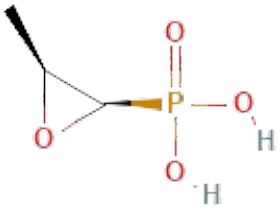
According to a modern definition, an antibiotic is a substance that inhibits the growth and replication of a bacterium or kills it outright. Antibiotics target bacterial infections within (or on) the body [12].

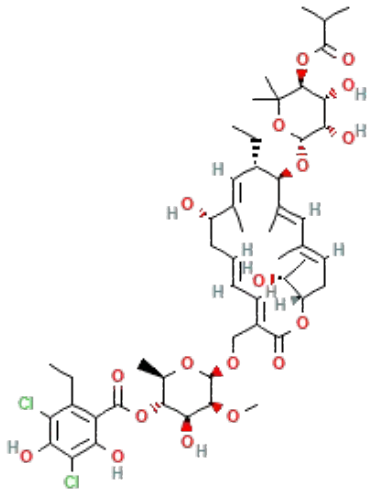
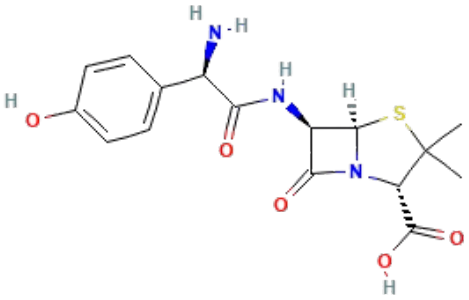
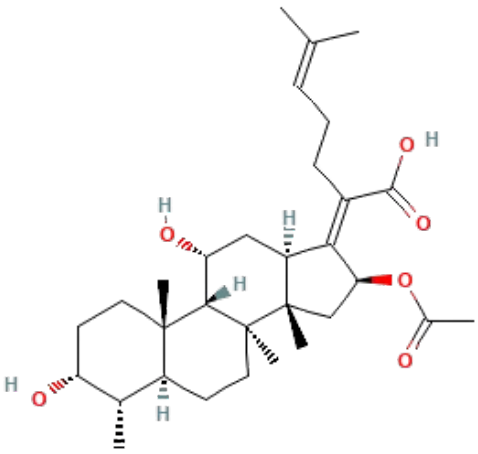
Antibiotics have been an indispensable part of human life through the centuries. According to Eber's papyrus, in 1550 B.C., moldy bread and medicinal soils were used for the treatment of infected wounds and burns in ancient Egypt, Greece, China, and, Serbia [13,14]. Traces of tetracycline were found in human bones (350-550 A.D.) collected during archeological excavations in Sudan [15]. In 1871, Joseph Lister discovered the inhibitory effects of *Penicillium glaucum* on bacterial growth. At the same time, Louis Pasteur noticed that the growth of *Bacillus anthracis* was inhibited when co-cultivated with "common" aerobic bacteria. Despite several observations of antagonisms between microorganisms, no antimicrobial molecule was purified. In 1909, Paul Ehrlich synthesized arsphenamine, an arsenic derivative active against the *Treponema pallidum* spirochaete bacterium, which causes syphilis. This antibiotic was commercialized in 1911 under the name Salvarsan® . In 1928, while working on *St. aureus,* Alexander Fleming discovered that the growth of *St.aureus* was inhibited by a fungus in contaminated culture plates exposed to air [16]. After culturing the mould - *Penicillium notatum*, a molecule called penicillin had been purified [13,16]. In 40s, several major antibiotics and antifungals, such as actinomycin (from Streptomyces spp.) [17], streptomycin (from *Streptomyces griseus*) [18], neomycin (from *Streptomyces fradiae*) [19], fumigacin (from *Aspergillus fumigatus*) and clavacin (from *Aspergillus clavatus*) [20] were discovered by Selman A. Waksman. The period between the 1940s and the 1970s is known as a "golden age" of antibiotic discoveries since the majority of currently used antibiotics were discovered during this time [13]. The major classes of clinically approved antibiotics [21] and the chemical structures of their representatives are listed in Table 1. Currently, the speed of discovery of new classes of antibiotics has substantially slowed down due to financial and technical difficulties.

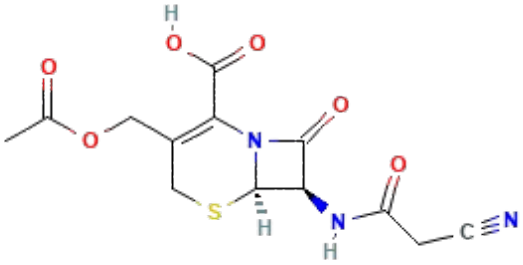
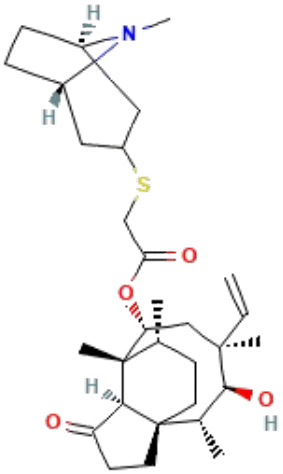
Class	Discovery reported	Introduced clinically	Example (and producing organism)	Molecular target

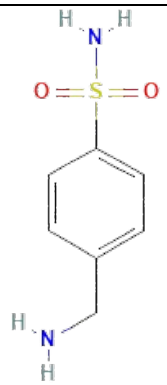
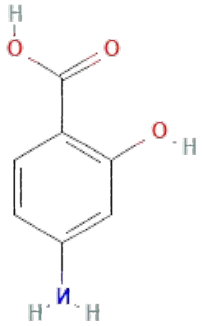
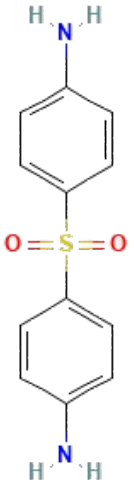
Antibiotics from actinomycetes				
Aminoglycosides	1944	1946	Kanamycin A (*Streptomyces kanamyceticus*) 	Protein synthesis: 30S ribosome subunit
Tetracyclines	1948	1948	Tetracycline (*Streptomyces aureofaciens*) 	Protein synthesis: 30S ribosome subunit
Amphenicols	1947	1949	Chloramphenicol (*Streptomyces venezuelae*) 	Protein synthesis: 50S ribosome subunit

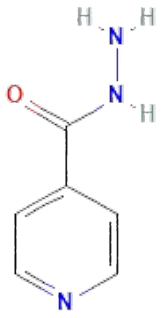
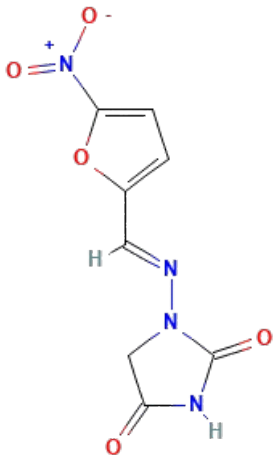
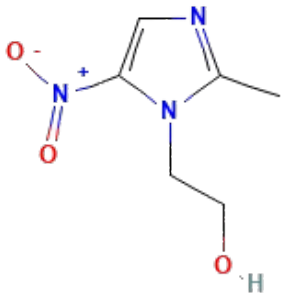
Macrolides	1952	1952	Erythromycin (*Saccharopolyspora erythraea*) 	Protein synthesis: 50S ribosome subunit
Carbapenems	1976	1985	Meropenem Synthetic molecule based on thienamycin (*Streptomyces cattleya*) 	Protein synthesis: 30S and 50S ribosome subunits (binds to the intersubunit bridge B2a)
Lincosamides	1962	1963	Clindamycin Semi-synthetic derivative of lincomycin (*Streptomyces lincolnensis*)	Protein synthesis: 50S ribosomal subunit

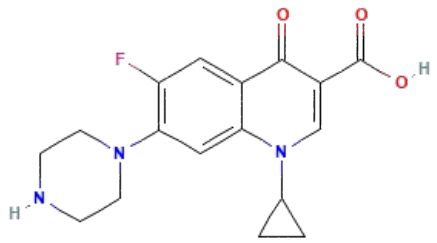
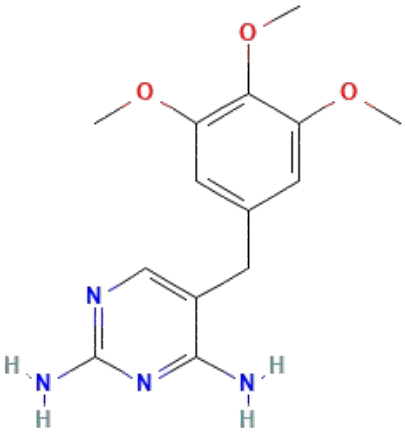
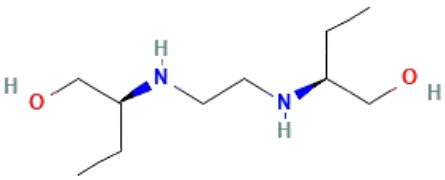
				
Ansamycins	1959	1963	Rifamycin SV Semi-synthetic derivative of rifamycin (*Amycolatopsis rifamycinica*) 	Nucleic acid synthesis: RNA polymerase
Phosphonates	1969	1971	Fosfomycin (*Streptomyces fradiae*) 	Cell wall synthesis: MurA (UDP-GlcNAc-3-enolpyruvyltransferase) inhibition
Lipiarmycins	1975	2011	Fidaxomicin (*Dactylosporangium aurantiacum* subsp. *hamdenesis*)	Cell wall synthesis: penicillin-binding proteins

				
Antibiotics from fungi				
Penicillins	1929	1943	Amoxicillin Semi-synthetic derivative of penicillin (*Penicillium chrysogenum*) 	Cell wall synthesis: penicillin-binding proteins
Fusidic acid	1958	1962	Fusidic acid (*Fusidium coccineum*) 	Protein synthesis: elongation factor G

Cephalosporins	1948	1964	Cefacetrile Semi-synthetic derivative of cephalosporin C (*Acremonium chrysogenum*) 	Cell wall synthesis: penicillin-binding proteins
Pleuromutilins	1951	2007	Retapamulin Semi-synthetic derivative of pleuromutilin (*Pleurotus mutilus*) 	Protein synthesis: 50S ribosomal subunit
Synthetic antibiotics				
Sulfonamides	1932	1936	Mafenide	Folate synthesis: inhibition of dihydropteroate synthetase

				
Salicylates	1902	1943	4-Aminosalicylic acid 	Folate synthesis: prodrug that inhibits dihydrofolate reductase
Sulfones	1908	1945	Dapsone 	Folate synthesis: inhibition of dihydropteroate synthetase
Pyrazinamides	1952	1952	Isoniazid	Cell wall: prodrug that inhibits the synthesis of mycolic acids

				
Nitrofurans	1945	1953	Nitrofurantoin 	DNA synthesis: DNA damage
Azoles	1959	1960	Metronidazole 	DNA synthesis: DNA damage
(Fluoro)quinolones	1962	1962	Ciprofloxacin	DNA synthesis: inhibition of DNA gyrase, and topoisomerase IV

				
Diaminopyrimidines	1950	1962	Trimethroprim 	Folate synthesis: inhibition of dihydrofolate reductase
Ethambutol	1962	1962	Ethambutol 	Cell wall: arabinosyl transferase inhibition
Thioamides	1956	1965	Ethionamide	Cell wall: prodrug that inhibits the synthesis of mycolic acids

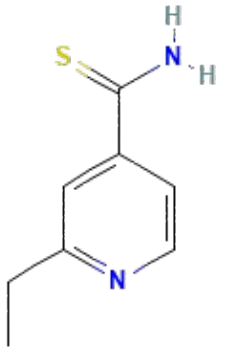
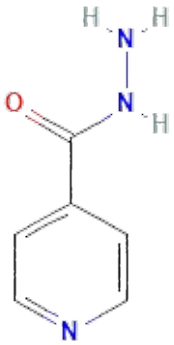
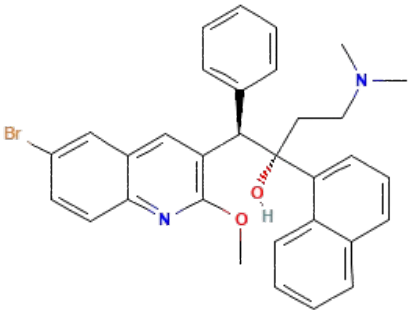
				
Oxazolidinones	1987	2000	Linezolid 	Protein synthesis: 50S ribosomal subunit
Diarylquinolines	2004	2012	Bedaquiline 	ATP synthesis: proton pump inhibition

Table 1. Major classes of clinically used antibiotics, their sources and targets.

[a] Classes are defined by origin, structure, and/or mechanism of action. (Modified from Hutchings et al. 2019).

2.2. Characteristics of the main components of bacterial cells affected by antimicrobial agents.

2.2.1. Bacterial envelope.

The bacterial envelope (comprising the cell wall and cytoplasmic membrane) is an essential component, defining the shape, promoting cell-cell signaling, protecting the cell from the environmental stress, and/or allowing the cell to quickly adapt to it. Based on cell wall composition, bacteria are divided into two major groups: Gram-positive and Gram-negative bacteria. Gram-positive bacteria (often mentioned as monoderms) have a thick peptidoglycan (PGN) layer (which retains the crystal violate dye during Gram staining) and a single layer of cytoplasmic membrane [22,23], while Gram-negative bacteria (diderms) possess a thin layer of PGN (unable to retain Gram-stain) and the second, outer layer of phospholipid membrane, containing lipopolysaccharides (LPS) (Fig. 2 A,B) [24]. Below, the main structural and functional peculiarities of Gram-negative and Gram-positive bacterial envelopes will be discussed.

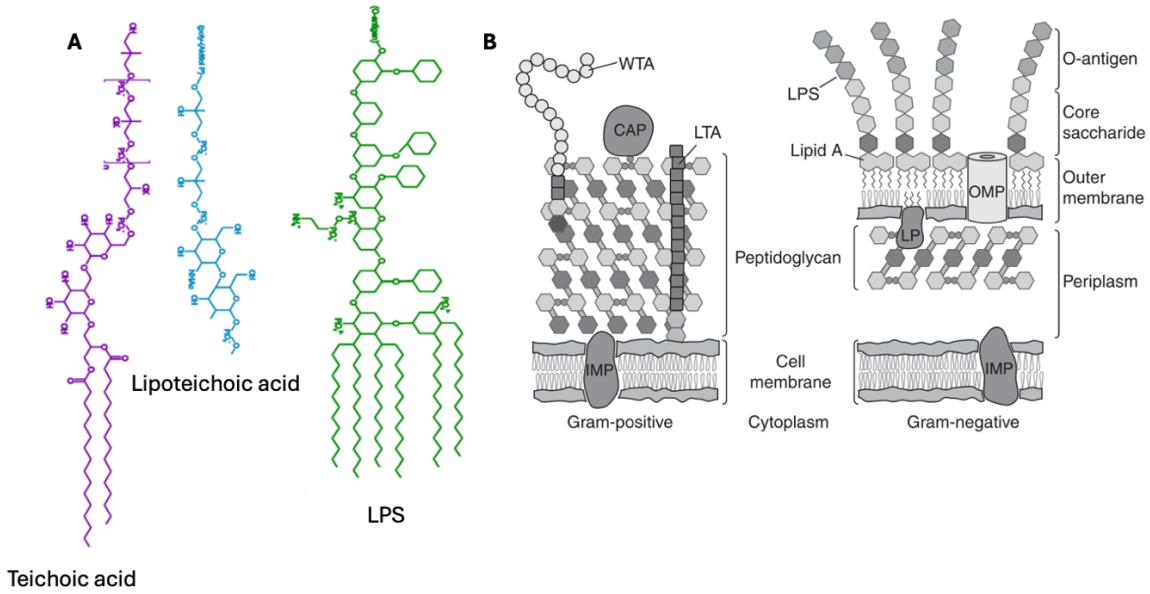


Figure 2. An illustration of bacterial cell envelope.

A. Chemical structures of the main components of the outer bacterial membrane. B. The structure of Gram-positive and Gram-negative bacterial envelopes: CAP = covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, outer membrane protein; WTA, wall teichoic acid. (From Silhavy et al. 2010) [23].

2.2.1.1. The envelope of Gram-negative bacteria.

The envelope of Gram-negative bacteria is composed of an outer membrane (OM), PGN mesh, periplasmic space, and inner membrane [25–27].

The OM is composed of lipopolysaccharides (LPS) (Fig. 2 A), lipoproteins, and β-barrel proteins (Fig. 2 B).

LPS plays a critical role in the barrier function of the OM and typically consists of a hydrophobic domain known as lipid A (or endotoxin), a "core" oligosaccharide, and a distal polysaccharide (or O-antigen) [23,28]. LPS is responsible for the endotoxic shock associated with septicemia caused by Gram-negative organisms [28]. The human innate immune system is sensitized to this molecule because it is a sure indicator of infection [23].

Most of β-barrel proteins also known as Outer membrane proteins (Omps) are porins through which small molecules such as mono- and disaccharides, amino acids, maltose, maltodextrins and phosphates passively diffuse across the OM. Additionally, there are Omps, that serve as gated channels in the high affinity transport of large ligands such as Fe-chelates or vitamins such as vitamin B-12 [29].

OM is anchored to the underlying PGN layer by lipoproteins (Lpp, Pal) (Fig. 3) [30]. Lpp-dependent OM–PGN connection plays an important role in controlling the stiffness of the cell envelope and its sensitivity to drugs [30].

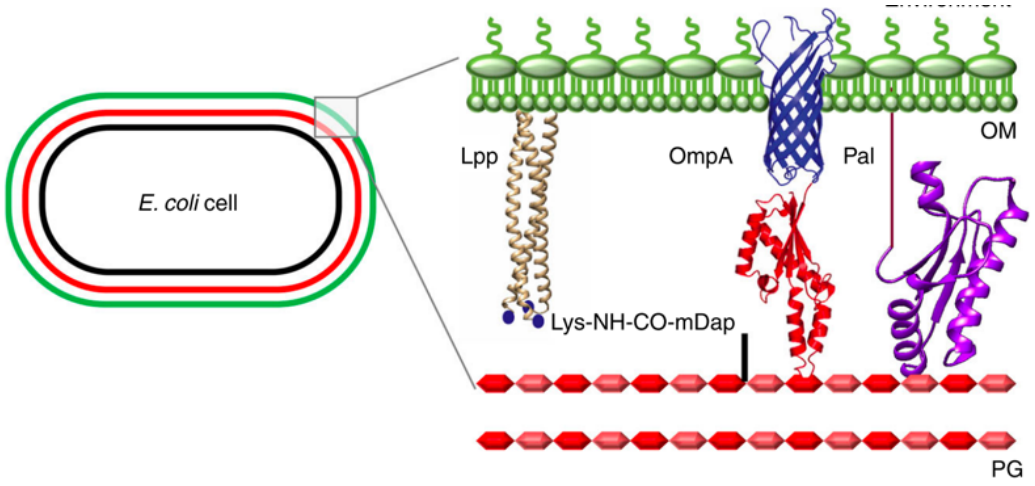


Figure 3. The envelope showing the presence of OM proteins connected to the PGN.

Lpp- lipoprotein; mDap - diaminopimelic acid; OmpA- outer membrane protein A; Pal- PGN-asscociated lipoprotein. The Lpp (PDB: 1EQ7) is the only OM lipoprotein covalently crosslinked to PGN while being inserted in the OM by a lipid moiety. Pal (PDB: 1OAP) interacts noncovalently with the PGN. OmpA is a representative of β- barrel protein inserted in the OM

(predicted structure represented in blue) and a periplasmic domain that interacts noncovalently with the PGN (represented in red: PDB: 4ERH). (Image from Mathelié-Guinlet et al. 2020).

PGN - the main determinant of bacterial cell shape is made up of repeating linear glycan strands composed of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues linked by glycosidic bond (Fig. 4). Each molecule of MurNAc contains a peptide stem through which crosslinking between glycan strands takes place [25]. Peptidoglycan fine structure (the types of peptide stem, glycan strands, or crosslinking between them) may vary depending on the strain, growth phase and the presence of nutrients [31]. It was shown that cells grown in the absence of some PGN synthesis precursors reduce the amount of peptidoglycan per surface unit down to one half the normal value, maintaining a typical morphology and growth parameters [31,32].

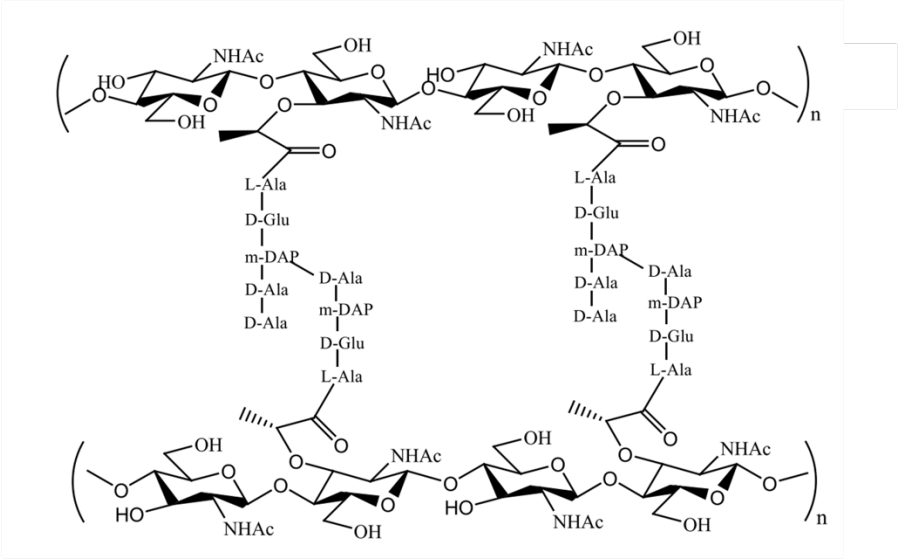


Figure 4. Schematic view of glycan strands united by peptide bridges. (From Kale et al. 2014) [33].

The OM and IM are divided by inner periplasmic space (Fig. 2 B). Periplasm accommodates peptidoglycan-remodeling enzymes, efflux pumps, nutrient transport proteins, and osmoprotectants, reflecting its roles in maintaining structural stability, cell wall integrity, and osmotic balance [27]. Periplasm also participates in scavenging and degradation of harmful degradative enzymes such as RNAse and alkaline phosphatase [23]. It also contains periplasmic binding proteins, which function in sugar and amino acid transport and chemotaxis, and chaperone-like molecules that function in envelope biogenesis [23,27].

IM is mainly composed of phospholipid bilayer, where most of the membrane proteins that function in energy production, lipid biosynthesis, protein secretion, and transport are located. [23]. IM is composed of glycerophospholipids i.e., phosphatidylethanolamine (PE) (~80%),

phosphatidylglycerol (PG) (~15%), and cardiolipin (CL) (~5%) [34–36], (Fig. 5). By using dyes with different lipid specificities it was demonstrated that different phospholipid species are not homogeneously distributed in the bilayer. For bacterial cytoplasmic membranes, it was observed that PE and CL preferentially occupy the inner leaflet, while PG is found on the outer face of the lipid bilayer [35,37,38]. Also, it was noticed that PG and CL are both localized at bacterial poles [35,39]. Such distribution of anionic phospholipids is supposed to be related to bacterial cell negative curvature at poles and septal regions (during bacterial division) [36,38,40]. Interestingly, a relatively small head group and a relatively large tail group give the CL molecule an intrinsic negative curvature, which can explain its localization at bacterial poles [36,40]. It was demonstrated that CL and PG were found to be associated with, and stabilize a variety of vitally important membrane proteins (ion channels, transporter, division proteins, and others) [40–42].

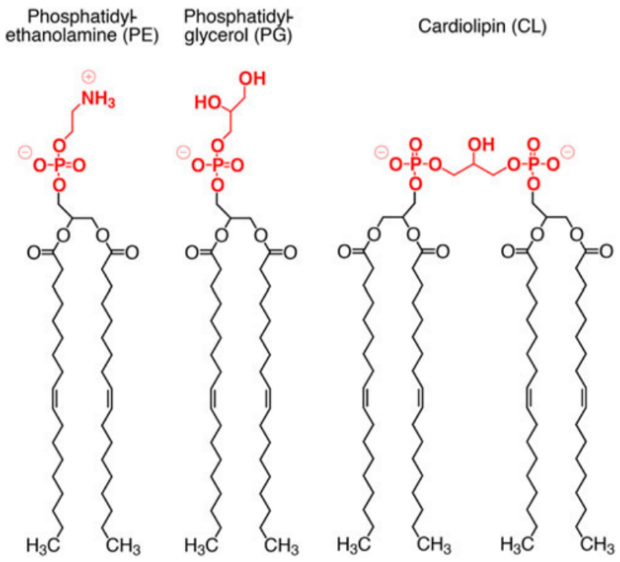


Figure 5.Chemical structures of three major classes of phospholipids found in cytoplasmic bacterial membrane. (From Lin et al. 2016) [43].

2.2.1.2. The envelope of Gram-positive bacteria.

The major difference between Gram-positive bacteria and Gram-negative is the absence of OM. The cell wall of Gram-positive bacteria is made of many PGN layers of about 40–80 nm [23,44]. Another peculiarity for Gram-positive bacteria is the occurrence of teichoic acid in the cell wall that can be linked to PGN (WTA) or via a glycolipid anchor with the plasma membrane (LTA) [44] (Fig. 2 B). Teichoic acids and surface proteins (CAP), which are attached to teichoic acids or PGN participate in the recognition and adherence to host tissues, resulting in the initiation of infection [45].

The IM is composed of phospholipids. Their types, headgroups and fatty acid moieties vary among species. Also, Gram-positive bacteria havea larger fraction of negatively charged PG compared to Gram-negative bacteria [23,44].

2.2.2 Bacterial cytoplasm.

Bacterial cytoplasm is a gel-like matrix composed of water, nutrients, wastes, gases and generally contains a nucleoid, plasmids (optional) and ribosomes.

2.2.2.1. Nucleoiod

Nucleoid is represented by a bacterial chromosome (single, topologically constrained, covalently closed circular DNA) and is usually complexed with RNA polymerase (RNAP), topoisomerases, and nucleoid-associated proteins (NAPs). The *E. coli* nucleoid (Fig. 6 A, B) is composed of two parts: a core consisting of a dense network of DNA and an outer periphery that extends into the cytoplasm and is associated with numerous soluble proteins [46]. Replication, transcription and translation are thought to occur in the outer periphery of the nucleoid [46–48].

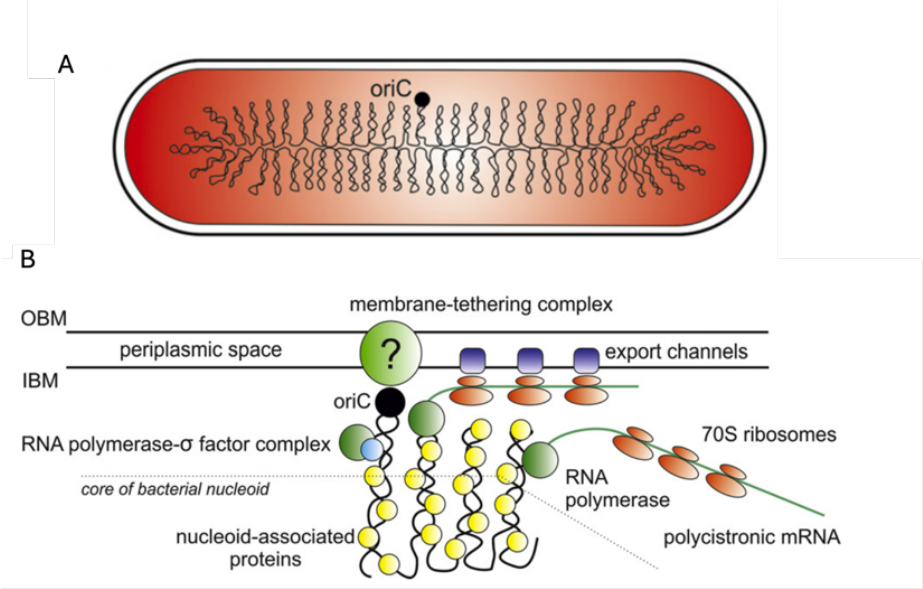


Figure 6. The *E.coli* nucleoid.

A. The overall illustration of nucleoid architecture. B. Schematic representation of the main nucleoid components. The origin of replication (oriC) is indicated by a black dot. OBM – outer bacterial membrane, IBM – inner bacterial membrane. DNA is shown as black strands; RNA as green strands. Yellow circles- represent several nucleoid-associated proteins (NAPs), green circles- RNAP, red ellipses represent ribosomes. (From Ambro et al. 2012) [46].

2.2.2.2.Topoisomerases

Topoisomerases are ubiquitous enzymes that control the topological state of DNA in the cell [49]. The four known topoisomerases of *E. coli* can be classified into two groups based on mechanisms of action. Type I topoisomerases, represented by *E. coli* topoisomerase I and

topoisomerase III, break single strands of duplex DNA, pass another single DNA strand through the break, and then reseal the break [50,51]. In contrast, type II topoisomerases, represented by *E. coli* DNA gyrase (also referred to as topoisomerase II) and topoisomerase IV, break both strands of duplex DNA, pass another DNA duplex through the break, and reseal both breaks coordinately [50,51]. Among four topoisomerases, DNA gyrase is the only known topoisomerase able to generate negative supercoils in the bacterial chromosome, which are essential for chromosome condensation, leading to proper chromosome segregation during cell division [52]. Topoisomerase I functions as a counterpart of DNA gyrase by removing negative superhelical twists from circular DNA. Both topoisomerase I and topoisomerase III have the ability to unlink or decatenate interlocked DNA circles during DNA replication [53]. Deletion of *topB* gene encoding Topoisomerase III although leaves the mutants viable, increases the rates of spontaneous deletions in chromosomal DNA [54].

Within the bacterial cell, topoisomerase IV appears to be the principal enzyme that resolves interlocked daughter DNA circles occurring at the completion of a round of DNA replication, allowing segregation of daughter chromosomes into daughter cells [40, 41]. Under special circumstances, hyperexpression of DNA gyrase may complement defects in Topoisomerase IV [55], and hyperexpression of Topoisomerase IV may complement defects in Topoisomerase I [50]. Both of the type II Topoisomerases of *E. coli* are essential for cell survival, as evidenced by conditional lethal mutations in their genes [56], and thus are promising targets for the development of antimicrobial drugs. The overview of reactions catalyzed by Topoisomerase IV and DNA gyrase is presented in Fig. 7.

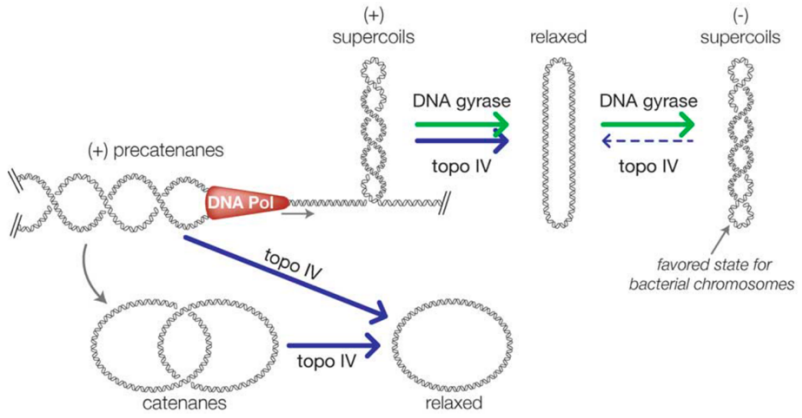


Figure 7. Reactions catalyzed by DNA gyrase and topoisomerase IV.

Gyrase (green arrows) relaxes positive supercoils and introduces negative supercoils to maintain bacterial genomes in a slightly underwound state. Topoisomerase IV (blue arrows) removes

positive supercoils, catenanes, and knots, and can also relax negative supercoils to a modest extent (dotted arrow) (From Corbett et al. 2005) [57].

2.2.2.3.NAPs and RNAP.

Together with topoisomerases, NAPs, and RNAP participate in the maintenance of DNA topological state through all major bacterial events.

NAPs (HU, H-NS (H1), H, HLP1, IHF, and FIS) are small, basic bacterial proteins involved in maintaining DNA architecture (Fig 6), [58] and play important roles in gene regulation [59–61]. They show minor similarities with eukaryotic histones at the sequence or the structural level [62]. Each of these proteins contributes to bacterial DNA folding into a compact structure by bridging, bending, or wrapping depending on the cell growth phase. Additionally, NAPs maintain genomic stability by protecting DNAs from DNAase I-mediated degradation (HU) and reactive oxygen intermediates (HLP) [63–65].

RNA polymerase (RNAP) is tightly associated with nucleoid and plays an important role in its remodeling and influences global gene expression. Multisubunit RNAP is responsible for the synthesis of all RNAs in the cell [66].

2.2.2.4. Ribosomes.

Translation of the mRNA-encoded genetic information into proteins is catalyzed by the intricate ribonucleoprotein machine, the ribosome. The bacterial ribosome (70S) is composed of two asymmetric subunits, the 30S and the 50S subunit, which assemble at the ribosome binding site on the mRNA during translation initiation. Each subunit contributes to specific functions in protein synthesis. In *E. coli* the small 30S subunit is composed of the 16S rRNA consisting of and 21 proteins [67]. It mediates the step of initiation and contains the binding sites for the three initiation factors as well as the messenger decoding center, where the respective codons of the mRNA are base-paired with the anticodon of the cognate tRNA [67]. The large 50S subunit consists of two different rRNAs, the 23S rRNA and the 5S rRNA and 34 proteins. It catalyzes peptide bond formation at the peptidyl transferase center, provides the binding sites for the elongation factors, and comprises the exit tunnel for the nascent peptide chain [68].

2.3. Mechanisms of action of conventional antibiotics

Antibiotics are commonly classified based on their mechanism of action. Four major antibiotic targets are: bacterial cell wall synthesis, protein synthesis, DNA and RNA synthesis, and folic acid (vitamin B9) metabolism (Fig. 8) [69].

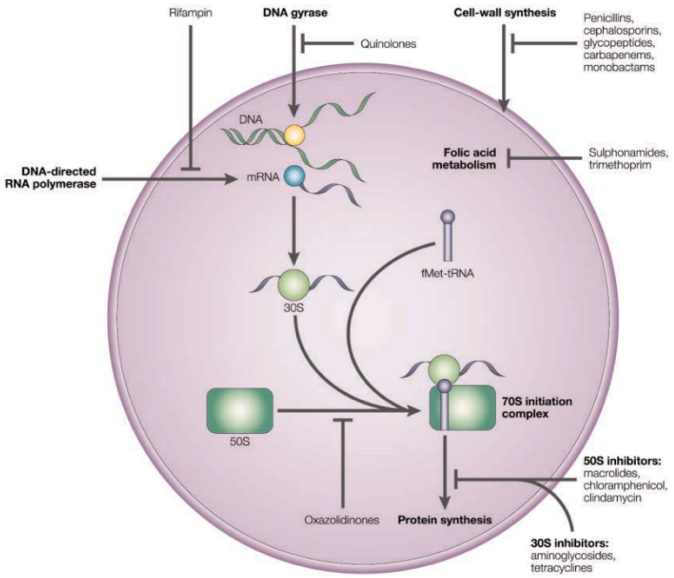


Figure 8. Main antibiotic drug targets. (Image from Coates et.al.2002) [70].

2.3.1 Inhibition of cell wall synthesis.

A major example of antibiotics targeting bacterial cell wall synthesis is represented by β-lactam antibiotics. β-lactam antibiotics contain β-ring in their structure, and the most common group is represented by penicillin derivatives, cephalosporins, and carbapenems [71]. They easily translocate to bacterial periplasm through membrane OmpF porin, where they covalently bind to penicillin-binding proteins (PBPs) (Fig. 9)- the enzymes that are involved in the terminal steps of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria [72]. As a result, prevention of cell wall formation leads to the activation of autolytic enzymes and, subsequently, bacterial lysis [73,74].

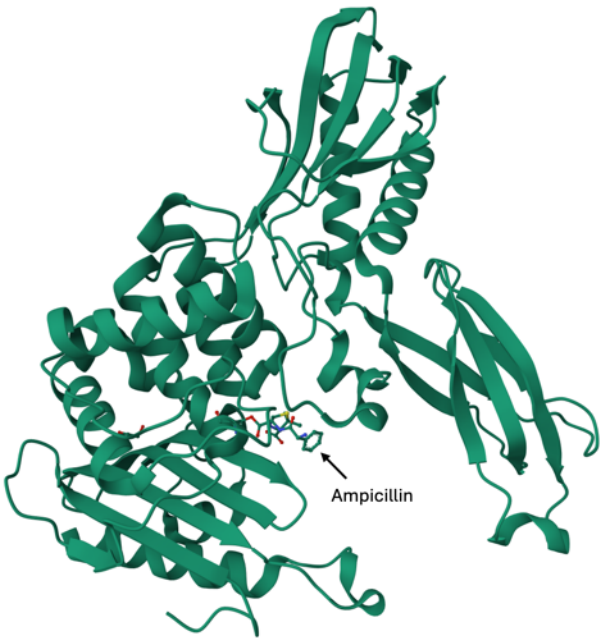


Figure 9. Crystal structure of penicillin-binding protein 4 (dacB) from *E. coli*, complexed with ampicillin.PDB entry: 2EX6.

2.3.2 Inhibition of protein synthesis.

Among antibiotics targeting protein synthesis are tetracyclines, chloramphenicol, aminoglycosides and others. All of them target either 50S or 30S subunits of bacterial ribosome (Fig. 10).

For example, aminoglycoside antibiotics (such as streptomycin and gentamicin (GEN) containing amino-modified glycoside in their structure impair bacterial protein synthesis by interaction with 30S ribosomal subunit [75]. GEN, for instance, binds to a conserved sequence of rRNA located near the codon-anticodon recognition site (A site) of 30S subunit. Aminoglycoside binding stabilizes the tRNA–mRNA interaction in the A site by decreasing tRNA dissociation rates, which interferes with proofreading steps that ensure translational fidelity [76,77]. Other classes of ribosome targeting antibiotics, such as macrolides and chloramphenicol, bind 50S subunit of 70S of bacterial ribosome and prevent the recognition of A site by tRNA [78,79].

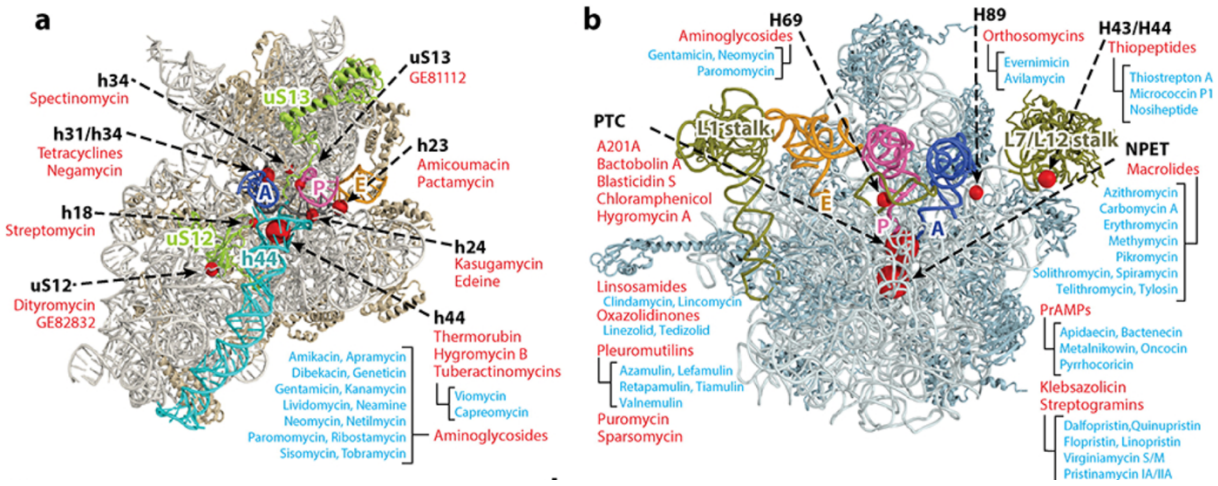


Figure 10. Major binding sites on the 30S (a) and 50S (b) subunits for ribosome-targeting antibiotics. Image from Lin et al. 2018 [80].

2.3.3 Inhibition of DNA replication.

The group of antibiotics targeting DNA replication is represented by quinolones sharing bicyclic aromatic core structure [81]. Quinolones only became widely used after the introduction of fluorine in their structure by chemical modification [82]. These second-generation compounds, fluoroquinolones (norfloxacin, ciprofloxacin, levofloxacin), displayed considerably improved activity due to better cell penetration and longer half-life [83]. Quinolones act by inhibiting the activity of two essential bacterial type II Topoisomerases: DNA gyrase and topoisomerase IV, which are involved in the modulation of the chromosomal supercoiling required for DNA synthesis, transcription and cell division [81,82]. Quinolones bind to DNA-Topoisomerase IV /gyrase and generate cleavage complexes (Fig. 11) physically blocking DNA strand religation, resulting in inhibition of DNA synthesis, which immediately leads to bacteriostasis (at low quinolone concentrations) and eventually to cell death (at lethal concentrations) [79,84].

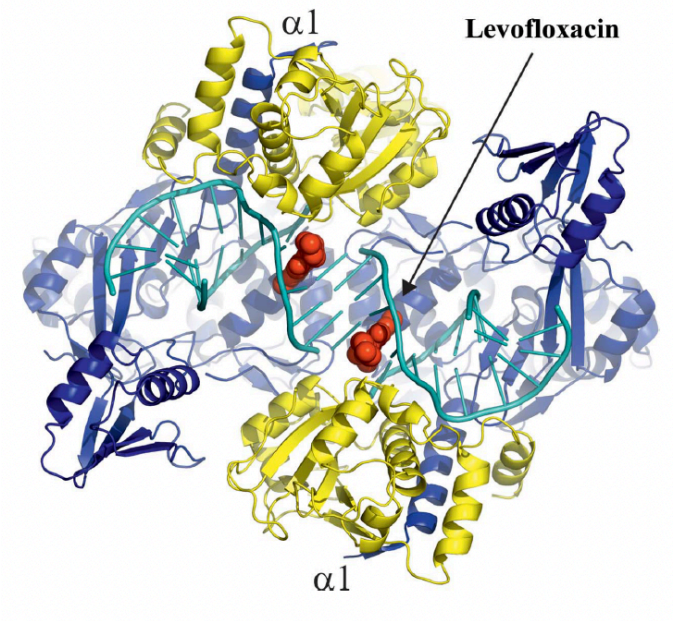


Figure 11. Topoisomerase IV cleavage complexed with levofloxacin. (From Veselkov et al. 2016) [85].

2.3.4 Inhibition of DNA transcription.

RNA polymerase (RNAP) is the key enzyme in bacterial gene expression [86]. The fact that bacterial RNAP subunit sequences are highly conserved among bacterial strains, less conserved eukaryotic RNAP I, RNAP II, and RNAP III represent an attractive target for antibiotics. Among commercially available antibiotics targeting bacterial RNAP, rifamycin (a product of fermentation from the gram-positive bacterium *Amycolatopsis mediterranei*) is the most studied representative [87]. Rifamycins bind within the cleft close to the active center of RNAP (Fig. 12), which sterically hinders the growth of the RNA product rather than DNA binding or RNA synthesis sites [88]. Rifampicin is effective against a broad range of bacteria, including *Mycobacterium tuberculosis*, making it a key drug in tuberculosis (TB) treatment [86,88].

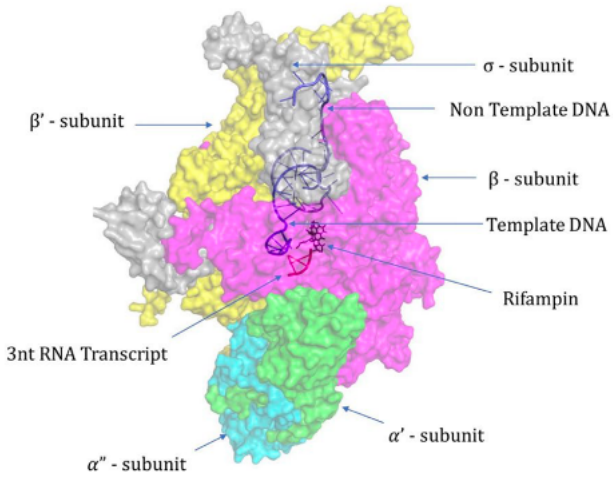


Figure 12. RNAP in complex with DNA, rifampin (a representative of rifamycin class antibiotics) and RNA transcript. (From Vedithi et al. 2018) [89].

2.3.5 Inhibition of folate synthesis.

Although folate is vital for humans and animals [90], only plants, fungi, and some bacteria (including pathogenic strains) have an elaborate folate biosynthesis pathway [91,92]. Synthesis of folate is crucial for bacterial growth and proliferation, in particular, folate cofactors are required for the formation of methionine, purines and thymine [91,92]. It has also been observed that *E. coli* with either genetically or pharmacologically impaired folate metabolism generated a lowered number of antibiotic-resistant cells [92], which is why folate biosynthesis represents a promising target for antibacterial drug design. Sulfa drugs act as competitive inhibitors mimicking the structure of a substrate (p-aminobenzoic acid) for folate synthesis and bind the active site of a corresponding enzyme (dihydropteroate synthase), preventing the production of folate precursors [91,93]. Antibiotic trimethoprim inhibits dihydrofolate reductase (DHFR), another key enzyme in the folate synthesis pathway which is required to generate an active form of folate involved in one-carbon transfer reactions necessary for the synthesis of nucleotides and amino acids [93] (Fig. 13).

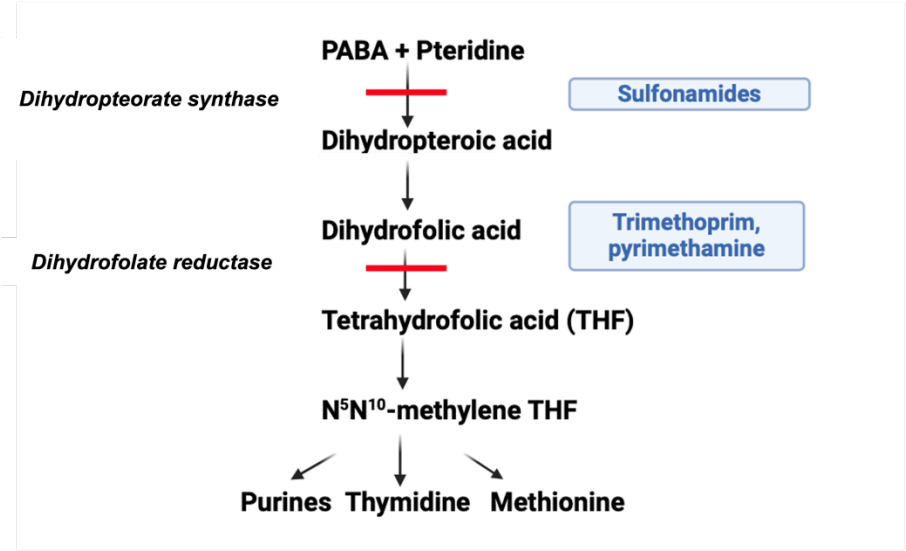


Figure 13. Folate synthesis inhibitors.

2.4. Mechanisms of antibiotic resistance.

Antibiotic resistance is the evolutionary response to the strong selective pressure that results from exposure to these antimicrobials [69]. Antibiotic resistance is not a modern phenomenon [94]. Recent studies conducted on ancient environmental and human samples have uncovered numerous antibiotic-resistance genes [95]. The analysis of bacterial DNA extracted from dental plaques of ancient human skeletons has shown the presence of resistance genes coding for multi-drug efflux pumps and resistance towards aminoglycosides, β-lactams and other antibiotics [94,96,97]. Susceptibility and resistance are usually measured as a function of minimum inhibitory concentration (MIC), the minimal concentration of a drug that inhibits the growth of the bacteria [98]. Prolonged exposure to sub-inhibitory concentrations of antimicrobials can lead to a selection of high-level resistance in successive bacterial generations and increase their ability to acquire resistance toward other antimicrobial agents [99]. The main mechanisms of antimicrobial resistance include limited uptake and drug efflux, drug target modification, and drug inactivation (Fig. 14) [98].

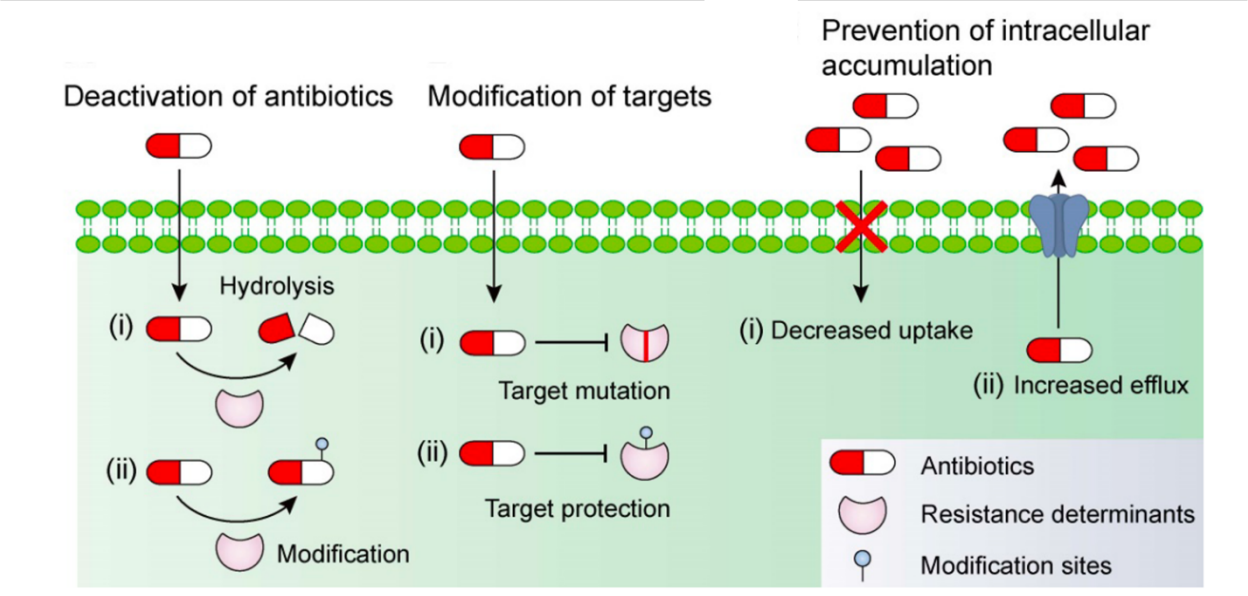


Figure 14. Schematic representation of main mechanisms of antibiotic resistance. (From Liu et al. 2021) [100].

2.4.1. Limited Antibiotic Uptake and Efflux.

Bacteria have developed mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target by decreasing the uptake of the antimicrobial molecule.

The decreased uptake of drugs is usually achieved by modification of porins, by regulation of porin expression, impairment of the porin function, and point mutations in genes encoding for porins [29,101,102]. Hydrophilic molecules such as β-lactams, tetracyclines, and some fluoroquinolones are particularly affected by changes in the permeability of the outer membrane since they generally use these water-filled diffusion channels to pass through the membrane of Gram-negative bacteria [103].

However, changes in porin permeability are frequently not enough to generate high resistance and are often combined with other mechanisms of resistance, such as increased expression of efflux pumps [29].

Efflux pumps are transmembrane proteins present ubiquitously in plasma membranes of bacteria (and other living organisms). Two major classes of bacterial efflux pumps are Major Facilitator Superfamily (MFS) Resistance Nodulation Division (RND) pumps and ATP binding cassette (ABC) transport proteins [104]. MFS and RNDs pumps act as secondary active transporters since they use proton motive force or sodium ion gradient as their energy source, while ABC pumps utilize ATP hydrolysis as the energy source for drug extrusion [105,106].

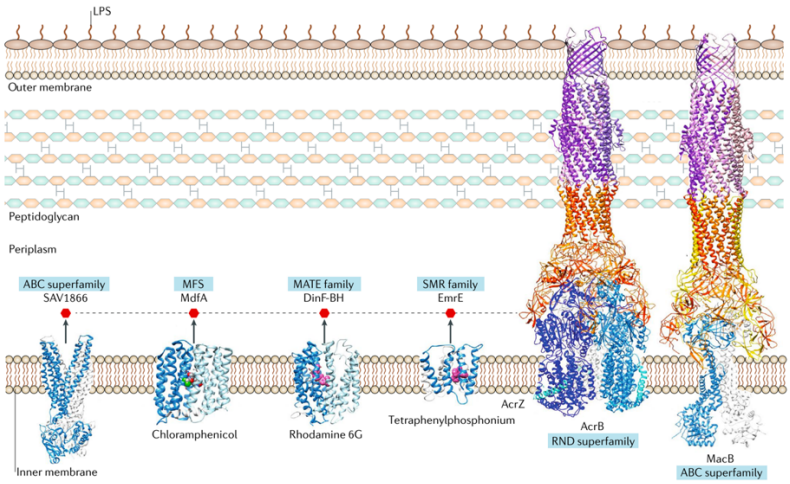


Figure 15. Structures of representatives of each of the transporter families.

ATP-binding cassette (ABC), major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE), small multidrug resistance (SMR), and resistance-nodulation (RND) families.The ligands for the MFS, MATE and SMR representatives are indicated. The RND superfamily bind substrates at the outer leaflet of the inner membrane and periplasm and efflux them to the cell exterior. Members of the other families of transporters translocate substrates across the membrane bilayer [107].

The description of an efflux system (MFS) able to pump tetracycline out of the cytoplasm of *E. coli* dates back to 1980s and was among the first to be described [108]. Since then, many classes of efflux pumps have been characterized in both Gram-negative and Gram-positive pathogens (Fig.15). These systems may be antibiotic specific (for tetracycline and macrolides) or have broad substrate specificity, which is usually found in MDR bacteria [109]. The described mechanism of resistance applies to a wide range of antimicrobial classes including aminoglycosides, fluoroquinolones, β-lactams, carbapenems, and polymyxins [105].

2.4.2 Drug Target Modification

There are multiple components in the bacterial cell that may be modified by the bacteria to enable resistance to antibacterial agents. For example, alteration of structure and/or number of PBPs (the key players of bacterial cell wall synthesis [110]), is used by Gram-positive bacteria against β-lactam drugs [111]. This results in a decreased affinity for the antibiotic or a decrease in the total number of PBPs [98,110,112].

Resistance towards drugs targeting ribosomal subunits may occur via mutations in ribosomal proteins (aminoglycosides, oxazolidinones) or ribosomal subunit methylation (aminoglycosides, macrolide, oxazolidinones, streptogramins) [80]. Both of these mechanisms interfere with the ability of the drug to bind the ribosome. The level of drug interference varies greatly among antibiotics [80,98].

For drugs that target nucleic acid synthesis (fluoroquinolones), resistance is achieved via modifications in DNA gyrase (Gram-negative bacteria) or Topoisomerase IV (Gram-positive bacteria). These mutations cause changes in the structure of gyrase and topoisomerase which decrease or eliminate the ability of the drug to bind the target sites [82,98,113] .

2.4.3. Drug inactivation.

There are two main ways in which bacteria inactivate drugs: by actual degradation of the drug or by transfer of a chemical group to the drug [98]. The β-lactamases (originally called penicillinases and cephalosporinases) inactivate β-lactam drugs by hydrolyzing a specific site in the β-lactam ring structure, causing the ring to open and prevent the binding to PBP proteins [114–116]. The production of β-lactamases is the most common resistance mechanism used by Gram-negative bacteria against β-lactam drugs [98,117].

Drug inactivation is accomplished by tranferases, which transfer acetyl, phosphoryl, and adenyl groups to the target antibiotic. For example, aminoglycoside modifying enzymes (nucleotidyltranferases, phosphotransferases, or acetyltransferases) catalyze the modification at −OH or −NH2 groups, preventing the antibiotic from interacting with A site of bacterial 16S rRNA [118–120]. Interestingly, some aminoglycoside acetyltransferases have adapted to modify fluoroquinolones by N-acetylation of nitrogen of their aromatic core [120] resulting in their reduced affinity to their target.

2.5. Alternatives to conventional antibiotics.

Intense dissemination of antibiotic resistance mechanisms brought the necessity of the development of alternative approaches to combat bacterial infections. Below are discussed the existing alternatives to commercially available antibiotics. Some of them have already been approved for clinical use (for example, phages and antibodies), while others, such as the CRISPR-CAS system, are still under laboratory studies.

2.5.1 Phage therapy.

Phage therapy relies on the use of naturally occurring phages to infect and lyse bacteria at the site of infection. Most phages are infectious only to the bacteria that carry their complementary receptor [121,122]. Host specificity varies among phages, some of which are strain-specific, whereas others have demonstrated the capability of infection across a range of bacterial strains [123]. Human trials for phage therapy have taken place at several institutes in Eastern Europe, one of which is the Eliava Institute of Bacteriophage. The Eliava Institute has extensively used phages (and phage mixtures) in the treatment of common bacterial pathogens such as *S.*

aureus, E. coli, Streptococcus spp., P. aeruginosa, Proteus spp., S. dysenteriae, Salmonella spp., and Enterococcus spp.[124,125].

Among the most promising advances in phage therapy is the isolation of phage-encoded lytic enzymes - endolysins (discussed later in this chapter), which are functionally similar to the antimicrobial enzyme lysozyme [126]. Recently, endolysin ABgp46 was reported to have the ability to lyse several Gram-negative and multidrug-resistant pathogens, including *A. baumannii*, *P. aeruginosa*, and *Salmonella typhimurium* [127]. However, despite successful examples of phage therapy for the treatment of pathogen-induced infections, immunogenicity induced by the release of bacterial endotoxins [128,129] hampers its broad clinical application [130].

2.5.2. Probiotics.

Antibiotic treatment usually perturbs the composition of the human gut microbiota, and as a consequence, drug-resistant pathogens provoke secondary infections [131]. Probiotics and prebiotics have been used for the treatment of various gastrointestinal infections such as pseudomembranous colitis caused by *Clostridium difficile* and *Helicobacter pylori* [132,133]. *Lactobacillus* and *Bifidobacterium* have been used for the treatment of various gastrointestinal infections. The concept governing the use of probiotics is that, upon restoration of balance in the gut microbial flora, the commensal bacteria can outgrow and competitively exclude pathogenic strains [129].

2.5.3. **Antibodies.**

Antibodies could be used to treat bacterial infections either by directly targeting the bacterial surface or indirectly by neutralizing the bacterial toxins and the virulence factors that are responsible for infection [134,135]. A major drawback of using antibodies for antibacterial therapy is the cost of production and poor shelf life [129].

2.5.4. **Lysins.**

Similar to phage endolysins bacterial exolysins (produced by bacteria to kill cells of other strains or species) and autolysins (functional during remodeling of peptidoglycans during cell growth and division) might be considered as antibiotic alternatives [136,137]. Since these enzymes are genetically encoded, they can be produced using genetic engineering [129,138].

2.5.5. **CRISPR-Cas 9.**

CRISPR (clustered, regularly interspaced, short palindromic repeats) - Cas9 (CRISP-associated protein 9) system, which serves as a part of bacterial defense mechanism from viruses, is viewed as a promising approach to combat the resistance[139]. Several reports have shown a successful delivery of CRISPR-Cas9 in carbapenem-resistant strains of *E.coli* and *St. aureus* strains and prevented horizontal gene transfer by targeting drug-resistance genes [140,141].

2.5.6. Antimicrobial peptides (AMPs).

Due to their diverse properties AMPs represent one of the most promising alternatives to modern antibiotics. Natural AMPs (ribosomal and non-ribosomal) exhibit activities ranging from antibacterial, antifungal, antiviral, anticancer, antiplasmodial, antiprotozoal, insecticidal to antibiofilm, immunomodulatory, and anti-inflammatory [142]. AMPs attack multiple bacterial targets simultaneously, which makes the development of resistance complicated. Although natural AMPs – such as polymyxin B and colistin (lipopeptide obtained from *Bacillus polymyxa*), gramicidin (a linear polypeptide derived from *Bacillus brevis*), daptomycin (from *Streptomyces roseosporus*) – are being used in the clinic [143,144], they have not yet had full clinical success [145] due to high production cost, short half-life and degradation by proteases. To circumvent the latter, synthetic mimics (peptidomimetics) of natural peptides are being developed. Some examples include β-peptides, oligoureas, peptoids, oligoacyl-lysines and others [146]. AMPs under clinical trial are listed in Table 2.

The diversity and biological properties of AMPs will be reviewed in more details in the following section.

Product	Company	Indication	Status
Locilex (Pexiganan)	Dipexium Pharma	Diabetic foot ulcers	Failed Phase III Phase
CLS001 (Omiganan)	Cutanea Life Sciences	Rosacea, acne, atopic dermatitis, and genital human papillomavirus	Phase III–Phase II
AB103	Atox Bio	Necrotizing soft-tissue infections	Phase III
LL-37	Promore Pharma	Chronic leg ulcers	Phase III
LL-37	M. D. Anderson Cancer Centre	Melanoma	Phase III
NP213	Novabiotics	Onychomycosis Oral	Phase III
P-113	Pacgen Lifesciences	Oral candidiasis	Phase III
SGX 942	Soligenix	Oral Mucositis	Phase II (Fast track)
Brilacidin	Innovation Pharmaceuticals	Oral mucositis Skin infections	Phase II Phase
AMC-109	Amicoat AS AP	Impetigo Nasal decolonization	Phase II Phase
AP 138	Adenium Biotech	MRSA implant infections Narrow-spectrum	Phase I
Avidocin and purocin	Pylum Biosciences	Narrow-spectrum antibiotic Acne	Preclinical
HB 1345	Helix Biomedix	Acne	Preclinical
HB 1275	Helix Biomedix	*Trichophyton* infections	Preclinical
Plectasin	Adenium Biotech	Gram-positive	Preclinical
OG-716	Oragenics	*Clostridium difficile* infections	Preclinical
NP432	Novabiotics	Multibacterial infections	Preclinical
CSA-13	N8 Medical	Bacterial infections/fracture	Preclinical

Table 2. Antimicrobial Peptides, Synthetic Mimics and Lysins in Clinical Trials. (From Ghosh et al. 2019) [129].

2.6. Diversity and biological properties of AMPs.

2.6.1. Discovery of antimicrobial peptides.

The history of antimicrobial peptides goes in hand with the history of antibiotics. In 1922 (a few years earlier than penicillin was discovered), Alexander Fleming discovered lysozyme - a typical antimicrobial peptide [147]. However, due to toxicity and difficulties/high costs in production, this and other AMP compounds identified later were left without much attention, while penicillins found in the same period were put into production and use [147,148]. In 1939, René Dubos reported another AMP gramicidin, which was isolated from the soil bacterium (Gram-positive) *Bacillus brevis* [149]. Gramicidin was a heterogeneous mixture of six AMPs consisting of N-formylated polypeptides with alternating L - and D-amino acids [150]. It exhibited both bactericidal and bacteriostatic activities against a wide range of Gram-positive bacteria and was the first AMP to be commercially produced as an antibiotic despite its high cytotoxicity [151].

Subsequently, AMPs were gradually isolated from bacteria, fungi, animals, and plants. The first reported animal-originated AMP is defensin isolated from rabbit leukocytes in 1956 [152]. In the following years, bombinin from epithelia [153] and lactoferrin from cow milk [154] were described. During the same time, it was also proven that human leukocytes contain AMPs in their lysosomes [155]. In 1981, cecropin - the first insect AMP in history, was isolated from the hemolymph (plasma and blood) of the silk moth, which was cationic, α-helical peptide, active against many types of Gram-positive and Gram-negative bacteria and fungi [156]. Another important advance occurred when Zasloff and colleagues in 1987 isolated and characterized cationic AMPs, named ‘magainins’ from the African clawed frog *Xenopus laevis*. Afterwards, peptides of structure similar to cecropin and magainin were isolated from bovine neutrophils [157]. These AMPs were later united in a peptide family named “cathelicidins.” The name derived from the similarity of their highly conserved domain to that of cathelin, a protease cathepsin L inhibitor [158]. About 30 cathelicidin family members have been identified in mammalian species. Cathelicidins and defensins represent two major families of vertebrate antimicrobial host defense peptides (HDP) [159].

In the mid-1990s, the antimicrobial role of AMPs was confirmed for the fruit fly *Drosophila melanogaster*, where deletion of a gene encoding an AMP rendered the insect susceptible to a massive fungal infection [160].

Due to the spread of MDR strains, the discovery of AMPs has been substantially scaled up during the last decades. Currently, more than 15,000 of AMPs of different structures and origins

have been discovered or synthesized as reported in the Database of Antimicrobial Activity and Structure of Peptides - DBAASP [161].

2.6.2. Natural Sources of antimicrobial peptides.

Depending on their origin, AMPs can be divided into natural or synthetically produced. Natural AMPs can be found in almost all kingdoms of life, ranging from viruses to mammals. Synthetic/semisynthetic AMPs are generated either by modifications mimicking natural AMPs or by the design of novel sequences on bases on various bioinformatical approaches discussed later in this chapter.

2.6.2.1. Bacteriophage/Viral AMPs.

Endolysins and virion-associated peptidoglycan hydrolases (VAPGHs) are two main groups of phage/viral proteins showing antibacterial activity [130,162,163].

Endolysins (already mentioned above) ranging from 25 to 40 kDa in size are fast-acting phage-encoded peptidoglycan-hydrolyzing enzymes targeting microbial cell wall and permitting bacteriophage progeny to release [164]. Endolysins have a characteristic modular structure, often with multiple lytic and/or cell wall-binding domains (CBDs). They degrade the PGN with glycosidase, amidase, endopeptidase, or lytic transglycosylase activities and have been shown to synergize with other antimicrobials [126].

VAPGHs are released from phage tail tips at the initial stage of PGN penetration and have a C-terminal cell-wall-binding domain, which can link them to receptors on the bacterial cell surface [163]. Due to the high specificity of VAPGHs, remarkable thermostability, and modular organization, these proteins are potential candidates for new antibacterial agents against MDR pathogens [165,166].

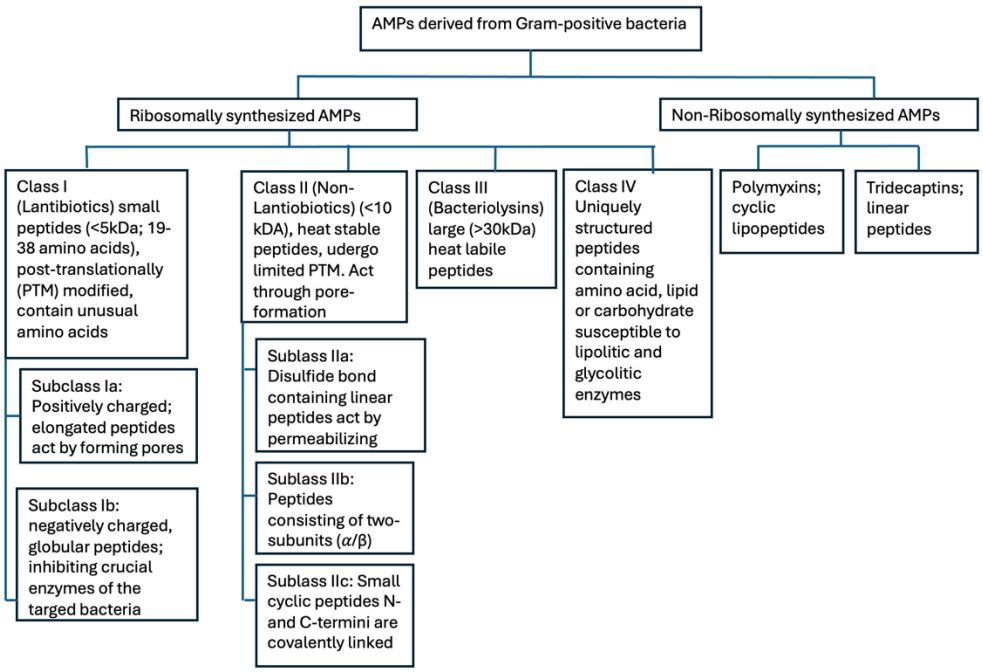
2.6.2.2. Bacterial AMPs.

Bacterial AMPs are of ribosomal or non-ribosomal origin [167]. Ribosomally synthesized bacterial AMPs from Gram-positive bacteria include: lantibiotics (< 5kDa translationally modified (PTM) peptides containing unusual amino acids), non-lantibiotics (<10kDa, heat stable disulfide-containing linear peptides and small cyclic peptides whose N- and C-termini are covalently linked), bacteriocins (> 30 kDa heat labile peptides possessing endopeptidase-like activity against peptidoglycan and uniquely structured bacteriocins susceptible to lipolyitc enzymes [168,169]. Non-ribosomal AMPs from Gram-positive bacteria include cyclic lipopeptides (heptapeptides with a tripeptide side chain linked to an N-terminal fatty acyl tail and linear peptides (13 amino acids tridecaptins possessing non-proteinogenic residues and having a chiral lipid tail [168,170]).

Bacteriocins isolated from Gram-negative bacteria include colicins (>10kDa peptides produced predominantly by *E.coli*, colicin-like microcins - structurally and functionally similar to *E. coli* colicins but are produced by several other species, including *P. aeruginosa* and *Klebsiella genus*, microcins (small peptides <10 kDa) and phage tail-like bacteriocins high molecular weight cylindrical peptides, so named due to their high similarity to the phage tail structure [171], Table 3.

The mechanisms of action exerted by bacterial AMPs include; interference with cell wall synthesis (nisin and epidermin [172,173]), inhibition of crucial enzymes of the targeted bacteria (lacticin 481, cytolysin, and salivaricins [174]) targeting DNA and RNA synthesis (colicins E2, E7, E8, cloacin DF13, and E9, microcin J25)[175,176], formation of pores in bacterial membranes leading to efflux of small molecules and dissipation of membrane potential (such as nisin, AS-48, Pep5 [177–179]).

A.



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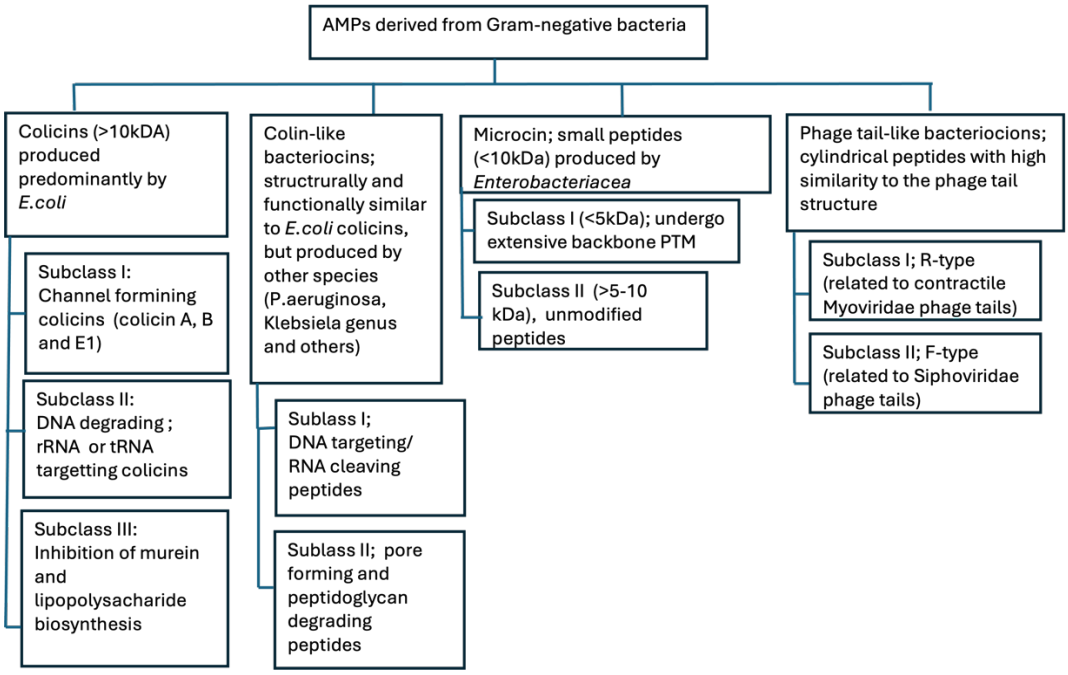


Table 3. Classification of bacteriocins. (Adapted from Hafeez et al. 2021) [168].

2.6.2.3. Fungal AMPs.

Fungal AMPs can be divided into peptaibols and fungal defensins [168,180]. The most widely studied peptaibol alamethicin is active against Gram-positive and Gram-negative bacteria [181] and fungi [182]. The primary mechanism of action of all peptaibols is similar and primarily involves membrane disruption [183], however some of them (for example plestasin) act by binding directly to the bacterial cell-wall precursors and inhibiting cell wall biosynthesis [168,184].

2.6.2.4. Plant derived AMPs.

Plant antimicrobial peptides (thionins, hevein-likes peptides, snakins and others) have been isolated from roots, seeds, flowers, stems, and leaves from a wide variety of species and have demonstrated antifungal and antibacterial activities [168,185]. The mechanism of action of most plant AMPs involves interaction with outer membrane of pathogenic fungi due to a chitin-binding domain, which can damage the fungal cell wall. Some of them (α-hairpinins) bind to DNA, inhibit RNA and protein synthesis by inhibiting trypsin and inactivating ribosome activity [186,187].

2.6.2.5. Invertebrate AMPs.

Defensins and cecropins are two major types of AMPs in invertebrates (insects, molluscs, nematodes, crabs) [188]. The invertebrate defensins are up to 52 amino acid long cationic

peptides and are structurally and phylogenetically related to the vertebrate β-defensins [189,190]. The antibacterial mechanism of insect defensins is mainly the disorganization of bacterial membranes via the formation of oligomerization surface, causing membrane permeabilization and cell disruption [191].

Another class of invertebrate AMPs is represented by cecropins ~40 residue long, cationic linear antimicrobial peptides which aggregate on bacterial lipid bilayer membranes causing membrane disruption and subsequent bacterial cell death via a carpet-like model (discussed in the following section) [192,193].

2.6.2.6. Vertebrate AMPs.

More than 1000 AMPs have been purified from frogs and toads and include bombinins, buforin, cathelicidin, dermaseptins, esculentins, fallaxin, magainins, temporins and others [194]. In general, more than 50% of the amino acid residues form the hydrophobic part of the molecule and there are no conserved structural motifs responsible for activity [195]. The vast majority of the AMPs are cationic [195].

Another major group of vertebrate AMPs is represented by mammalian AMPs - cathelicidins and defensins [168]. All mature mammalian cathelicidin peptides are cationic with an amphipathic structure that assumes α-helical, β-hairpin, or elongated conformations [159,196]. The first cathelicidin precursor to be described was rabbit CAP18 which has shown a broad-spectrum bactericidal activity [197,198].

LL-37, the most well-studied cathelicidin, is active against a variety of Gram-positive and Gram-negative pathogens [196,199]. The antibacterial activity of LL-37 is due to either pore formation or interference with cell wall formation [200,201].

Vertebrate defensins sharing several common features, including a cationic net charge (+1 to +11), short polypeptide sequences (18–45 amino acids), are secreted by neutrophils (HNP1–4) [202,203], and Paneth cells located in the intestinal epithelium [204,205] (HD-5 and HD-6). Most of them are active against *St. aureus, B. subtilis*, *Staphylococcus epidermis*, and *E. coli* [323], resulting from the inhibition of DNA and protein synthesis [206].

2.6.3 Structural properties of AMPs.

Ribosomally synthesized AMPs contain only natural amino acids [142] and are divided into three distinct groups based on the types of secondary structures present, namely 1) α-helical, 2) β-sheets, 4) extended coils (Table 4, Fig.16) [168,207].

2.6.3.1. α-helical AMPs.

Cationic α-helical AMPs are the most studied structures, with cecropin, pleurocidin, melittin, magainin, and moricin being the best described [188,208]. The α-helices are often rich in Phe, Ala and Val [208].

Most α-helical peptides are unstructured in an aqueous solution and fold into an amphiphilic α-helix structure upon the interaction with membranes by separating the hydrophilic and hydrophobic residues [209,210].

Another feature of α-helical peptides is that the C-terminus is mostly amidated, which enhances the electrostatic interaction between positively charged peptide and the negatively charged bacterial membrane. Stabilization of the helical structure on the membrane interface contributes to antibacterial activity and prevents a peptide from enzymatic degradation [211].

2.6.3.2. β-sheet AMPs.

AMPs adopting β-sheet structure are composed of at least two β-stands with linear structures adopting β-hairpin-like conformations [188]. This group of peptides is often Arg-rich and Cys-rich. While cysteines can form disulfide bonds to stabilize the β-sheet structure, Arg-s are critical for antimicrobial activities [208,209]. Unlike α-helix AMPs, β-sheet AMPs appear structured in an aqueous solution and do not undergo significant structural changes upon the interaction with the membrane [212,213].

2.6.3.3. Extented coils.

Some AMPs adopt a structure, which is often referred to as non-αβ AMPs or loop AMPs [214]. Most of these types of AMPs are rich in Proline, Glycine, and Tryptophan in the sequence. Proline-rich AMPs act on intracellular targets and have strong activity against Gram-negative bacteria [168,208]. Due to their short length, a simple residue substitution can lead to broad changes in both their structural and functional properties.

Together with AMP structure, net charge, hydrophobicity, amphipathicity, and solubility are the main and crucial physiochemical properties for the antimicrobial activities of AMPs [215].

Category[a]	Peptides	Unique Structural/Sequence Feature	Source
α helical peptides	Aurein 1-2	Amidated C-terminus	Frogs
	Mellitin	Amidated C-terminus	Bees
	Bervinin		Frogs
	Maculatins	Amidated C-terminus	Frogs
	Citropin	Amidated C-terminus	Frogs
	Bufforin II		Toad
	Cathecilidins		
	• LL-37[b]	Amidated C-terminus	Humans
	• BMAP-27,28,34[b]		Bovine
	• Magainins		Frogs
	• Cecropin	Amidated C-terminus	Insect
β sheet peptides	Cathecilidins		
	• Protegrins	Cysteine rich	Pigs
	• Bactenicin	Disulfide froming loop/Arginine rich	Bovine
	Defensins[c]		
	• α defensins	Three disulfide bonds	Mammals
	• β defensins	Three disulfide bonds	Mammals
	• θ defensins	Three disulfide bonds, cyclic	Gorilla
	Tachyplesins	Cysteine/arginine rich and amidated C-terminus	Horse Crab
	Polyphemunsin	Cysteine/arginine rich and amidated C-terminus	Horse Crab
Extented/flexible	Cathecilidins		
	• PR-39[b]	Proline and arginine rich	Pigs
	• Tritrpticin	Tryptophan and arginine rich	Pigs
	• Indolicidin	Tryptophan and amidated C-terminus	Bovine
	• Crotalicidin	Lysine rich	Snakes
	• Histatins	Histidine rich and amidated C-terminus	Humans

Table 4. Class of AMPs based on structure. (From Kumar et al. 2023) [209].

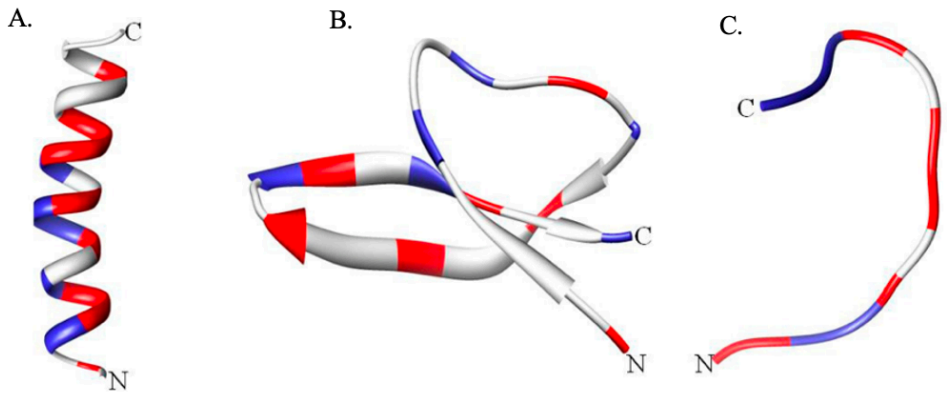


Figure 16. Structural diversity of AMPs. (A) *α*-helical magainin, B. β-sheet human defensin 5, C. extended coil indolicidin. Positively charged residues are colored blue. Hydrophobic residues are red. (From Chen et al. 2023) [207].

2.6.4. Physico-chemical properties of AMPs.

2.6.4.1. Net charge.

Most AMPs are positively charged (+2 to +13 net positive charges) [208,209]. The positive charges are derived primarily from Lys and Arg which form a specific cationic domain in the sequence [216]. Studies have demonstrated the correlation between charge and antimicrobial activity of AMPs [217,218]. For example, increasing the charge of magainin from +3 to +5

improved the antibacterial activity against both Gram-positive and Gram-negative bacteria, but an increase to +6 or +7 resulted in high hemolytic activity and loss of antimicrobial activity [219,220].

Although most AMPs are positively charged, negatively charged, anionic AMPs (AAMPs) also have been reported [221,222]. AAMPs come from various sources; humans (Fibrinopeptide A/B, thymosin-β4), insects (MD pep5, Gm anionic peptide 1) amphibia (AA-2-5, Temporin-1Ja), plants (Mi AMP2a, hevein) [223]. AAMPs have a net charge range of − 1 to − 8 and contain from 5 to 70 amino acid residues [224]. Most of these peptides are fragments of proteolysis, however a few anionic AMPs are the small molecules encoded by genes [213,224].

2.6.4.2. Helicity.

Helicity represents the ability of an AMP to form spin structure and determines the toxicity against eukaryotic cells [225]. Reducing helicity by incorporating D-amino acids into the primary sequence has been shown to lower the hemolytic effect, while the antimicrobial effect was retained [226]. For example, some α-helical peptides were modified by replacing 35% of the L-amino acids with D-amino acids, and it was found that this modification eliminated the hemolytic activity [227].

2.6.4.3.Hydrophobicity.

Hydrophobicity has also been shown to influence the activity and selectivity of AMP molecules. Almost 50% of amino acids in the primary sequence of natural AMPs are hydrophobic residues [213]. In most cases, an increase in hydrophobicity increases antimicrobial activity, while decreasing hydrophobicity can reduce antimicrobial activity [228]. The hydrophobicity of the peptides determines to a great extent, the ability to penetrate the lipid bilayer [229]. However, excessive hydrophobicity can also lead to decreased antibacterial activity and increased mammalian toxicity. Due to poor solubility in aqueous solutions, highly hydrophobic peptides are more prone to bind and destroy eukaryotic cell membrane [207,230,231].

2.6.4.4. Amphipathicity.

An amphipathicity that segregates basic and hydrophobic residues into a polar and a nonpolar face is recognized as a prerequisite for α-helical AMP activity [207]. The presence of amphiphilicity makes peptide conformations largely flexible, able to form α-helices, β-sheets, or a mixture of both when interacting with target microbial membranes [228]. In general, at least 7–8 amino acids are required to form an amphiphilic structure [232].

2.6.4.5. Solubility.

Another important feature of AMPs is solubility. Aggregation of AMP molecules might impair interaction with bacterial membrane [215]. For example, a hybrid synthetic AMP composed of

cecropin and melittin has a tendency to form dimers. Substituting a Lys residue on the non-polar face of this AMP hybrid prevents dimerization, leads to reduced hemolytic activity and enhances its ability to incorporate into microbial membranes [233].

2.6.5 Mechanism of action of AMPs.

In contrast to traditional antibiotics that accomplish their function by targeting specific bacterial proteins or pathways, AMPs don't act via any particular mechanism but rather exert their antibacterial activity by simultaneous disruption of several bacterial targets. Thus the development resistance against AMPs becomes more complicated [142,207,209].

Also, compared with conventional antibiotics, the killing is extremely rapid (less than 1 hour *in vitro* [234]. In contrast, most traditional antibiotics such as ampicillin, need from 4 h to 24 h to kill bacteria [235,236].

The antimicrobial activity of AMPs is extremely broad (Gram-negative and Gram-positive bacteria, viruses, fungi, and protozoa) [237] and can be displayed at micromolar and sub-micromolar concentrations [188].

2.6.5.1. Interaction with bacterial membrane.

Molecular dynamics simulations and experimental results indicate that some random coil peptide CM15 [238], helical LL-37 [239] and mellitin pass through OM without disrupting it and kill bacterial cell by lysing the inner phospholipid membrane [34,238].

On the other hand, polymixins have been shown to bind to OM lipopolysaccharides and permeabilize the outer membrane to self-promote uptake into Gram-negative bacteria. Because cationic peptides have higher LPS affinities compared to the native divalent cations Ca^{2+} and Mg^{2+}, they competitively displace these ions and disrupt the normal barrier property of the outer membrane [240] finding their way to inner bacterial membrane. The disrupted OM is visualized as surface blebbing [241] (discussed later in this chapter).

2.6.5.2.Proposed models of action of membrane-active AMPs at high concentrations.

As mentioned above, the interaction of AMPs with inner bacterial membrane is greatly explained by their cationicity and hydrophobicity. This feature ensures the initial electrostatic interaction with the negatively charged cell membrane and the insertion into the membrane interior. Several models explain how AMPs exert the disruption on the inner bacterial membrane (Fig. 17).

Carpet-Like Model

In this model AMPs arrange parallel to the cell membrane. Their hydrophilic end faces the extramembrane space, and their hydrophobic end faces the phospholipid bilayer [242]. AMPs

cover the membrane surface similar to a carpet and at a high peptide:lipid ratio destroy the cell membrane in a 'detergent'-like manner (Fig. 17 A) [242,243]. Human cathelicidin LL-37 and several β-sheet proteins exhibit their activity through this mechanism [244,245].

Barrel-Stave

According to this model antimicrobial peptides aggregate into a barrel-like structure in which a central aqueous pore surrounded by peptides is formed (Fig. 17 B). Hydrophobic peptide regions face lipid core while hydrophilic parts face the interior region of the pore [243]. This pore increases in diameter through the progressive recruitment of additional monomers and results in cytoplasm leakage [192,225]. In severe cases, AMPs can induce cell membrane collapse and lead to cell death [246]. For instance, alamethicin and protegrin-1 perform their pore-forming activity by this mode [182,247].

The Toroidal Pore Model

The toroidal pore model is also known as the wormhole model, according to which AMPs accumulate and get vertically embedded in the cell membrane and then bend the membrane to form a ring hole with a diameter of 1–2 nm (Fig. 17 C) [248]. The typical examples of this model are magainin 2, TC19, TC84, and BP2 [248,249].

These three models do not exhaust the types of membrane disruptions that have been observed for membrane active antimicrobials [250].

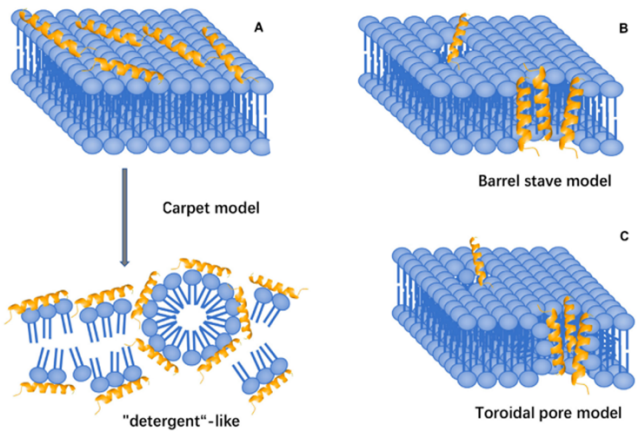


Figure 17. Models of AMPs' membrane activity. (From Y. Huan et al. 2020) [242].

2.6.5.3. Proposed models of action of membrane-active AMPs at low concentrations.

At low peptide concentrations, most peptides are believed to bind to the bacterial membrane surface and induce blebbing/vesiculation, phospholipid redistribution, and/or interact with inner targets.

2.6.5.3.1. Blebbing/vesiculization and phospholipid redistribution.

Bacterial membrane vesicles are membrane-derived vesicles discharged by both Gram-positive and Gram-negative bacteria in response to stress (high temperature, osmotic dysregulation, antibiotic or AMP treatment [251]. Membrane vesicles (MVs) were first found to be produced through controlled blebbing of the outer membrane of Gram-negative bacteria and are, therefore, often referred to as outer membrane vesicles (OMVs) [252]. During outer membrane blebbing, Gram-negative bacterial cells continue their physiologic activities, while vesiculation helps the cells to maintain homeostasis [251].

Later it was reported that MVs from *P. aeruginosa* contain DNA, confirming earlier studies demonstrating the presence of DNA and RNA in Gram-negative MVs [253,254]. It was proposed that the PGN layer of the bacterial cell is weakened by autolysins so that the inner membrane protrudes into the periplasm, allowing cytoplasmic contents such as DNA to enter the vesicle, which is eventually pinched off from the cell surface together with a surrounding outer membrane. [252,255]. The existence of double bilayer vesicles (termed Outer Inner Membrane Vesicle -OIMV) was later proved by transmission electron microscopy [256].

'Explosive cell lysis' is another possible route for the formation of MVs containing chromosomal DNA [257]. Cell lysis is triggered by DNA damage and results in cell envelope fragments that recircularize and thereby enclose the released DNA [252,258]. In support of this, a recent study showed that treatment of MDR bacteria *Stenotrophomonas maltophilia* with ciprofloxacin not only induces the SOS response and, consequently, cell lysis but also stimulates the production of OIMVs [259] (Fig. 18).

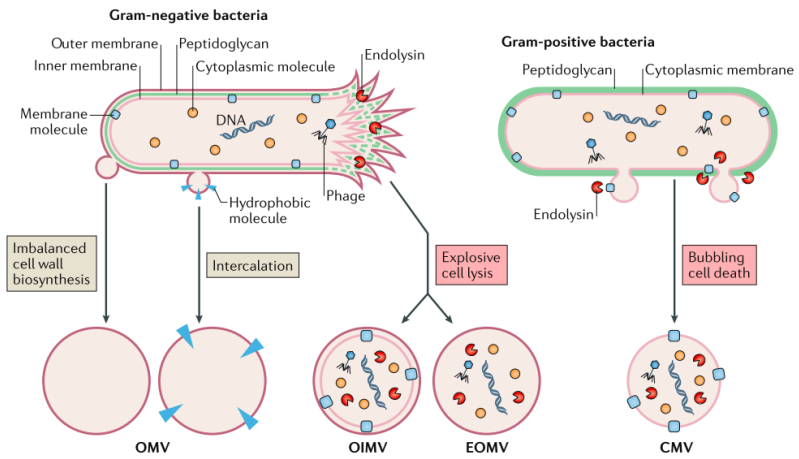


Figure 18.Types of bacterial membrane vesicles.(From M.Toyofuku et al.2019)[252].

Recently B22 and B22a (derivatives of cathecilidin) were reported to induce disruption in membrane-bound aerobic respiratory electron transport chain and release O_2- from the complex [260], leading to the accumulation of ROS and disruption of PGN biosynthesis, which subsequently caused membrane-bound blebs containing displaced cytoplasmic material in *St. aureus* [260]. Together with blebbing, several AMPs were reported to induce perturbation in phospholipid bilayers of bacterial membranes. Antimicrobial fragment of LL-37 was shown to perturb bacterial membrane by segregation of anionic and zwitterionic lipids in lipid films [261,262]. Lactoferricin-derived N-acylated lipopeptides (6-MO-LF11-322 and O-LF11-215) were shown to induce the redistribution of CL-domains on the whole cell surface from septum and poles in *E.coli* [263]. Since phospholipid domains play a significant role in bacterial physiology, their dislocations and redistributions impair bacterial growth and division [36,263].

2.6.5.3.2. Interaction with inner bacterial targets.

Since the discovery of AMPs and their membrane targeting mechanisms of action, it was proposed that they should have intracellular targets [264] (Fig. 19). Indeed, later, it was revealed that some AMPs at their minimal effective concentrations translocate through bacterial membrane (without causing its permeabilization) and form complexes with anionic macromolecules (DNA, RNA, proteins, LPS and others) leading to the inhibition of DNA replication, protein synthesis, cell division, cell wall metabolism, etc.) [209,213,265].

2.6.5.3.2.1 AMPs acting on nucleic acids.

The best studied examples of peptides that interact with DNA are buforin II and indolicidin.

Buforin II is a 21-amino acid derivative of the 39-amino acid buforin I, isolated from the Asian toad *Bufo bufo gargarizans* [266]. Fluorescein isothiocyanate (FITC)-labeled buforin II was found to penetrate the *E. coli* cytoplasmic membrane and accumulate inside, even below its MIC without membrane disruption or cell lysis [267]. Through gel retardation studies it was shown that buforin II was able to form complexes with DNA which might be explained by 37/39 amino acids of homology with the N-terminal region of the DNA-binding nuclear protein histone H2A from *Xenopus* [266,267].

Indolicidin - a 13-amino acid linear tryptophan-rich (39%) peptide from bovine neutrophils, a member of the cathlecidin group is another example of DNA targeting LCAMP [268]. It has been revealed that indolicidin induces filamentation of E.coli cells (pointing to inhibition of cell division) and inhibits DNA synthesis (as indicated by reduced incorporation of thymidine into the cell) [269]. Other study has shown that indolicidin is able to form covalent complexes with viral DNA and inhibit human topoisomerase I mediated DNA relaxation [270].

2.6.5.3.2.2. AMPs that target proteins.

Insect-derived proline-rich antimicrobial peptides (PrAMPs) (Pyrrhocoricin [271], Apidaecin [272], Oncocin [273] , and others) are reported to bind heat shock proteins, DnaK and/or GroEL, impair their folding and inhibit ATPase activities (Pyrrhocoricin, Apidaecin) [274–276]. By using *E. coil* proteome microarray, it was revealed that Lfcin B (an AMP derived from pepsin digestion of lactoferrin) targets proteins related to the metabolism of pyruvate, leading to the accumulation of the latter in *E.coli cells* [277,278].

2.6.5.3.2.3. AMPs, acting on protein synthesis.

Bacterial ribosomal subunits are another targets for LCAMPs [265]. For example, together with DnaK targeting (described above), PrAMPs - Onc112 interacts with 70S subunit of a ribosome and blocks the binding site for an incoming aminoacyl-tRNA, thus effectively trapping the ribosome in an inactive initiation complex on the mRNA [279,280].

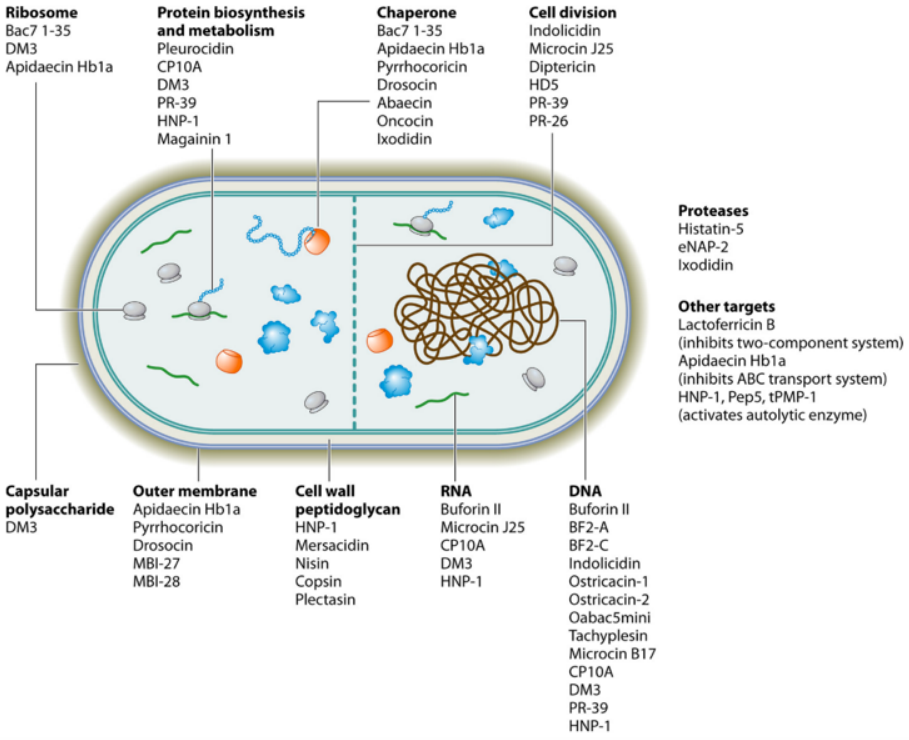


Figure 19. Major pathways targeted by AMPs in bacterial cells. (From Cheng-Foh et al. 2017).

2.6.5.3.2.4. AMPs impairing bacterila cell division.

The cell division process of bacteria is a new and attractive target to find antibacterial drugs [281]. The impairment of cell division was first associated with a number of filament-forming temperature-sensitive (*fts*) genes found in the mutated *E. coli* strain [282]. Proteins encoded by these genes participate in the formation of the septum and initiation of the division [283]. Among *fts* -encoded proteins, filamentous thermosensitive protein Z (FtsZ), possessing guanosine triphosphatase (GTPase) activity essential for its polymerization and formation of Z-ring, - (a

structure acting as a matrix for the recruitment of other cell division proteins) is involved in septum formation and subsequent cell division (Fig. 20) [284–286]. Mutants lacking FtsZ cannot divide but continue to elongate into filaments, eventually resulting in bacterial death [287].

A variety of FtsZ-targeting AMPs have been described to be effective against different pathogens, including MRSA and *E. coli* [285]. Most of these peptides target GTP-ase activity of FtsZ by blocking the assembly (for example MciZ, cathelin-related antimicrobial peptide (CRAMP)) or disassembly (PC190723, Temporin L and its analogs) of the Z-ring [288,289].

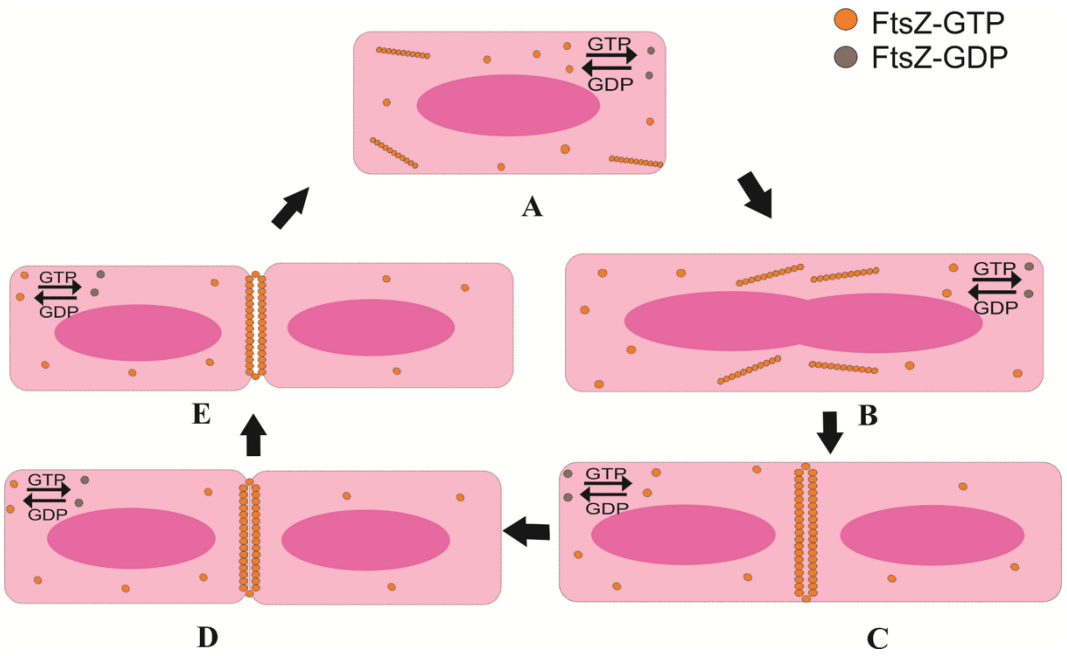


Figure 20. Representation of Z-ring formation and cell division.

(A) Bacterial cell prior to the onset of cell division with FtsZ protofilaments dispersed in the cell and undergoing continuous nucleotide exchange between GTP-bound FtsZ and GDP-bound FtsZ with rapid equilibrium, favoring GTP-bound FtsZ (B) Polymerization begins, cell elongation and, localization of FtsZ protofilaments at the mid-cell. (C) Generation of Z-ring: the 'steady-state turnover'-GTP hydrolysis in continuous competition with protofilament growth during polymerization. (D) Formation of septum (E) Contraction of the Z-ring followed by membrane alteration to bring about cell division. (From S.Tripathy et al. 2019).

2.6.5.4. Antibiofilm Activity of AMPs.

According to recent data, biofilms are responsible for 70% of all microorganism-induced infections in humans [290]. Biofilms are composed of complex microbial communities attached to biological or abiotic surfaces and embedded in the matrix of extracellular polysaccharides, proteins, nucleic acids, and other small cellular molecules [291].

Microorganisms in biofilms are capable of tolerating high concentrations of antimicrobials even though they are totally sensitive in planktonic conditions [292]. It has been shown that some AMPs can inhibit the adhesion of bacteria to surfaces by reducing bacterial motilities [293,294].

For example, synthetic AMP 1037 has been shown to stimulate "swarming", a type of motility known to promote the disassembly of biofilm [295]. Interestingly another AMP- human β-defensin 3 can down-regulate certain genes involved in cell signaling and quorum sensing, the latter being known to play a role in biofilm formation and/or in the organization and communication of bacteria within the biofilm in *Staphylococcus epidermis* [294,296].

2.7 Limitations of natural AMPs.

While several natural lipo- and glycopeptides (e.g. colistin, vancomycin, daptomycin) have been approved by the Food and Drug Administration (FDA) (Table 5) as antibiotics, most natural AMPs have not been proven to be suitable for the treatment of drug-resistant bacterial and fungal infections mainly due to their cytotoxicity, low stability towards proteases and high manufacturing costs [142,144].

Peptide Name	Origin	Mechanism of action	Indication
Nisin	Bacteria (*Lactococcus lactis*)	Membrane depolarization	Bacterial infections
Gramicidin	Bacteria (*Brevibacillus brevis*)	Membrane depolarization	Bacterial conjunctivitis
Melittin	Insect (*Apis mellifera)*	Membrane disruption	Anti-inflammatory applications
Daptomycin	Bacteria (*Streptomyces roseosporus*)	Membrane depolarization	Skin infections
Lactoferricin	Mammals	Membrane depolarization	Anti-inflammatory applications
Histatin	Humans	Inhibition of respiration	Fungal infections

Table 5. List of natural AMPs in clinical practice. (From Moretta et al. 2021) [297].

2.7.1. Cytotoxicity.

An AMP can be considered an effective antibiotic only if it is highly selective towards bacterial cells and, at the same time, is not cytotoxic towards eukaryotic cells [143,156,298]. Many membrane active AMPs are not completely selective towards microbial cells and may present potential toxicity [178,299] to eukaryotic cells due to higher hydrophobicity [228,300]. For example, gramicidin S, alamethicin, and melittin, despite their potent activity, have significant hemolytic toxicity, restricting their clinical use to topical applications [299,301,302].

2.7.2. Low stability.

The activity of many AMPs appears to be inhibited under physiological salt and serum conditions [303,304]. In addition, these peptides are readily degraded by both endogenous human proteases and proteases secreted by invading microbes [305]. Among the endogenous human proteases, trypsin and chymotrypsin are the greatest threats to AMPs. Both attack peptides at basic (Lys and Arg) and hydrophobic (Trp and Phe) residues. It has also been reported that LL-37 is destroyed by *St. aureus* aureolysin [306].

2.7.3 High manufacturing costs.

Screening of new natural AMPs requires high-throughput experimental research, which is labor-intensive and time-consuming. The cost of chemical synthesis of peptides is generally much higher than that for 'conventional' antibiotics and requires a complex purification step to isolate the desired peptide from its contaminants [307].

2.8. Approaches implemented to improve the efficacy of AMPs.

To overcome these problems, researchers try to develop synthetic and semi-synthetic peptide analogs (peptidomimetics) with improved biological properties of known natural AMPs [207], combine natural/synthetic LCAMPs with each other or other antimicrobials or develop new sequences (*ab initio*) with desired features using computer-assisted approaches [308].

2.8.1. Chemical modifications improve the protease stability of AMPs.

In order to improve the stability of peptides against proteolytic digestion, various chemical modifications of AMPs have been introduced. Among them, the most common are: caping (amidation/acetylation), cyclization, truncation and substitution of natural amino acids with D-amino acids, β-amino acids, and others) (Fig. 21), [242,309].

Capping involves the modification or addition of specific motifs to the terminal or side chains of AMPs and is an effective strategy for enhancing the stability and efficacy of AMPs [310].

Common AMP capping techniques include C-terminal modification (amidation) and N-terminal modification (acetylation, methylation, and lipidation) (Fig. 22-23), [242,310–312].

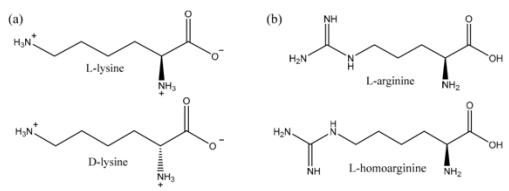
In the process of amidation, the C- terminal end of a peptide —the carboxyl group (–COOH) is converted into a carboxamide group (–CONH2) by replacing a hydroxyl group with a nitrogen atom [313]. This improves the antimicrobial potency of peptides in two ways: by increasing the net charge of the peptide and by enhancing proteolytic stability, thus improving half-life times *in vivo* [314,315].

For example, C-terminal carboxyl-amidated aurein 2.5 (GLFDIVKKVVGAFGSL- CONH2) exhibits increased antimicrobial potency against *K. pneumoniae* compared to its C-terminal carboxylated (GLFDIVKKVVGAFGSL-COOH) analog [316].

Acetylation of the N-terminus also increases the proteolytic stability of peptides as it blocks the activity of aminopeptidases however, this leads to the removal of a positive charge which in most cases decreases the antimicrobial activity (as reported for β-defensin-3) [317].

The functions of N-terminal lipidation include: increasing LPS neutralization, increasing stability to proteases and peptidases, and reducing cytotoxicity [312]. N-terminal lipidated analog C4VG16KRKP shows enhanced antibacterial activity against various Gram-negative bacteria [242].

Substitution is another commonly applied technique in the modification of AMPs [221]. Native amino acids can be substituted with non-natural amino acids to introduce unique chemical and structural properties into the peptide. For example, replacing a native amino acid with a non-natural amino acid, such as a halogen (fluoride) N-methyl amino acid, or β-amino acid, can affect peptide conformation and resistance to enzymatic degradation [318,319]. Similarly, incorporation of non-natural D-amino acids into AMP sequences reverses the stereochemistry of the peptide and hence prevents protease degradation [318,320]. For example in order to reduce trypsin proteolytic digestion of MPI (Lys rich AMP from the venom of the social wasp (*Polybia paulista*) [321], two peptides were designed: one with all the amino acids replaced with D-amino acids (D-MPI), and the other peptide with only the Lys residues substituted with D-amino acid, (D-lys-MPI) (since trypsin cleaves after positively charged amino acids such as Lys [209,322]). Both the peptides, D-MPI and D-lys-MPI were resistant to trypsin digestion, however only D-MPI was equipotent in terms of activity when compared to MPI [322]. Effective D-amino acid substitutes were also reported for D-BMAP28, (a peptide from bovine myeloid) [209,323]. Interestingly, it was found that the D-form of KLKLLLLLKLK-NH_2 peptide exhibited enhanced membrane permeability in *S. aureus* through showing higher affinity towards peptidoglycan compared to its L-form [320].



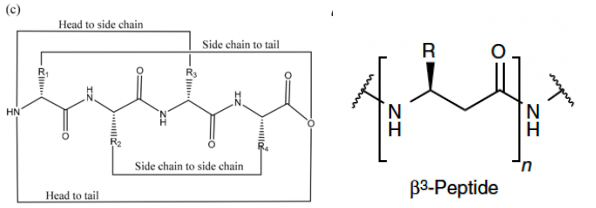


Figure 21. Chemical modifications of AMPs. (From Kumar P. 2018) [209].

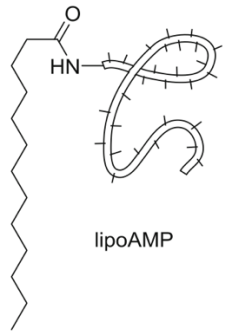


Figure 22. Lipidation of AMPs.(From B. Albada et al.)[324].

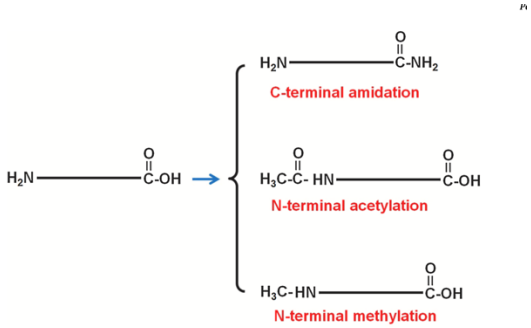


Figure 23. Schematic of N-/C-terminal modifications of peptides. (From Y.Han 2021) [325].

2.8.2 Semi-synthesis reduces the toxicity of AMPs.

Although chemical synthesis partially tackles the problem of cytotoxicity, another approach to reduce the toxicity and enhanced antibacterial activity of AMPs is to isolate them from natural sources and subsequently chemically modify them [221].

In order to achieve more specificity towards negatively charged bacterial membrane, inclusion of lipophilic substituents and/or the installation of cationic moieties, elimination of sugar N-terminal acylation or alkylation [326,327] are the most common approaches. These modifications were used to obtain semi-synthetic analogs of glycopeptides eremomycin, vancomycin, and teicoplanin. Several semi-synthetic glycopeptides such as such as oritavancin,

telavancin, and dalbavancin are [328] (Fig. 24). Another frequently semi-synthetically modified AMP is the lanthipeptide nisin. Its modifications often include proteolytic digestion followed by attachment of other constructs such as vancomycin, lipophilic chains, or pore-forming peptides [221,329].

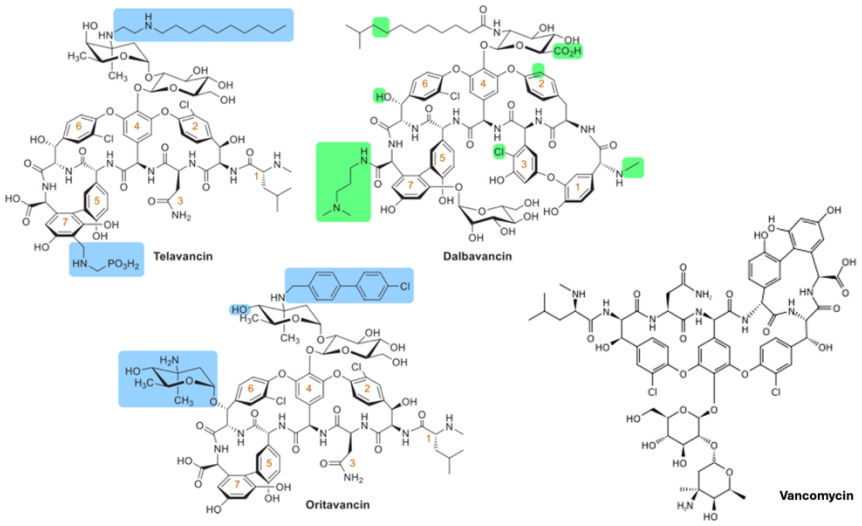


Figure 24. Structure of two FDA-approved semi-synthetic lipoglycopeptides (derivatives of vancomycin).

Structural differences of telavancin and oritavancin compared to vancomycin are indicated in blue. Structural differences of dalbavancin compared to teicoplanin are indicated in green. The amino acids of the peptides are numbered in orange, starting at the N-terminus. (From Van Groesen 2022) [327].

2.8.3 Peptidomimetics reduce the costs of AMPs.

The observation that the key requirement for antibacterial activity are defined by sufficient cationic charge balanced by hydrophobic elements has recently allowed the design of diverse AMP mimics (peptidomimetics) with maintained and even improved bioactivity over their native counterparts [330]. Generally, the term peptidomimetic is used in a broad sense, referring to essentially any oligomeric sequence designed to mimic a peptide's structure, physico-chemical properties and/or function but whose backbone is not solely based on α-amino acids. One advantage of peptidomimetics over typical α -peptides (i.e., peptides made exclusively from α -amino acids) is that they can be structurally simpler and, therefore, cheaper and easier to synthesize [221]. Peptidomimetics display improved antimicrobial activity, improved stability (both metabolic and proteolytic), and reduced toxicity compared to unmodified α-peptides [219].

Two most prominent examples of peptidomimetics are peptoids and ceragenins - cationic steroid antimicrobials (CSA) [221,331].

Peptoids - isomers of peptides in which the side chain is bonded to the backbone nitrogen instead of the α -carbon, making them resistant to protease degradation (Fig.25) [332]. Peptoids derived from pexiganan (synthetic analog of magainin) [333] have been shown to mimic structure, function and mechanism of action of pexiganan. Circular dichroism (CD) studies confirmed that peptoids adopt α helical structure in the presence of phospholipids, whereas X-ray analysis showed peptoids bind to the membrane and are membrane-active [332,334].

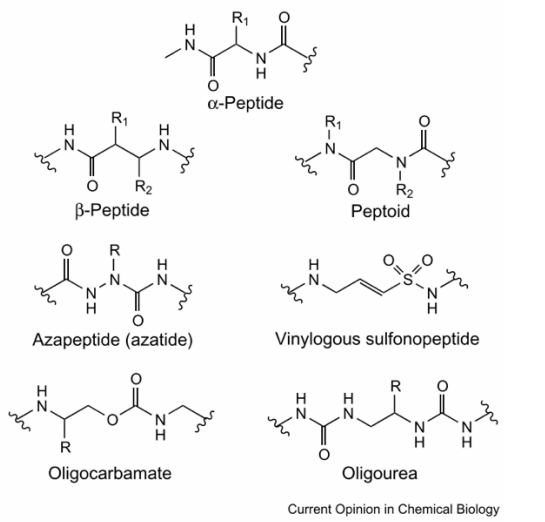


Figure 25. Representatives of peptidomimetic oligomers. (From Patch et al. 2002) [335].

Ceragenins - synthetic non-peptide molecules were designed to mimic the properties of naturally occurring cationic antimicrobial peptides (LCAMPs). This class of AMPs mimics has been developed by the synthesis of steroids (derivatives of bile acids) with covalently attached amine groups [331,336].

CSA-13 (a mimic of LL-37) (Fig.26) was demonstrated to have potential for treatment of *H. pylori* infections. Contrary to LL-37 it maintained strong bactericidal activity in the presence of pepsin at low pH (conditions optimal for *H.pylori*) [337].

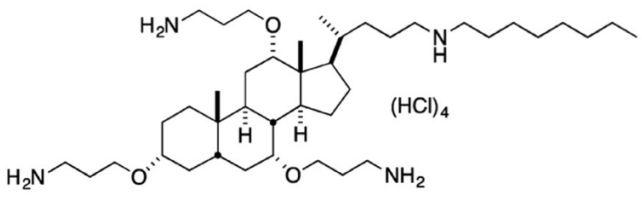


Figure 26. Structure of LL-37 derived ceraginine CSA-13.

2.8.4. Synergy as approach to reduce AMP toxicity and lower costs.

It is well established that AMPs act synergistically with other AMPs and antibiotics. If two drugs act synergistically, lower doses of each drug could potentially be used, which allows to preserve

the desired effects of drugs and reduce the unfavorable ones [338]. Lowering the effective concentrations of antimicrobial would directly translate into decreased toxicity and lower drug costs.

Since synergy studies constitute a substantial part of the present work, the mechanisms underlying synergistic properties of AMPs will be discussed briefly.

Synergy is a phenomenon when the effect of the two antimicrobials is greater than the sum of their individual activities [339].

Substantial deviations from synergy are defined either as additivity or antagonism [338]. An additive interaction has a combined activity no greater than the sum of the activities of each component, while antagonism shows lower activity of agents in combination than that of the most active component [340].

The most widely used technique employed to evaluate the combination of two antimicrobials for the presence or absence of synergy is the Checkerboard Assay [341]. Serially diluted compounds are combined in 96-well plates at different concentrations and incubated with bacterial culture. Synergistic combinations are assessed on the basis of calculations of Fractional Inhibitory Concentration indices (FICi) -s, which represent the sum of the FICs for AMP-AMP or AMP-antibiotic combinations.

In order for the combination to be considered synergistic, equal or >4-fold reductions in the MICs of both compounds in combination, compared to the MICs of the compounds alone, are needed.

General mechanisms by which antibacterial synergy can arise include enhancement of the uptake of one of the compounds, inhibition of common or different biochemical pathways, inhibition of protective bacterial enzymes and cell wall synthesis [342,343]. Antagonism could arise as a result of competition of compounds for binding sites or interaction between compounds leading to their inactivation [344,345].

2.8.4.1. Synergy between natural antimicrobial peptides.

In vivo, studies have shown that during immune response, multiple antimicrobials are released at the same time [346–348]. It has been long hypothesized that combinations of two or more types of AMPs may generate synergism however, only a few examples of synergistic AMPs have been identified [349–351] so far. Synergistic antibacterial combinations of AMPs could enable bacterial pores to stay open for longer durations, prevent pore repair, increase perturbation of bacterial intracellular functions, or convey other independent but complementary bacterial

killing mechanisms [349]. These mechanisms may potentially increase antimicrobial efficacy, decrease resistance, and reduce host toxicity if only low concentrations of each antimicrobial component are needed to carry out a large antimicrobial effect [346,352].

The most studied example of AMP-AMP synergy is the combination of PGLa and magainin 2 (MAG2), both of which are found in the skin of the African frog *Xenopus laevis* [353,354]. According to the proposed molecular model PGLa and MAG2 form functionally active self-assebled PGLa-MAG2 complex, consisting of a membrane-spanning antiparallel PGLa dimer that is stabilized by Gly-Gly contacts, and where each PGLa monomer is in contact with one MAG2 molecule at its C-terminus [355]. The synergistic effect is revealed in form of membrane potential dissipation [356] and membrane permeabilization [355].

Another evidence of AMP-AMP synergy was observed for MAG2 and Tachyplesin 1 (TP1). It was shown that these two AMPs similar in molecular mass but distinct in sequence and structure at an optimal concentration and ratio form membrane pore-forming heterooligomers in which the orientation of TP1 and MG2 within the aggregate structure can stabilize transmembrane pores [347]. A similar mechanism of synergism has been described for Temporin B and TemporinL [357].

2.8.4.2. Synergy between antimicrobial peptides and antibiotics.

AMPs can also synergize with antibiotics, and in some cases, overcome antibiotic dose-relayed and bacterial resistance [346]. One of the mechanisms by which AMPs improve antibiotic function is disrupting bacterial membranes to aid in the delivery of antibiotics into the bacterial cytoplasm, where antibiotics can act on intracellular targets [346] depending on their mechanism of action.

For example enhancing effect of the cathelicidin BMAP-28 on vancomycin activity observed against Gram-positive cocci has been attributed to the increased access of the antibiotic through the cytoplasmic membrane [358]. A number of *in vitro* studies have shown that combinations of several AMPs and broad-spectrum antibiotics are synergistic against MDR *P. aeruginosa* strains.

Cathecilidin LL-37 (known to act via carpet-like mode [359]) was shown to act synergistically with azithromycin (AZM) which is known to bind the 50S large ribosomal subunit at the polypeptide exit tunnel, blocking protein synthesis [360].

The linear variant of bactenecin- Bac2A shown to interact with DnaK, and DNA polymerase [361] has revealed synergy with five different antibiotics including membrane-targeted polymixin B, protein synthesis targeted kanamicin, erythromycin, tetracyclines, and DNA synthesis inhibitor - ciprofloxacin [362].

Similarly to Bac2A, indolicidin known for multiple modes of action [363] reveals synergy with kanamicin and ciprofloxacin [349].

Another short *in silico* generated peptide LOP3 exhibits synergistic interactions with, meropenem, gentamicin, and erythromycin [362]. The exact mechanisms of this synergy are not yet fully understood.

Recently a hydrophobic arenicin-1, a 21- residue AMP, isolated from lugworm Arenicola marina [364] and reported to exhibit broad-spectrum antimicrobial activity in yeast and bacteria via membrane disruption and oxidative stress [365,366] was shown to synergize with ampicillin (cell wall synthesis disrupting antibiotic) against *St. aureus*, *Staphylococcus epidermidis*, *P. aeruginosa* and *E. coli* O-157 [344]. The observed synergy is largely attributed to the enhanced formation of hydroxyl radicals by arenicin-1 in combination with ampicillin. This was proved by the fact that thiourea reduced the death of bacterial cells treated with arenicin-1 alone and in combination with ampicillin [344].

Considering the above-mentioned, exploration of the ability of AMPs to synergize with other AMPS or antibiotics represents a promising approach to enhance the antimicrobial effectiveness of both agents and minimize the chances of resistance development.

2.8.5. Computer-aided design of AMPs.

Whereas finding new potent AMPs and/or evaluating chemically modified AMPs and their mimics through an experimental approach is still a long and expensive process, various computer-aided methods (such as machine learning (ML), linguistic models, pattern insertion methods, and genetic algorithms) can be useful for rapid generation and preliminary evaluation of potential antimicrobial properties of a given sequence [367]. ML allows the generation of novel AMP sequences with specific properties based on amino acid position preference, composition, and frequency [318,368]. It utilizes the strategy of quantitative structure-activity relationship (QSAR) [369], which uses physicochemical descriptors (such as hydrophobicity, charge, isoelectric moment, etc.) to predict the biological activity of peptides from their amino acid sequences [370–372]. Screening millions of available peptide sequences and identifying similar functional motifs and properties enables the training of ML models to predict the potential antimicrobial activity of a given amino acid sequence against a specific pathogen [368–370] (Fig. 27).

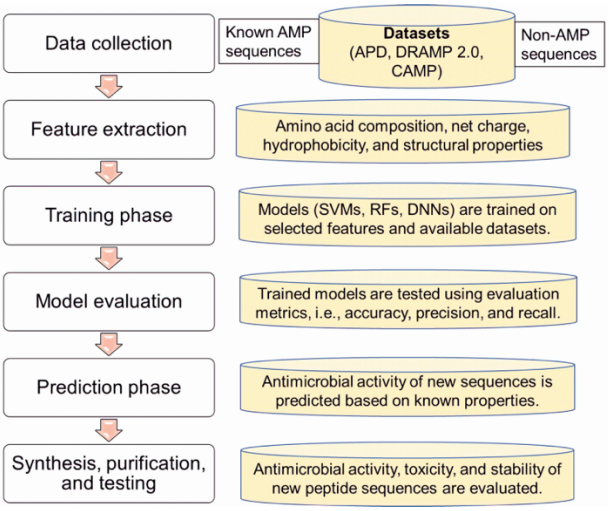


Figure 27. General overview of AMP design using ML. (From J.Mwangi et al. 2021).

In order to generate successful prediction models diverse AMP datasets are required [318]. These datasets are usually extracted from AMP databases. Databases contain extensive information on the peptide structure, chemical modifications, bioactivities and classification. The AMP databases are classified into two main groups: general databases and specific databases. General databases contain different types of AMPs irrespective of a given peptide class, while specific databases cover information related to a certain class of AMPs (e.g. only defensins or cyclotides) [373]. Among general databases, DBAASP v.3 - developed by the Laboratory of Bioinformatics at the I. Beritashvili Center for Experimental Biomedicine is one of the biggest repositories of peptides [161]. Currently, it contains more than 21000 entries and provides information on ribosomal, non-ribosomal, and synthetic peptides that show antimicrobial activity as monomers, multimers, and multi-peptides. The database also comprises the records with data on synergistic activities. Among the tools introduced in DBAASP v3 that differentiate it from other AMP databases is an application to make predictions of peptide activity against selected microbial strains. These features have made DBAASP a widely used resource to develop predictive models of AMPs and to facilitate the *de novo* design of novel bioactive peptides.

2.9 Aims and objectives of the present study.

Standard antibiotics are becoming less effective for clinical use due to the emergence and spread of antimicrobial resistance. LCAMPs might potentially represent new therapeutic agents in the fight against this global healthcare problem. Since naturaly available LCAMPs have several draw-backs (instability, toxicity, high production costs) hampering their use in clinical application, the *in silico* development of new antimicrobial peptides on the basis of the computer-

aided design represents a high through-output, less time-consuming approach to combat the problem of antimicrobial resistance.

The aim of the present study was to experimentally evaluate the biological properties of *in silico* generated LCAMPs (predicted by the Antibacterial Peptide Prediction algorithm (APP) of DBAASP) to be active against *E.coli ATCC 25922* and have low hemolytic activities.

The objectives of this study were:

1. Investigate the antibacterial properties of *de novo* LCAMPs.
2. Investigate hemolytic activity and cytotoxicity of *de novo* LCAMPs.
3. Investigate the stability of *de novo* LCAMPs towards proteases.
4. Investigate the properties of *de novo* LCAMPs to act synergistically with commercial antibiotics of different mechanisms of action.
5. Select several promising *de novo* LCAMPs on the basis of favorable therapeutic potentials (antimicrobial activity, low toxicity, stability to proteolytic degradation, and the ability to synergize with antibiotics)
6. Investigate mechanisms underlying the antibacterial activity of the promising *de novo* LCAMPs. In particular, to evaluate their membrane-active properties and the ability to interact with inner bacterial targets.

Chapter 3 : Materials and Methods.

3.1. *De novo* LCAMPs used in these studies.

13 Linear Cationic Antimicrobial Peptides (LCAMPs) -13 amino acids (aa) long, were *de novo* designed using the target-specific APP tool of DBAASP [374]. The length of 13 aa was chosen on the basis of the fact that most short, natural (ribosomal) peptides have a length of 13 aa (Fig. 28). Also, 13 aa long peptides have enough resources to adopt the α-helical structure, which is crucial for the activity of many AMPs [375,376]. *De novo* designed LCAMPs were predicted to be active against Gram-negative *E. coli ATCC 25922* and have low hemolytic activity against human erythrocytes. Peptides' aa sequences and their physico-chemical features are presented in Table 6.

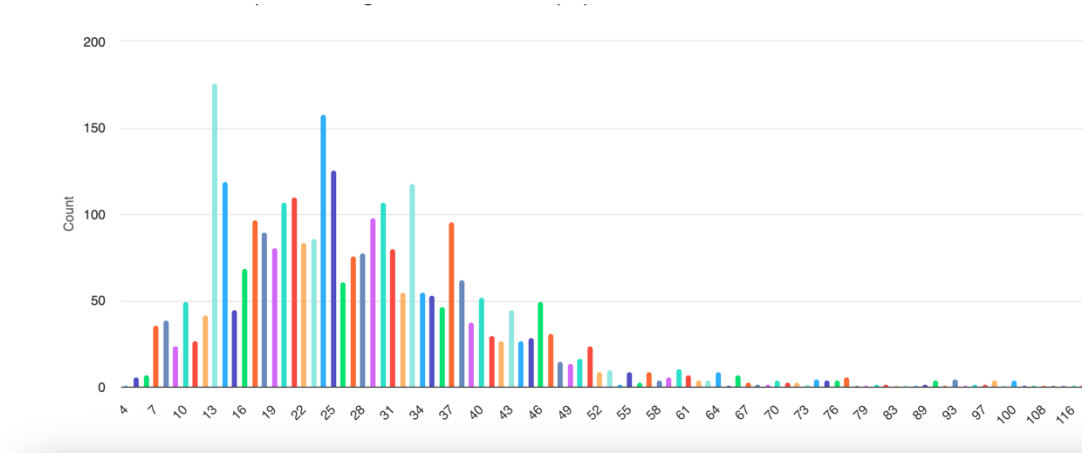


Figure 28. Distribution of length for ribosomal peptides. (From DBAASP v.3) [161]. https://dbaasp.org/statistics?page=general-statistics).

Name	Sequence	Length	Net Charge	Normalized Hydrophobicity	NHM*	Amphiphilicity Index
24L	RWIRWVWRKKLR	12	+7	0.13	0.72	3.16
24D	rwirwvwrkklr	12	+7	0.13	0.72	3.16
L1L	AIKIRKLFKKLLR	13	+7	-0.15	1.71	1.51
L1D	aikirklfkkllr	13	+7	-0.15	1.71	1.51
LCAPL2	GIKIRKLFKKLLR	13	+7	-0.02	1.61	1.51
LCAPL4	GIKFFLKKLKKH	12	+6	0.43	1.4	1.65
LCAPL8	VARFLKRIIKALF	13	+5	-1.08	1.69	0.94
LCAPL9	GFIKIVRKLLRLF	13	+5	-1.08	1.87	0.94
LCAPL10	IIKRILIQLKKLL	13	+5	-0.82	1.58	1.13
LCAPL14	WKKLKLWLKWKLW	13	+6	-0.15	0.51	3.54
LCAPL15	KKFLGKWKLRFGW	13	+6	-0.03	0.82	2.38
ST1L	LVWKLWWRLRWLK	13	+5	-0.88	0.65	3.07
ST1D	lvwklwwrlrwlk	13	+5	-0.88	0.65	3.07
L-Temporin	FVQWFSKFLGRIL	13	+3	-1.35	1.66	1.1

Table 6. Amino acid sequences and physicochemical properties of LCAMPs used in this study.

*NHM- normalized hydrophobic moment.

De novo designed peptides were purchased through the custom peptide synthesis service of GenScript Biotech Corporation. All peptides are C-terminally amidated and the labeled versions of 24L, 24D, L1L and L1D have fluorescein isothiocyanate (FITC)-Ahx tag on their N-terminal end. Peptides were HPLC-purified and MS-verified (MALDI) were delivered as lyophilized salts of hydrochloride. All peptides were dissolved in sterile ddH2O to 2 mg/mL stock solutions and stored at −80 °C.

3.2 Reagents.

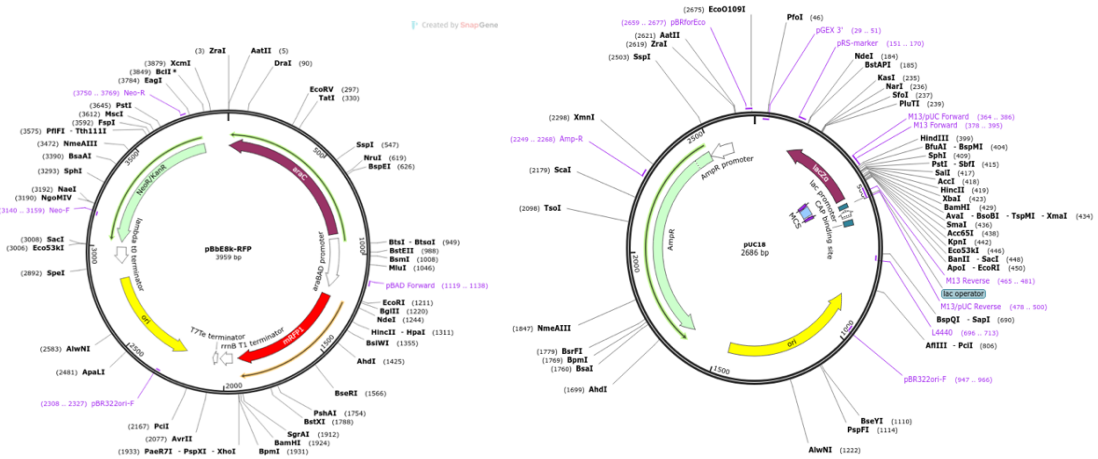
Temporin-L was purchased from CRB Discovery; ampicillin sodium salt and kanamycin sulphate were purchased from Carl Roth; N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide (FM-4-64) was purchased from Thermo Fisher Scientific, rabbit polyclonal anti-FtsZ antibody – from Agrisera, secondary mouse anti-goat FITC-conjugated antibodies were from Santa Cruz Biosciences, 3,6-bis(dimethylamino)-10-nonyl-acridinium bromide (NAO), gentamicin, penicillin G, levofloxacin, nalidixic acid, bacteriological agar and Lisogeny- Broth (LB) medium components (Trypton and Yeast Extract) were purchased from Sigma Aldrich. All other reagents were of molecular biology grade.

Antibiotic stocks were dissolved in distilled water at a concentration of 100 μg/mL and filter-sterilised using a 0.2 μm pore syringe filter (Nalgene), with the exception of gentamicin which was provided in form of liquid 50 mg/mL. Temporin-L was dissolved in DMSO to a concentration of 1 mg/mL.

3.3 Plasmids.

3.3.1 Plasmids used in this work.

Plasmids: pBbE8k-RFP – containing (Red fluorescent protein (RFP) insert under arabinose inducable BAD promotore and bearing kanamycin resistance gene) was a gift from Jay Keasling (Fig. 29A) and pUC18 (29B).



A. B.

Figure 29. Plasmid maps.

A - a map of pUC18; B – a map of pBbE8k-RFP. Figures taken from https://www.addgene.org [377].

3.3.2 Plasmid DNA purification.

Plasmid E.coli DH5alfa was grown overnight (O/N) in 3 mL of LB-medium supplemented with ampicillin (100 µg/mL). Plasmid DNA was isolated by Fast-n-Easy Plasmid Mini-Prep Kit (Jena Bioscience) according to manufacturer's instructions. Briefly, 1 mL of bacterial culture was harvested by centrifugation at 5000g for 3 min at room temperature (RT) and the pellet was resuspended for in 300 µL of Lysis Buffer. After vortexing 300 µL of RNase containing Neutralization Buffer was added and the sample was centrifuged at 10000g for 5 min at RT. The supernatant was applied into the activated Binding column and centrifuged at 10000g for 30 sec. After washing the column with 500 µL of Washing Buffer, plasmid DNA was eluted with 30 µL sterile ddH2O.

The purified plasmid DNA concentration was determined using NanoDrop 1000 spectrophotometer.

3.3.3 Plasmid transformation experiments.

3.3.3.1.Preparation of competent cells *E.coli DH5α cells*.

E.coli DH5α cells from glycerol stocks were streaked on fresh LB-agar plates. A day before the experiment single colony was inoculated into 3 mL of fresh LB medium and left for O/N incubation at 37°C at 250 rpm. The obtained O/N culture was diluted 1:100 in 10 mL of LB medium and incubated at 37°C at 250 rpm for 3-4 hours till OD_{600}=0.4. The culture was then placed on ice for 10 minutes (min) and then centrifuged at 5000 rpm for 5 min at 4°C. The supernatant was removed and the pellet was resuspended in 10 mL of cold 0.1 M CaCl. The

suspension was left on ice for 20 min and centrifuged at 5000 rpm for 3 min at 4°C. The competent cells were either resuspended in 5 mL of cold 0.1 M CaCl and used straight away for transformation or resuspended in 0.1 M CaCl supplemented in 15% glycerol, aliquoted and stored at -80 °C.

3.3.3.2. Transformation.

E.coli DH5α competent cells (100 µL) were placed on ice, and 100 ng of pBbE8k-RFP plasmid was added. The mixture was left on ice for 15 min and then transferred at 42°C for 1.5 min and returned to ice for 5 min. After that, 1 mL of LB medium was added and the cells were incubated for 1h at 37 °C in a thermomixer with shaking at 1010 rpm. The cells were centrifuged at 4000 rpm for 5 min. The pellets were resuspended in 1 mL of LB medium and plated on LB-agar plates containing 50 µg/mL of KAN. A single colony of transformed cells was picked with a loop and inoculated in 3 mL of LB-medium supplemented with 50 µg/mL of KAN and 0,2% arabinose to verify the expression of RFP. From this culture 500 µL was removed, mixed with 50%glycerol/LB and stored at -80°C.

3.4. Antibacterial activity studies.

3.4.1.Culturing and quantification of *E.coli ATCC 25922* cells. Generation of bacterial cell number calibration curve.

E.coli ATCC 25922 strains were streaked on Petri LB-agar plates from glycerol stocks and left at 37°C O/N. Bacterial liquid cultures were prepared by picking 3-5 colonies from fresh LB-agar plates with a sterile loop and inoculated in 3 mL of LB-medium (per Liter -10 g Tryptone, 5g Yeast Extract, 10 g NaCl.) The culture was grown at 37°C at 250 rotations per minute (rpm) in a shaking incubator for 6 hours and subsequently split for Optical Density (OD) measurement and colony counting.

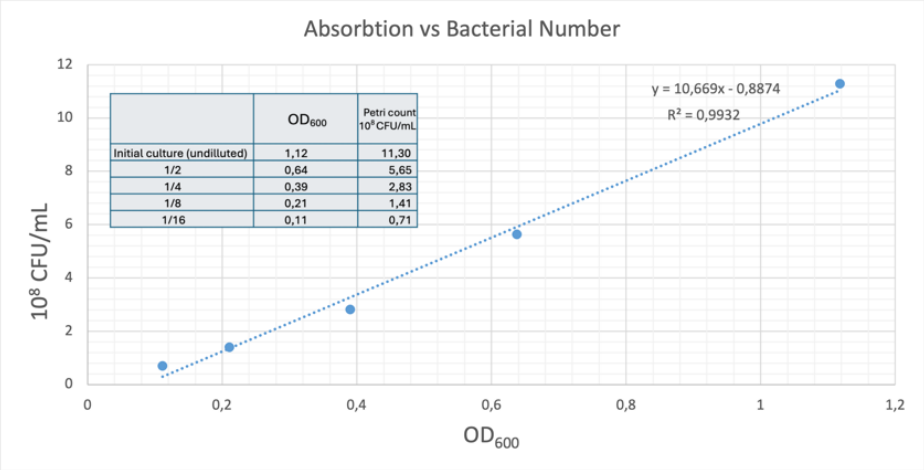
For OD measurements, the initial culture was 2-fold serially diluted in fresh medium and for each dilution absorbance at 600 nm (OD_{600}) was measured in a spectrophotometer using 2 mL glass cuvettes. LB media was used as blank.

For plate counting the initial culture was 10 fold serially diluted and 50 µL of each dilution (from 10^{-5} to 10^{-9}) was spread over LB-agar plates. The plates were left at 37°C O/N. Petris containing 30-50 colony forming units (CFU) were counted and the number of bacteria per mL (CFU/mL) was estimated using the following formula:

$N = C * 20/10^{-D}$

where, N = CFU/mL; C = number of colonies per plate; D = number of the 1:10 dilution.

The value of OD_{600} measurements and the estimated bacterial count were used to build OD_{600} vs CFU/mL calibration curve (Fig. 30), which was used to determine bacterial load in antibacterial activity experiments.



	OD_{600}	Petri count 10^8 CFU/mL
Initial culture (undilluted)	1,12	11,30
1/2	0,64	5,65
1/4	0,39	2,83
1/8	0,21	1,41
1/16	0,11	0,71

Figure 30. Calibration curve for determination of bacterial count.

After each antimicrobial susceptibility experiment described below, in order to monitor/verify bacterial inoculum load, the bacterial cultures were further 10-fold serially diluted to 10^3 CFU/mL from which 50 µL was spread on LB-agar plates. The plates were incubated at 37°C and the colonies were counted the next day.

3.4.2 Determination of Minimal Inhibitory Concentration (MIC).

Antimicrobial susceptibility testing for antibiotics and *de novo* LCAMPs was carried out using broth microdilution assay which is the most commonly used technique to determine the minimal inhibitory concentration (MIC) of antimicrobial agents, including antibiotics and other substances that inhibit the growth of bacteria [378]. MICs for *de novo* LCAMPs as well as commercially available antibiotics were determined at two bacterial densities: 5×10^5 CFU/mL and $5x10^3$ CFU/mL.

A range of 2-fold serial dilutions of antimicrobials (200-0,098 µg/mL) in LB medium were prepared, and 100 µL of each dilution was transferred into each well of 96-well microtiter flat bottom polypropylene plate (Greiner-Bio). Priority to polypropylene over polystyrene was given since cationic peptides were reported to bind polystyrene surfaces) [379]. The OD_{600} of *E.coli ATCC 25922* exponential culture (grown as described for calibration curve) was measured and the equation was used to estimate bacterial concentration CFU/mL. Bacterial culture was adjusted to 10^6 CFU/mL or 10^4 CFU/mL and aliquotes of 100 µL of a corresponding culture was added to the wells containing serially diluted antimicrobials. For positive control 100 µL of

bacterial culture was added to 100 μL of LB medium, while for the sterility control, wells were filled with 200 μL of LB medium. The plates were incubated in a plate-shaking incubator at 1000 rpm at 37℃ O/N.

The percentage of bacterial growth was calculated based on OD_{600} readings obtained from Biotek ELX800 microplate reader. Growth in positive control wells was considered as 100%. The MIC was defined as the minimal concentration of antimicrobial with less than 10% bacterial growth. Each test was reproduced at least three times.

3.4.3 Determination of MICs for dividing and non-dividing bacterial cultures.

E.coli cells were grown in LB-medium at 37℃ at 250 rpm O/N. The culture was harvested by centrifugation at 1500 g and bacterial cells were brought to 10^6 CFU/mL by diluting the pellet in fresh LB (for dividing bacteria experiments) or 1xPBS(for non-dividing bacteria experiments).

The bacterial suspensions (100 μL) were aliquoted in 1.5 mL Eppendorf tubes (Roth) and mixed with an equal volume of antimicrobials that had been twofold serially diluted in LB medium (for dividing bacteria) or 1xPBS(for non-dividing bacteria) with concentrations ranging from 100 to 1.56 μg/ mL. All samples were incubated for 1 h at 37 ℃ in a shaking incubator at 1000 rpm and then centrifuged at 1500 g for 5 min to remove antimicrobials. Subsequently, the pellets were resuspended in fresh LB and left at 37 ℃ at 1000 rpm in a shaking incubator O/N. The MIC values were determined as described above. Each test was reproduced at least three times.

3.4.4. Checkerboard Assay. Determination of Fractional Inhibitory Concentration Indices (FICi).

The antimicrobial effect of AMPs in combination with commercially available antibiotics was determined by two-dimensional broth microdilution checkerboard assay [380] (Fig. 31 A,B) against *E.coli ATCC 25922* at two inoculum densities of 5×10^5 CFU/mL and $5x10^3$ CFU/mL. Three antibiotics having different mechanisms of action were selected: ampicillin (cell wall synthesis inhibitor), gentamicin (protein synthesis inhibitor), and levofloxacin (DNA gyrase inhibitor).

Bacterial exponential culture was grown in fresh LB medium for 6 h and resuspended in fresh LB medium to reach 10^6 CFU/mL. LCAMPs and conventional antibiotics were twofold serially diluted in LB medium in polypropylene 96-well plates to a final volume of 100 μL in each well. Subsequently, bacterial suspension at final concentrations of 5×10^5 CFU/mL or 5×10^3 CFU/mL was applied to each well. Rows containing only AMP or only antibiotic were used to confirm the MICs of the individual compounds. Additionally, one row contained only LB-medium (for

sterility control) and one row for positive control (bacterial culture without antimicrobial). The plates were incubated at 37 °C in a shaking incubator O/N at 1000 rpm.

Synergistic interactions between LCAMPs and NAO were evaluated against *E. coli ATCC 25922* at 5×10^5 CFU/mL as described above for LCAMPs and antibiotics.

OD_{600} measurements were obtained from Biotek ELX800 microplate reader. MICs were determined as described above. FICi were determined using the following formula:

FICi = FIC A + FIC B = (MIC AB/MIC A) + (MIC BA/MIC B),

where MIC A and MIC B are individual MICs of LCAMPs and antibiotics, respectively,

MIC AB and MIC BA are the MICs of LCAMPs and antibiotics in combination.

The following types of interaction were defined:

FICi ≤0.5 (Synergy).

0.5 < FICi ≤0.625 (Potentiation)

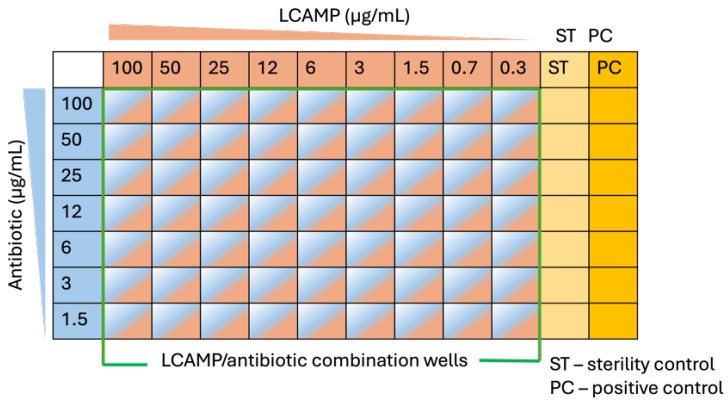
0.625 < FICi ≤1.0 (Additivity).

1.0 < FICi ≤4.0 (Indifference)

FICi >4.0 (Antagonism).

Each test was reproduced at least three times.

A.



B.

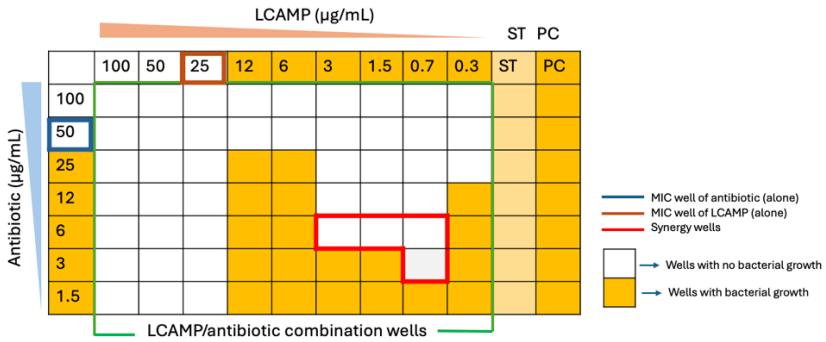


Figure 31. Illustration of Checkerboard assay.

A. 96-well plate setup scheme. B. Representation of Checkerboard assay results.

3.5. Toxicity studies of *de novo* LCAMPs.

3.5.1. Evaluation of toxicity of *de novo* LCAMPs against human erythrocytes. Hemolytic activity assay.

Since the erythrocyte membrane is considered to be a generalized model of the mammalian cell membrane, hemolysis (a release of hemoglobin in response to the destruction of membrane integrity of red blood cells (RBC) is a universal approach to rapidly assess the initial toxicity of LCAMPs.

Fresh human blood (500 µL) was collected into 2 mL microcentrifuge tubes containing heparin (30 units) and immediately centrifuged at 1200 × g for 5 min at RT. The pellet containing erythrocytes was washed 3 × 5 min in 1x PBS at 1200 g, and the pellet containing red blood cells (RBC) was diluted with 1x PBS to obtain 2% RBC suspension. 250 µL of this suspension was added to equal amounts of serially diluted (100 -3.125 µg/mL) LCAMPs in 2 mL polypropylene microfuge tubes (Deltalab). The microtubes were incubated in thermomixer at 37 °C, 300 rpm for 1 h . The samples were centrifuged at 1200× g for 5 min and the supernatant (200 µL) was removed from each microtube and transferred into 96-well polystyrene (Deltalab) microplate. 0.1% Triton X-100/1xPBS (SantaCruz) was used as a positive control, while 1x PBS used as a negative control (NC). The degree of hemolysis was determined by measuring the absorbance of released hemoglobin at 450 nm in Biotek ELX800 microplate reader. The following formula was used to calculate the percentage of hemolysis.

$$\% \text{ hemolysis} = OD_{450} (\text{LCAMP}) - OD_{450} (\text{NC}) / OD_{450} (0{,}1\% \text{ Triton X-100}) - OD_{450}(\text{NC})$$

Hemolytic activity was defined as a concentration of LCAMP at which at least 10% RBC are lysed.

Each experiment was repeated three times.

3.5.2. Evaluation of cytotoxicity of LCAMPs against mammalian cell culture.

3.5.2.1.Culturing of Hepa 1-6 cells.

Hepa 1-6 (murine hepatic carcinoma cells) were stored at -80°C in freezing medium containing 50% Fetal Bovine Serum (FBS, Gibco), 40% Dulbecco's Modified Eagle Medium (DMEM) and 10% dimethyl sulfoxide (DMSO). The cells were thawed at RT, dispensed in microfuge tubes and resuspended 1:1 in DMEM containing (1% penicillin, 1% streptomycin and 1% glutamine). The cells were centrifuged at 1500 rpm for 5 min, resuspended in complete DMEM (10% FBS, 1% penicillin, 1% streptomycin, and 10% glutamine), and transferred for culturing in 25 cm^2 culture flasks at 37°C under 5% CO_2. After reaching 70-80 % confluency the cell culture medium was carefully aspirated and a solution of 0,25% trypsin/ 0,53 mM EDTA/1xPBS (pH 7,8) was added for 3-4 min at 37°C to allow the cells to detach. After incubation, 2 mL of fresh DMEM was added to the flasks, resuspended with Pasteur pipette, and transferred into a 15 mL tube. The cells were counted in a hemocytometer, brought to a concentration of 10^6 cells/mL with a complete DMEM, and seeded in a 96-well cell-culture plate at a density of 10^5 cells per well. The cells were incubated for 24 h at 37°C under 5% CO^2.

3.5.2.2. 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay.

MTT reagent is able to translocate through the cytoplasmic membrane of cells and interact with various components (including the components of glycolytic pathways) of metabolically active cells. As a result MTT gets reduced and accumulated in intracellular organelles and/or on cell membrane in the form purple formazan crystals [381].

After the aspiration of DMEM the cells were washed with 1xPBS. A solution of LCAMPs in DMEM (without FBS and antibiotics) was added to the wells at final concentrations ranging from 100 to 3,125 μg/mL. To the wells serving as a negative control, only DMEM was added. The cells were incubated O/N as described above. LCAMP-containing media was removed and MTT reagent at a concentration of 0.5 mg/mL /1xPBS was added to the cells and left for 4 h incubation at 37°C under 5% CO^2 in the dark (to allow viable cells to reduce the yellow tetrazolium salt (MTT) into formazan crystals). 100 μL of DMSO was added to each well to dissolve the crystals and the plates were incubated at RT for 15 min with shaking. The absorbance (OD_{490}) was measured in a plate reader BioTek ELx800.

The following formula was used to calculate cell viability:

Viability (%) = (OD490 value of treated cells / OD490 value of negative control) × 100.

The peptide was considered toxic if cell viability was reduced by more than 80%.

Each experiment was repeated three times.

3.6. The proteolytic stability towards proteases.

The resistance of LCAMPs to α-chymotrypsin (a bovine serine protease catalyzing the hydrolysis of peptide bonds on the C-terminal side of tyrosine, phenylalanine, tryptophan, and leucine) and proteinase K (a serine protease derived from mold, cleaving the peptide bond adjacent to the carboxylic group of aliphatic and aromatic amino acids) was analyzed with 16% Tricin SDS-PAGE which provides the optimal resolution for peptides in the range of 1-7-kDa [382]. 10 µg of each peptide was incubated with, α-chymotrypsin or Proteinase K at a starting peptide:enzyme molar ratio of 1000:1 or 500:1 in Digestion Buffer (50 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$), the latter molar ratio was tested only for LCAMPs which remained undigested when treated at 1000:1. Bovine Serum albumin (BSA) (diluted in ddH_2O at a concentration of 1 mg/mL and filtered through 0.22 µm syringe filter) at a corresponding correspond molar ratios was used as a negative control.

The reaction mixture (60µL) was incubated at 37 ℃ for 16 h. The reaction was stopped by incubating the samples at 95 ℃ for 3 min and 12.5 µl of 5x LaemmLi loading buffer (0.05% Coomassie Brilliant blue G 250 (VWR), 150 mM Tris-HCl, pH 7.0, 12% SDS, 30% glycerol) was added. 10 µl of each sample was loaded in 16.5 % Acrylamide-Bisacrylamide (AB), (4% stacking gel, 10% spacer gel and 16% separating gel) gel mounted in the vertical electrophoresis apparatus. Anode Buffer (0.1 M Tris-HCl, pH=8.9) was used as lower electrode buffer and Cathode Buffer (0.1 Tris-HCl, 0.1 Tricine, 0.1% SDS, pH=8.25) was used an upper buffer. Initially, the electrophoresis started at 30 V. After the samples entered 10% gel voltage was increased to 80V and finally, the constant voltage of 120 V was applied. After the run was complete the gel was transferred to Fixing solution (100mM ammonium acetate, (VWR) 10% acetic acid, 50% methanol) for 1 h and then stained O/N in a solution containing 0.15% Coomassie Brilliant Blue G 250 (VWR) and 10% acetic acid. The gel was destained with 10% acetic acid for 3 h. Peptide degradation was considered positive if the peptide bands disappeared from the gel after protease treatment.

3.7. Fluorescence microscopy studies.

3.7.1. Determination of the antimicrobial activity of FITC-labeled LCAMPs by microcolony technique.

RFP-expressing *E. coli K12 DH5α* culture from glycerol stock was streaked on LB-agar plates containing 50 µg/mL KAN. A few colonies were transferred into a fresh LB-medium (supplemented with 50 µg/mL KAN and 0.2% arabinose) and grown O/N at 37℃ at 250 rpm. The grown culture was diluted 100-fold with fresh LB medium and mixed with 1% low

melting point agarose (LMA) at a ratio of 1:3 (v/v). 20 μL of this suspension was immediately applied onto 1% agarose pre-coated microscope slides, covered with coverslips (18x18 mm) and left at RT for 5 min to allow agarose to solidify. Coverslips were then removed and the slides were transferred onto a slide moat at 30°C. Twofold serial dilutions (ranging from 100 to 3.125 μg/mL) of FITC-labeled LCAMPs in 1× PBS were pipetted on top of agarose-encapsulated bacterial cells and incubated for 1 h. The slides were rinsed 3 times with 1xPBSand left in LB at 30 °C for O/N incubation. Before the observations LB was removed and a coverslip (22x22mm) was placed onto an agarose area. The presence of microcolonies was viewed under 100x oil objective using brightfield microscopy and with TRIC filter cube of Olympus BX41 fluorescence microscope.

3.7.2 Quantification of blebbed bacterial cells.

RFP-expressing *E. coli K12 DH5α* was grown O/N as described above. Since quantification of bacterial blebbing in liquid is challenging due to the fact that most blebbs rapidly detach from bacterial cell surface and move away from bacterial vicinity, the cells were encapsulated in 1% LMA, where the agarose matrix limited the motility of bacteria and kept the detached blebs in close proximity to the bacterial cell. This, in turn, made the quantification more accurate.

The slides containing LMA encapsulated cells (prepared as described above) were washed in 1xPBSand incubated at RT for 30 min to remove the traces of the medium. Subsequently, the slides were placed onto a slide moat, and LCAMPs (in the presence or absence of 100 mM thiourea (TU)) diluted in 1xPBSto their ½ MIC concentrations, were added to agarose-encapsulated bacterial cells and left at 30°C for 10 min. The slides were then washed in 1xPBSfor 30 min and observed under Olympus BX41 fluorescence microscope. The percentage of blebbed cells was determined by counting 50 cells on each slide. Each test was reproduced at least three times. Statistical significance between groups was determined by two-tailed, unpaired Student's T-Test.

3.7.3 . Induction of aggregate formation in *E.coli ATCC 25922*.

To induce the formation of aggregates inside the bacterial cell, *E. coli ATCC 25922* was grown O/N (as described above), diluted 1:100 in fresh LB medium and incubated at 47°C for 15 min [383]. The presence of aggregates was verified under the brightfield. The cells were then encapsulated in 1% LMA and incubated with FITC-labeled LCAMPs as described below for *E. coli K12 DH5α* and *E. coli ATCC 25922* strains.

3.7.4. Localization of FITC-labeled LCAMPs in the bacterial cell.

(RFP)-expressing *E. coli K12 DH5α* or *E. coli ATCC 25922* were grown O/N in LB medium, encapsulated in 1% LMA (as described above), and incubated with FITC-labeled LCAMPs at

their MIC concentrations for 1 h on a slide moat at 30 °C. The slides were briefly rinsed in 1 × PBS, covered with coverslips and observed with FITC filter cube under 100x oil immersion objective of Olympus BX41 fluorescence microscope. All the images were captured with CCD camera and processed using FIJI software.

3.7.5. NAO staining.

NAO is a green fluorescent dye extensively used for location and quantitative studies of cardiolipin (CL) in living bacterial and eukaryotic cells. A more recent study found the most important factor for targeting NAO to CL to be the insertion of the nonyl chain into the bilayer at the hydrophobic surface created by the four fatty acid chains [384].

An O/N culture of *E. coli ATCC 25222* was diluted with fresh LB medium to final OD600 = 0.5. Bacteria were then incubated with LCAMPs at their ½ MIC concentrations in the presence of NAO at a concentration of 0.235 μg/mL for 1 h at 37°C with shaking at 1000 rpm. The samples were harvested by centrifugation at 5000g for 3 min. The pellets were washed twice with 1xPBSand resuspended in 100 μL of fresh LB. 5 μL of the resulting suspension was spotted onto 1% agarose pre-coated microscope slides, covered with coverslips (22x22mm), and examined under Olympus BX41 fluorescence microscope. Images were captured and processed as described above.

3.7.6. FM-4-64 staining.

FM4-64 – a lipophilic red fluorescent dye reported to be used to track changes in bacterial cell morphology and dynamics of membrane lipids [39].

E. coli ATCC 25222 was grown O/N and diluted with fresh LB to a final OD600 = 0.5 and incubated with LCAMPs at their ½ MIC concentrations in the presence of FM4-64 at a concentration of 10 μg/mL for 1 h at 37°C with shaking at 1000 rpm. The samples were immediately spotted onto 1% agarose pre-coated microscope slides and examined under an Olympus BX41 fluorescence microscope. Images were captured with CCD camera and processed using FIJI software.

The percentage of bacterial cells with visible lipid domain redistributions and the measurements of cell length and circularity were performed for at least 50 cells per slide.

Statistical significance between groups was determined by a two-tailed, unpaired Student's T-Test. Each experiment was reproduced at least three times.

3.7.7. *In situ* immunofluorescence studies.

For coating with poly-L-lysin, coverslips 18x18 mm were cleaned by washing in Alkaline buffer (10% NaOH, 60% ethanol) for 2 h, thoroughly rinsed with water and left in pure ethanol for 30 min. Each slide was then picked with tweezers and flame sterilized. 250-300 µL of 0.1 mg/mL poly-L-lysine (70 000 kDa (Sigma) was applied on top of each coverslip and left at RT for 2 h. Poly-L-lysine was removed and the coverslips were rinsed with sterile ddH_2O and dried O/N in Biosafety Cabinet.

E. coli ATCC 25922 were grown O/N and diluted with fresh LB medium 1:100 and incubated with LCAMPs at ½ MIC concentrations for 30 min at 37°C and immediately fixed with 2% paraformaldehyde (PFA)/1xPBS (10 mM Na_3PO_4, 150 mM NaCl, 15 mM KCl (pH 7.4)) for 10 min at RT and then transferred on ice for another 20 min. To remove PFA the samples were centrifuged at 4000g for 5 min at 4°C and the pellets were resuspended in 1xPBS and washed 2 x 5 min. Finally, the pellet was resuspened in 300 µL of GTE buffer (50 mM glucose, 10 mM ethylenediaminetetraacetic acid (EDTA), 20 mM Tris-HCl pH 7.5) and pipetted onto a poly-L-lysine coated coverslips (18x18mm) and left for attachment at RT for 15 min. The liquid was gently aspirated and 300 µL of 0,1% Triton X-100/1xPBS was added. The samples were left at RT for 10 min, washed with 1xPBS, and then blocked with 2% (BSA)/1xPBS for 30 min at RT. The blocking solution was aspirated and the cells were incubated with primary anti-FtsZ antibody diluted 1:100 in 2% BSA O/N at 4°C. Subsequently, the cells were washed with 0.01% Tween-20/1xPBS and incubated with secondary FITC-conjugated antibodies for 1 h at RT. The samples were washed with 0.01% Tween-20/1xPBS and mounted onto a drop of anti-fade mounting medium Fluoroshield (Sigma) on microscope glass slides. The edges were sealed with nail polish. The samples were observed under Olympus BX41 fluorescence microscope. Images were captured with CCD camera and processed using FIJI software. The percentage of cells revealing disrupted Z-rings was estimated by counting at least 150 cells on each slide. Each experiment was performed three times. Statistical significance between groups was determined by two-tailed, unpaired Student's T-Test.

3.8. Interaction of LCAMPs with DNA.

3.8.1. Electrophoretic mobility shift-assay (EMSA).

600 ng of pUC18 DNA in 10 mM Tris-HCl buffer (pH 7.4) was mixed with LCAMPs at a molar ratio of 1:1000 or 1:500 to a final volume of 60 µL and incubated at 37 °C for 1 h. Subsequently, 6 × loading buffer (30% glycerol, 1 mM EDTA, 0.25% bromphenol) was added and aliquots of 15 µL were loaded into 1% agarose gel. The gel was run in 1×TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) at 0.6 V/cm. The gel was stained with ethidium bromide (10 µg/mL)

and visualized under ultraviolet illumination. Only peptides showing full retardation of plasmid DNA at 1:1000 DNA : LCAMP molar ratio were tested at a molar ratio of 1:500.

3.8.2. Determination of DNA double-strand brakes (DSBs) by pulsed-field gel electrophoresis (PFGE).

A bacterial culture of *E. coli ATCC 25922* grown for in LB (as described above) (OD_{600} = 0.8) was pelleted at 1500 g for 5 min and diluted in 1xPBS to 1.5× 10^9 CFU/mL. The bacterial suspension was then mixed with 1.5% LMA at a ratio of 1:2 and approximately 80 µL of the solution was dispensed into each slot of a plastic plug mold (Bio-Rad) and left to solidify at RT. The blocks containing bacterial cells were then transferred into 2 mL tubes (one block per tube) containing 500 µL of solutions of peptides (100 µg/mL), levofloxacin (0.5 µg/mL), or ampicillin (50 µg/mL) in 1xPBS and incubated at 37 ℃ for 2 h (peptides and ampicillin) or 1 h (levofloxacin) in the presence or absence of 100 mM TU without agitation. Following the exposure to antimicrobial agents, the solutions were removed and the blocks were treated with proteinase K (1 mg/mL) in digestion buffer (100 mM EDTA, 50 mM Tris-HCl, 1% sodium lauryl sarcosine, pH 8) and incubated O/N at 50 ℃ without agitation. The blocks were then washed 4 × 45 min in 1×TE (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) and stored at 4 ℃ in TE buffer until PFGE. The plugs containing bacterial chromosomal DNA were loaded into wells of 1% agarose gel. Electrophoresis was run in 0.5×TBE buffer (45 mM TRIS, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 300 V for 6 h at 12 ℃ with a pulse time of 25 s using Gene Navigator Pulsed Field Gel Electrophoresis System (Amersham Biosciences). The gel was stained with ethidium bromide and visualized under ultraviolet illumination.

Chapter 4 : Results.

4.1. Biological Activity Studies.

4.1.1. All *de novo* designed LCAMPs reveal antibacterial activity against *E.coli ATCC 25922*.

The MICs of 10 *de novo* designed LCAMPs, D-enantiomers of three of these LCAMPs (24L, L1L and ST1L), 6 commercially available antibiotics (ampicillin, kanamycin, levofloxacin, penicillin G, nalidixic acid, gentamicin) and Temporin-L (a natural AMP, sharing some similarities with our *de novo* peptides in terms of cationicity, the number of amino acids and linearity) [15,16], were determined. It has been reported that the antibacterial activity of several AMPs and peptidomimetics, was significantly affected by bacterial inoculum density [385]. In our studies, the antibacterial properties of *de novo* LCAMPs against *E. coli ATCC 25922* were determined at two bacterial inoculum densities 5×10^5 CFU/mL (an inoculum density recommended by the Clinical and Laboratory Standards Institute (CLSI) for *in vitro* testing of antimicrobial agents) and 5×10^3 CFU/mL) in LB medium as described in Materials and Methods section.

A 4-fold or greater change in the MIC of antimicrobials resulting from a 100-fold change in bacterial concentration was considered as an inoculum effect (IE) [386,387]. At lower cell densities compared to a standard inoculum density of 5×10^5 CFU/mL, only the MIC of peptide L1L decreased 8-fold, while most of the tested LCAMPs showed 2- to 4-fold decrease in their MIC values. In contrast, the MIC values of only one of the 6 tested antibiotics gentamicin changed in response to the changes in the bacterial concentration (see Table 7). Surprisingly, Temporin-L did not show antimicrobial activity at any tested concentrations at either inoculum density (Table 7.)

Antibiotic / Peptide	Sequence	MIC (μg/mL)	
		5×10^3 CFU/mL	5×10^5 CFU/mL
Ampicillin	—	6.25-12.5	6.25-12.5
Penicillin G	—	25-50	50
Gentamicin	—	0.78-1.56	3.125-6.25
Kanamycin	—	6.25	6.25
Levofloxacin	—	0.024	0.024
Nalidixic Acid	—	3.125-6.25	3.125-6.25
24L	RWIRWVWRKKLR	6.25-12.5	25-50
24D	rwirwvwrkklr	3.125	3.125-6.25
L1L	AIKIRKLFKKLLR	12.5	50-100
L1D	aikirklfkkllr	3.125-6.25	12.5-25
LCAPL2	GIKIRKLFKKLLR	12.5	50
LCAPL4	GIKFFLKKLKKH	50	100
LCAPL8	VARFLKRIIKALF	3.125-6.25	6.25
LCAPL9	GFIKIVRKLLRLF	6.25	6.25
LCAPL10	IIKRILIQLKKLL	6.25-12.5	25-50
LCAPL14	WKKLKLWLKWKLW	50	100
LCAPL15	KKFLGKWKLRFGW	6.25	12.5
ST1L	LVWKLWWRLRWLK	100	100
ST1D	lvwklwwrlrwlk	25-50	50
Temporin-L	FVQWFSKFLGRIL	>100	>100

Table 7. Minimal inhibitory concentrations (MIC) of antimicrobials against *E. coli ATCC 25922* at two bacterial concentrations.

4.1.2. Antibacterial activity of *de novo* LCAMPs does not dramatically depend on bacterial division state.

Since there is plenty of data reporting that bacterial susceptibility toward antimicrobial agents might depend on the metabolic state of a bacterial culture [40,41], we decided to assess the MIC values of *de novo* designed LCAMPs against dividing and non-dividing cells of *E. coli ATCC 25922*. Since it was observed that the MICs of LCAMPs did not significantly differ between 1 h and O/N incubation in LB medium (compare Tables 7 and 8), in this set of experiments, O/N culture of bacteria was pre-incubated with LCAMPs in 1xPBS or LB for 1 h at 37 °C and then recovered in fresh medium O/N. As shown in Table 8, at a standard inoculum density, MIC values against dividing and non-dividing cells were not significantly different for the majority of the tested LCAMPs. However, in 1×PBS, MIC values markedly decreased for LCAPL14 (>32-fold), and Temporin-L (being inactive in LB) exhibited antimicrobial activity at 12.5–25 μg/mL. The revival of the antimicrobial properties of Temporin-L and the lowered MIC for LCAPL14 in 1xPBS might most probably be attributed to their antagonistic interactions with some components of the LB. Overall, the obtained results indicate that all the tested *de novo* LCAMPs retain their antibacterial activity regardless of the division state of the bacteria.

Antibiotic / Peptide	MIC (μg/mL)	
	PBS	LB
Ampicillin	>100	>100
Gentamicin	25	50
24L	100	50-100
24D	25-50	12.5
L1L	12.5	50-100
L1D	12.5-25	12.5-25
LCAPL2	12.5-25	50
LCAPL4	50-100	≥100
LCAPL8	6.25	6.25
LCAPL9	1.56	6.25
LCAPL10	12.5	25-50
LCAPL14	1.56	50-100
LCAPL15	12.5	12.5-25
ST1L	≥100	≥100
ST1D	50	50
Temporin-L	12.5-25	>100

Table 8. Minimal inhibitory concentrations (MIC) of antimicrobials against dividing (LB) and non-dividing (PBS) cells of *E. coli ATCC 25922.* Bacterial cell density – 5×10^5 CFU/mL.

4.1.3. Only D-enantiomers of *de novo* LCAMPs are stable against proteases.

It's widely known that proteolytic instability of AMPs often diminishes their antimicrobial properties. Therefore, the stability of *de novo* LCAMPs against proteases was evaluated.

De novo LCAMPs were exposed to Proteinase K and α-chymotrypsin (as described in the Methods section). As expected, D-variants of 24 L and L1L were not subjected to protease cleavage by any enzyme at any tested molar ratios (Table 9, Fig 32).

At a peptide/enzyme molar ratio of 1000:1, 6 out of 11 LCAMPs were fully digested by both α-chymotrypsin and Proteinase K. 24L was partially digested with both proteases at both (1000:1 and 500:1 peptide:enzyme) molar ratios. LCAPL 4 was partially digested by proteinase K but fully digested with α-chymotrypsin, while LCAPL14 was partially digested by α-chymotrypsin and fully cleaved by proteinase K at a ratio of 500:1. Antibacterial properties of partially digested peptides were further tested by MIC assay, however none of them was able to inhibit bacterial growth of *E.coli ATCC 25922* at any tested concentration (up to 100 μg/mL, data not shown).

	Peptide/Protease Molar Ratio			
	Proteinase K		*α*-chymotrypsin	
Peptide	1000:1	500:1	1000:1	500:1
24L	PD	PD	PD	PD
24D	ND	ND	ND	PD
L1L	D	NT	D	NT
L1D	ND	ND	ND	ND
LCAPL2	D	NT	D	NT
LCAPL4	PD	PD	D	NT
LCAPL8	D	NT	D	NT
LCAPL9	D	NT	D	NT
LCAPL10	D	NT	D	NT
LCAPL14	PD	D	PD	PD
LCAPL15	D	NT	D	NT
BSA	D	D	D	D

Table 9. Proteolytic stability for the *de novo* designed peptides.

D—Digested; NT—not tested (if a peptide digested by a protease in a lower concentration of the protease, the experiment for the higher concentration was not carried out); PD—Partially digested; ND—not digested.

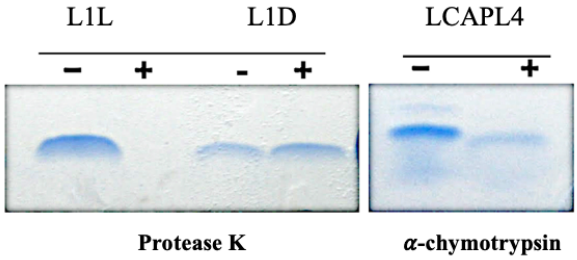


Figure 32. A representative image of Coomassie-stained SDS-PAGE gel of proteolytic analysis of LCAMPs.

(-) LCAMP non treated

(+) LCAMP treated with either protease K or *α*-chymotrypsin.

4.1.4. Toxicity of *de novo* LCAMPs.

Toxicity of *de novo* LCAMPs was assessed by Hemolysis and MTT assays. Hemolytic activity of LCAMPs was evaluated by their ability to induce hemolysis of human erythrocytes. The cytotoxicity of LCAMPs on the viability of murine 1-6 Hepa cells was assessed by MTT assay.

4.1.4.1. Several *de novo* LCAMPs reveal hemolytic activity against human erythrocytes.

In this work, LCAMP was considered hemolytic if it produced more than 10% hemolysis at its MIC concentration (at a standard inoculum density).

Among the tested *de novo* LCAMPs, L8, L9, L10, L14, and ST1L appeared hemolytic at their MIC concentrations (Table 10). Interestingly, L1D LCAMP appeared to be hemolytic at its 2x

MIC concentration, compared to its non-hemolytic L1L variant. At the same time, for 24D, the substitution of L-amino acids with D variants didn't result in increased hemolytic activity. Overall, more than half tested *de novo* LCAMPs revealed low (less than 5%) or no hemolysis at their MIC concentrations against standard inoculum density and thus were considered as non-hemolytic in this work.

Peptides	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.125 µg/ml
24L	0	0	*0	0	0	0
24D	0	0	0	0	0	0
L1L	2,440	0,39	1,64	0,49	0,77	0,40
L1D	31,40	15,55	5,82	0,69	1,29	0,08
LCAPL2	4,48	1,19	0,51	0,00	0,00	0,00
LCAPL4	0,73	0,06	1,03	0,06	0,83	0,00
LCAPL8	100,00	100,00	100,00	80,90	39,42	9,49
LCAPL9	100,00	99,70	67,00	38,15	19,05	5,15
LCAPL10	94,89	64,30	32,56	13,86	3,85	0,90
LCAPL14	58,08	39,33	26,30	16,69	9,94	6,22
LCAPL15	25,44	13,80	7,31	3,85	2,01	1,02
ST1L	90,70	73,18	68,51	28,63	10,27	4,98

Table 10. Mean hemolytic activities of *de novo* LCAMPs represented as a percentage of total hemolysis of human erythrocytes caused by 0.1% Tritonx-100. The hemolytic concentrations corresponding to MICs are framed. Each experiment was repeated three times.

4.1.4.2. The majority of *de novo* LCAMPs are not cytotoxic at their MIC concentrations.

In this work, an LCAMP was considered non-cytotoxic if its MIC concentration did not compromise the viability of more than 80% of cells after treatment.

As shown in Table 11, the only LCAMP showing cytotoxicity at its MIC concentration against murine 1-6 Hepa cells was L8. The rest of *de novo* were considered non-cytotoxic.

Peptides	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml	3.125 µg/ml
24L	53,5	93,6	83,4	89,0	90,7	90,5
24D	25,4	58,1	73,8	97,1	97,2	91,3
L1L	83,0	97,7	95,1	83,3	89,0	88,5
L1D	66,3	74,75	81,95	81,65	101,9	80,25
LCAPL2	83,4	109,1	104,7	116,9	110,5	109,2
LCAPL4	83,3	108,1	93,8	101,3	114,3	102,2
LCAPL8	19,9	36,85	54,2	59,25	76,5	84,45
LCAPL9	58,3	71,0	80,1	77,8	81,0	93,5
LCAPL10	87,4	77,2	84,8	100,8	90,1	104,4
LCAPL14	89,8	82,4	100,55	106,55	104,45	103,7
LCAPL15	91,3	94,1	105,15	91,4	99,65	106,9

Table 11.Cytotoxicity of *de novo* LCAMPs evaluated by MTT assay.

Mean percentage viabilities of Hepa 1-6 cells treated with LCAMPs compared to non-treated cells. Cytotoxic concentration corresponding to MIC is framed. Each experiment was repeated three times.

4.1.4.3. Synergy between LCAMPs and conventional antibiotics depends on bacterial cell density.

Since there is no data regarding the effect of IE on AMP-antibiotic synergy, it was decided to conduct synergy studies between 11 *de novo* LCAMPs and 3 conventional antibiotics (having different mechanisms of action) against *E. coli ATCC 25922* at two bacterial inoculum densities using a Checkerboard assay. At a standard cell density, peptides 24L, L1L, and L1D showed synergistic effects with all the tested antibiotics (Fig. 33 and Table 13). The highest number of synergistic combinations were obtained for gentamicin, which, apart from 24L, L1L, and L1D, showed synergy with LCAPL2 and LCAPL10. In all synergistic combinations, the MICs of LCAMPs decreased 4 times, while the MIC values of some antibiotics decreased more significantly. For example, the MIC value of gentamicin in combination with L1D decreased 16-fold. At a cell density of 5×10^3 CFU/mL, synergy was observed only in combination with ampicillin for two LCAMPs (LCAPL2 and ST1L) with the 8-fold decreased MICs of both antimicrobial agents. At both inoculum densities, peptide-antibiotic combinations with FICi values of $0.5 < \text{FICI} \leq 0.625$ were labeled as potentiation.

The maximum decrease in peptide MIC (16-fold) was observed for LCAPL15 in combination with ampicillin at a standard inoculum density, while the rest of the LCAMPs showed a less significant decrease in their MIC values (2–8 fold). The changes in the MIC values of ampicillin were mostly in the same range as those of LCAMPs. However, levofloxacin and gentamicin showed a more prominent decrease in their MICs. For example, the MIC of levofloxacin decreased 32-fold in combinations with LCAPL2 and LCAPL10 at 5×10^3 CFU/mL and 5×10^5 CFU/mL, respectively, while the maximum (64-fold) decrease of MIC was revealed for gentamicin in combinations with ST1D and ST1L at 5×10^3 CFU/mL and 5×10^5 CFU/mL, respectively. No antagonistic interactions were observed in any antibiotic-peptide combinations.

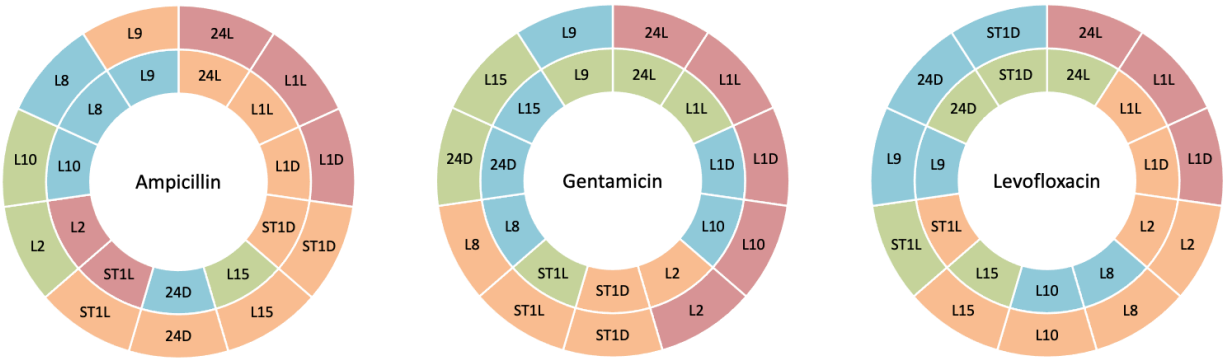


Figure 33. Synergistic interactions between peptides and antibiotics against *E. coli ATCC 25922* at two bacterial concentrations. Inner circle – 5×10^3 CFU/mL, outer circle – 5×10^5 CFU/mL; Colors: blue– indifference; green – additivity; orange – potentiation; pink – synergy.

	$5x10^3$ CFU/mL					$5x10^5$ CFU/mL				
Peptides	**MIC (A)**	**MIC (B)**	**MIC (AB)**	**MIC (BA)**	**FICI**	**MIC (A)**	**MIC (B)**	**MIC (AB)**	**MIC (BA)**	**FICI**
Ampicillin										
24L	12.5	6.25	6.25	0.78	0.625	25	12.5	6.25	3.125	0.5
24D	3.125	12.5	3.125	0.195	1.016	6.25	12.5	3.125	1.56	0.625
L1L	12.5	6.25	6.25	0.39	0.563	50	12.5	12.5	3.125	0.5
L1D	3.125	12.5	1.56	1.56	0.625	12.5	12.5	3.125	3.125	0.5
LCAPL2	12.5	6.25	3.125	1.56	0.5	50	12.5	25	3.125	0.75
LCAPL8	6.25	12.5	6.25	0.195	1.016	6.25	6.25	6.25	0.195	1.031
LCAPL9	6.25	6.25	6.25	0.098	1.016	6.25	12.5	3.125	1.56	0.625
LCAPL10	6.25	6.25	6.25	0.098	1.016	25	6.25	12.5	1.56	0.75
LCAPL15	6.25	12.5	3.125	3.125	0.75	12.5	6.25	0.78	3.125	0.563
ST1L	100	6.25	25	0.78	0.375	100	12.5	50	1.56	0.625
ST1D	25	6.25	3.125	3.125	0.625	50	12.5	25	1.56	0.625
Gentamicin										
24L	12.5	1.56	6.25	0.78	1	50	3.125	12.5	0.78	0.5
24D	3.125	1.56	3.125	0.024	1.015	6.25	3.125	3.125	1.56	1
L1L	12.5	1.56	6.25	0.39	0.75	50	3.125	12.5	0.78	0.5
L1D	3.125	1.56	3.125	0.195	1.125	25	6.25	6.25	0.39	0.313
LCAPL2	12.5	1.56	6.25	0.195	0.625	50	6.25	12.5	0.78	0.375
LCAPL8	3.125	0.78	3.125	0.024	1.031	6.25	3.125	3.125	0.098	0.531
LCAPL9	6.25	0.78	3.125	0.39	1	6.25	3.125	6.25	0.098	1.031
LCAPL10	12.5	1.56	12.5	0.195	1.125	50	3.125	12.5	0.78	0.5
LCAPL15	6.25	0.78	6.25	0.024	1.031	12.5	3.125	3.125	1.56	0.75
ST1L	100	1.56	50	0.78	1	100	3.125	50	0.049	0.516
ST1D	50	1.56	25	0.024	0.516	50	3.125	6.25	1.56	0.625
Levofloxacin										
24L	6.25	0.024	3.125	0.006	0.75	25	0.024	6.25	0.006	0.5
24D	3.125	0.024	1.56	0.012	1	3.125	0.024	3.125	0.001	1.031
L1L	12.5	0.024	6.25	0.003	0.625	100	0.024	25	0.006	0.5
L1D	6.25	0.024	3.125	0.002	0.563	25	0.024	6.25	0.003	0.375
LCAPL2	12.5	0.024	6.25	0.001	0.531	50	0.024	25	0.003	0.625
LCAPL8	3.125	0.024	3.125	0.006	1.25	6.25	0.024	3.125	0.003	0.625
LCAPL9	6.25	0.024	6.25	0.024	2	6.25	0.024	6.25	0.006	1.25
LCAPL10	12.5	0.024	12.5	0.001	1.031	25	0.024	12.5	0.001	0.531
LCAPL15	6.25	0.024	1.56	0.012	0.75	12.5	0.024	6.25	0.002	0.563
ST1L	100	0.024	12.5	0.012	0.625	100	0.024	25	0.012	0.75
ST1D	50	0.024	25	0.012	1	50	0.024	50	0.001	1.031

Table 12. Synergistic interactions between peptides and antibiotics against *E. coli ATCC 25922* at two bacterial concentrations.

MIC A – MIC of peptide alone; MIC B – MIC of antibiotic alone; MIC AB – MIC of peptide in combination with antibiotic; MIC BA – MIC of antibiotic in combination with peptide; FICi – fractional inhibitory concentration index.

4.2. **Mechanism of action studies.**

Four non-hemolytic and non-cytotoxic *de novo* LCAMPs, three of which (L1L, 24L, and L1D) revealed synergy with all tested antibiotics, and 24D - the enantiomer of 24L (having the highest antimicrobial activity) were selected to further study their effects on bacterial cell.

4.2.1. Localization of *de novo* LCAMPs in live bacterial cells.

Prior to the investigation of peptide localization in the bacterial cell, effective concentrations of the selected FITC-labeled LCAMPs were determined using the microcolony technique. Multiple microcolonies were observed for FITC-24L, FITC-24D, and FITC-L1L at concentrations lower than 25 μg/mL, while for FITC-L1D, the colonies appeared at concentrations lower than 12.5 μg/mL. Thus, 25 μg/ mL or 12.5 μg/mL were considered as their MIC values in this system and were used for the evaluation of LCAMP localization patterns in live bacteria. The treatment of *E. coli ATCC 25922* with FITC-labeled LCAMPs showed that the FITC signal was localized predominantly along the membrane surface of treated cells with slightly enhanced fluorescence at the bacterial poles and septal regions (Fig. 34 A). Similar results were obtained for *E. coli K12 DH5α* (data not shown). A different distribution pattern of LCAMPs was observed for RFP-expressing *E. coli K12 DH5α* (bearing inclusion bodies (IB)) (Fig. 34 B), where, as shown in Fig. 34C, FITC signal was predominantly localized in IBs. IBs are submicron proteinaceous aggregates (usually ranging from 50 to 800 nm), accumulated in cells as a result of stress or overexpression of recombinant proteins [388,389]. Under bright field microscope, IBs are visualized as dense dark areas in the cytoplasm at the poles and septal regions [390]. To define whether the accumulation of LCAMPs in IBs could be attributed to the presence of recombinant proteins or caused by the attraction and accumulation of LCAMPs by endogenously produced protein aggregates, we subjected *E. coli K12 DH5α* to heat shock at 47 °C. As shown in Fig. 34 D, heat shock promoted the generation of multiple aggregates inside the bacterial cells and the localization of FITC-labeled LCAMPs corresponded to the exact location of these aggregates.

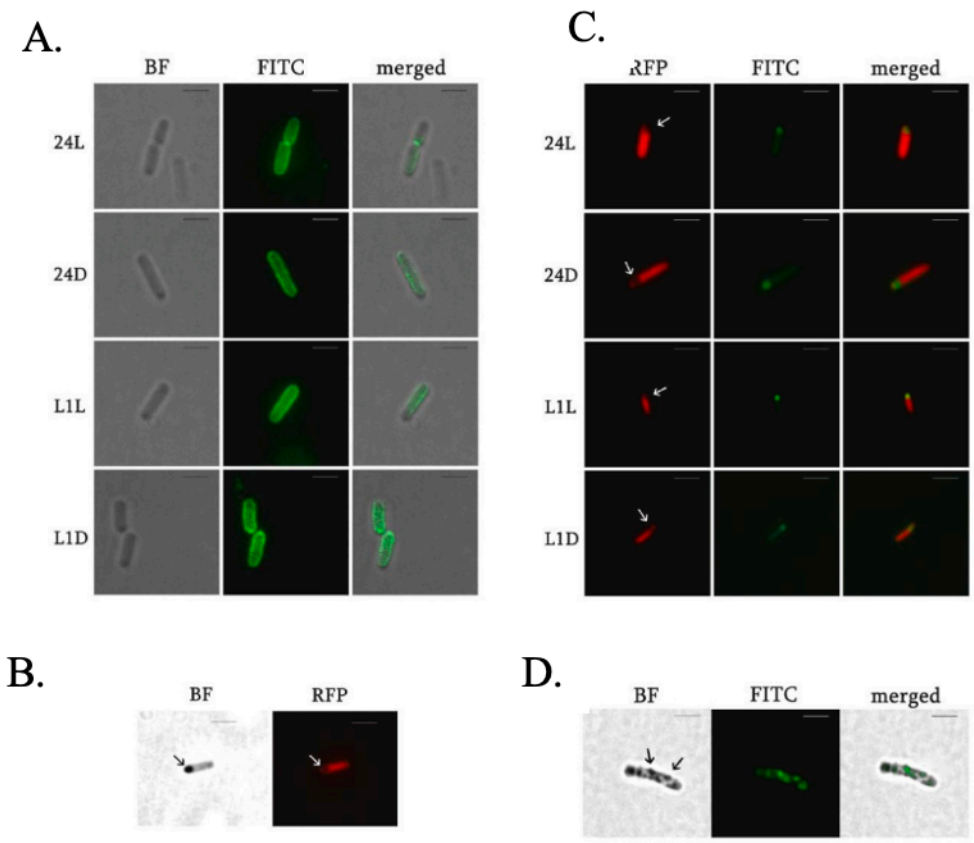


Figure 34. Presence or absence of IBs results in the different distribution patterns of FITC-labeled LCAMPs in *E. coli* cells.

(A) Representative images of LMA-encapsulated *E. coli ATCC 25922* cells treated with FITC-labeled LCAMPs at their MIC concentrations (as described in Materials and Methods). (B) Bright field and fluorescent images of RFP-expressing *E. coli K12 DH5α* cells bearing IBs. (C) Representative images of LMA-encapsulated RFP- expressing *E. coli K12 DH5α* cells treated with FITC-labeled LCAMPs at their MIC concentrations. (D) Representative images of heat-shocked E. coli DH5α cells treated with FITC-labeled peptide 24D. All images were captured with CCD camera of Olympus fluorescent microscope equipped with 100× oil-immersion objective lens. Scale bar – 2 μm. Arrows are pointing at IBs.

4.2.2. *De novo* LCAMPs induce redistribution of anionic phospholipids in bacterial membrane.

NAO is known to specifically bind to anionic phospholipids (preferentially cardiolipin) by an interaction between its quaternary amine and the phosphate residue of the phospholipids and by intercalation of its hydrophobic acridine moiety into the lipid bilayer [384,391]. Since higher cell densities were required for microscopy studies MICs for 24L/D and L1L/L1D against *E.coli ATCC 25922* at $5x10^7$ CFU/mL were defined (Fig. 35 A.). Also, before starting NAO staining experiments, the MIC of NAO against *E. coli ATCC 25922* was determined. An antibacterial effect of NAO was detected at 3.125 μg/mL, thus a 13-fold lower concentration in subsequent

experiments was used. In our experiments, untreated bacterial cells had an NAO-staining pattern similar to what has been observed in *E. coli ATCC 25922* cells treated with FITC-labeled LCAMPs. However, non-fluorescent LCAMPs and Temporin-L at their ½ MIC concentrations induced a significant redistribution of anionic phospholipids observed as discrete green fluorescent domains along the bacterial cell envelope (Fig. 35 B). Interestingly, when incubated with RPF-expressing *E. coli* cells, NAO was predominantly localized in IBs as it was observed for FITC-labeled LCAMPs (Fig. 35 C). Overall, the cells treated with LCAMPs were characterized by brighter staining due to better NAO uptake. These results led to the investigation of a potential synergy between NAO and LCAMPs. As shown in (Fig. 35 D), NAO indeed exhibited synergy when in combination with 24L, L1L, and L1D. Also, we noticed that all tested LCAMPs induced blebbing with intense NAO fluorescence at bleb origination sites (Fig. 35 E). Phospholipid redistribution noticed in NAO staining experiments could be attributed to the synergy between *de novo* LCAMPs and NAO, therefore it was decided to continue observations with a non-toxic lipophilic dye FM-4-64, which didn't reveal synergy with any of the four tested LCAMPs (data not shown). This allowed us to quantify the percentage of cells showing lipid redistribution. The experiments have shown that all four LCAMPs at their ½ and ¼ MIC concentrations induced lipid perturbations visible as red, bright fluorescent foci on the bacterial membrane. The highest percentage of cells with lipid redistribution was observed for L1L and L1D (~80%), while the lowest percentage (6%) was shown for 24D (Fig. 35 F).

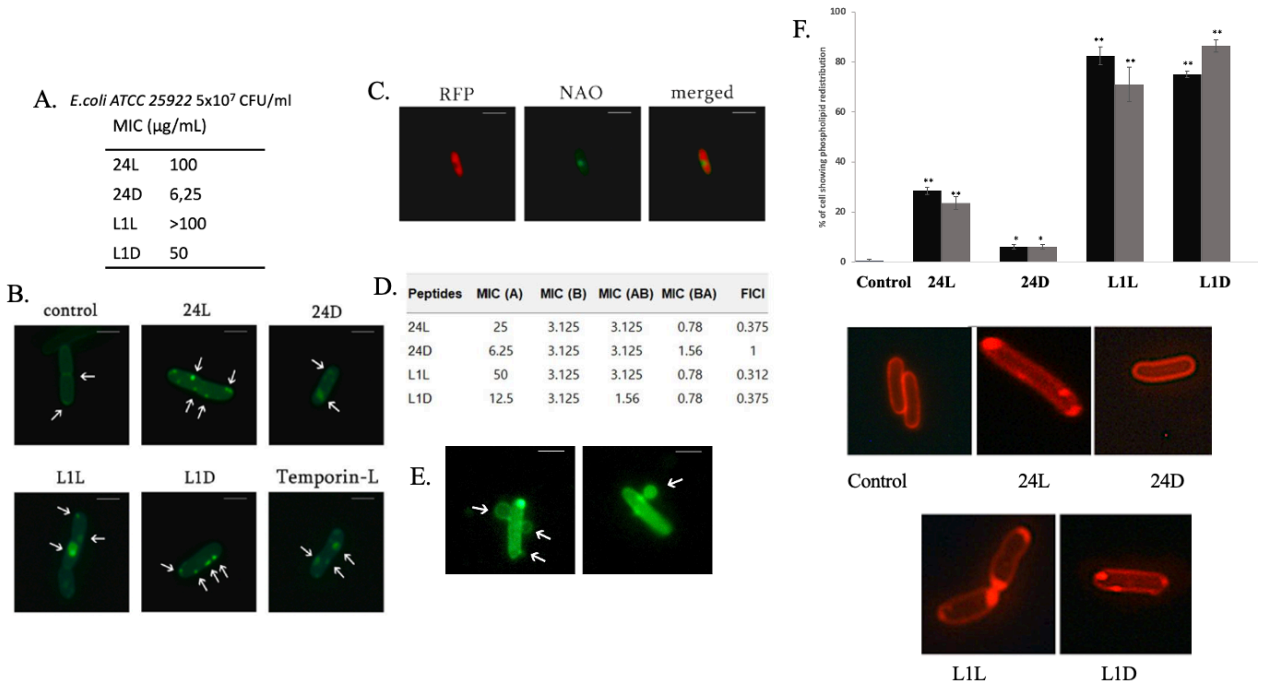


Figure 35. LCAMPs induce the redistribution of negatively charged phospholipids in *E. coli ATCC 25922* cells.

(A) MICs for 24L/D and L1L/D against 5x10^7 CFU/mL. (B) Representative images of *E. coli ATCC 25922* cells treated with NAO alone (control) or in combination with LCAMPs at their ½ MICs. Arrows are pointing at NAO accumulation sites. (C) Representative images of NAO distribution in RFP-expressing *E. coli K12 DH5α* cells. (D) Synergy analysis against E. coli ATCC 25922 between LCAMPs and NAO was performed by checkerboard method. MIC A and MIC B are the individual MICs of LCAMPs and NAO, respectively, and MIC AB and MIC BA are the MICs of LCAMPs and NAO in combination, respectively. (E) Representative images of LCAMP-induced blebbing of *E. coli ATCC 25922* cells stained with NAO. Arrows pointing at bleb origination sites.
(F) Quantification and representative images of FM 4-64 stained *E.coli ATCC 25922* cells showing phospholipid redistributions. *P value <0.05; ** P value < 0.01. ½ MIC (black) and ¼ MIC (grey)
All images were captured with a CCD camera of an Olympus fluorescent microscope equipped with a 100× oil- immersion objective lens. Scale bar – 2 µm.

4.2.3. *De novo* LCAMPs induce shape changes of *E.coli ATCC 25922* cells.

Together with phospholipid redistribution treatment with *de novo* LCAMPs resulted in morphological changes of bacterial cells. The shape and size of bacterial cells varied, depending on the LCAMP and their concentration (Fig. 36 A,B). The length of bacterial cells treated with 24L at ½ MIC increased 1.58 times compared to control cells (Fig. 36 A). The same effect, less prominent but statistically significant, was observed for its D-enantiomer at ¼ MIC

concentration. Opposite to 24L, cells treated with L1L appeared circular at both concentrations, while L1D did not induce any significant changes in cell shape (Fig. 36 B).

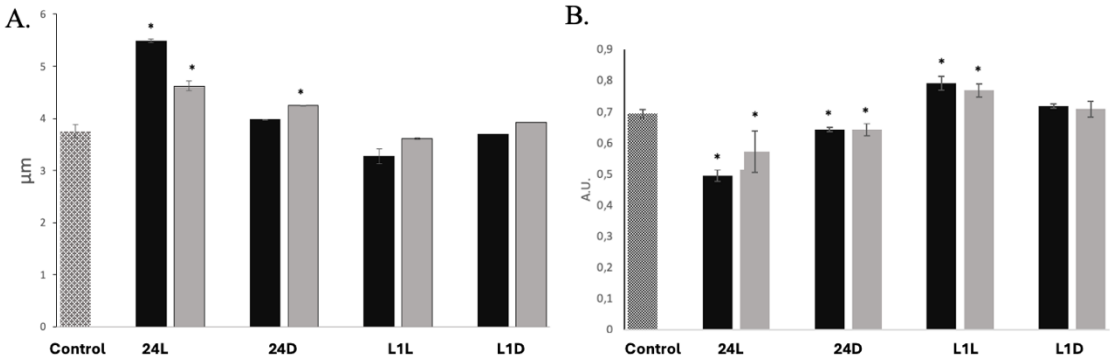


Figure 36. The effect of *de novo* LCAMPs on *E.coli ATCC 25922* cell shape.

½ MIC (black) and ¼ MIC (grey). (A). Length of bacterial cells treated with LCAMPs. (B). Circularity of bacterial cells treated with LCAMPs. * P value <0.05.

4.2.4. *De novo* LCAMPs promotes blebbing and inner membrane perturbations in RFP – expressing *E. coli* cells.

Since bacterial blebs detach from the cell surface in a time-dependent manner, bacterial cells were immobilized by encapsulating in LMA (as described in the Materials and Methods section). This approach allows newly formed vesicles to remain in the vicinity of the bacterial cells even after detachment. For these experiments, *E. coli K12 DH5α* with a cytoplasmic expression of RFP was used. Therefore, vesicles (if any) produced as a result of inner membrane disruption could be detected under a fluorescent microscope as the red fluorescence of cytoplasmic RFP leaked into vesicles. As shown in Fig. 37 A,B, all the tested LCAMPs (L1L, L1D, 24L and 24D) and Temporin-L induced blebs with strong red fluorescence. The maximum percentage of blebbed cells −53% was observed for L1D, while the minimum – 12%, was observed for Temporin-L. In untreated cells, the percentage of blebbed cells never exceeded 1%. Blebbing was accompanied with the overall decreased fluorescence intensity of *E. coli* cells pointing to the leakage of cytoplasmic content. In order to test whether ROS was involved in the generation of blebs, bacterial cells were treated with LCAMPs in the presence of TU, which is known to possess a radical scavenging activity [392]. As shown in Fig. 37 A, TU significantly reduced the percentage of blebbed cells for all the tested LCAMPs but not for Temporin-L.

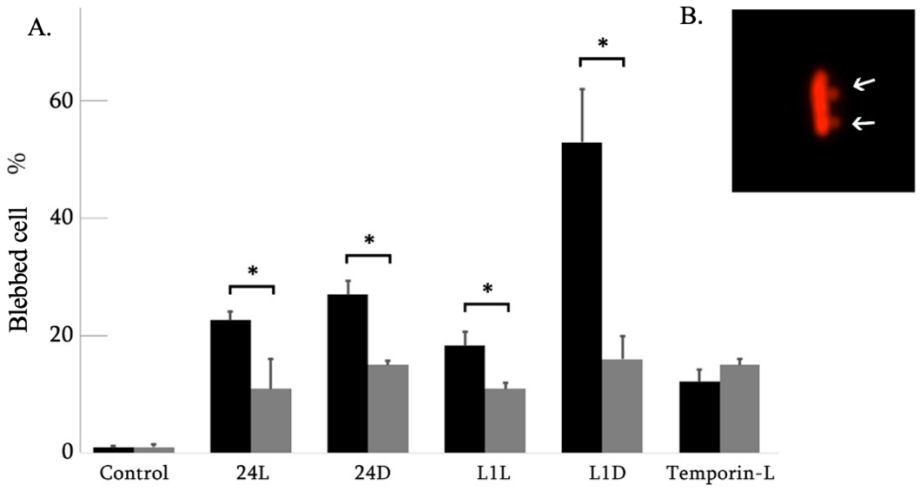


Figure 37. LCAMPs promote ROS-dependent blebbing of RFP-expressing *E. coli K12 DH5α* cells, accompanied by the leakage of cytoplasmic content.

(A) Percentage of blebbed cells after 10 min treatment with LCAMPs or Temporin-L in the absence (back) or presence (gray) of TU. The results are the means of at least 3 independent experiments. Error bars indicate the standard error of the mean. * ($p < 0.05$). (B) Representative image of blebbed RFP-expressing *E. coli K12 DH5α* cell.

4.2.5. *De novo* LCAMP - 24D impairs bacterial cell division.

Anti-FtsZ antibody was used to monitor the formation of the Z-ring, the structure indispensable for septum synthesis and post-divisional separation of newly formed bacteria. The cells treated with 24D at ½ MIC lacked an assembled FtsZ-ring and showed dispersed FtsZ signals inside the bacterial cell (Fig. 38 A,B). The rest of the LCAMPs didn't induce any significant impairments in the Z-ring compared to control cells.

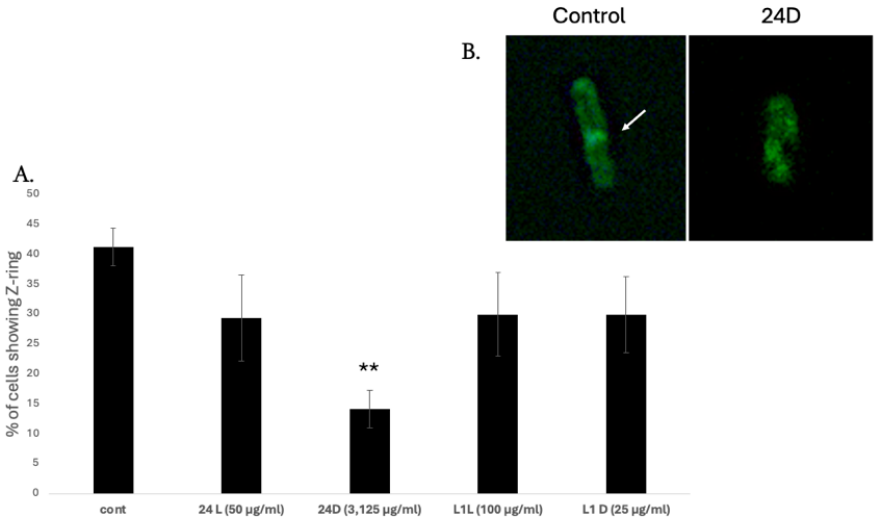


Figure 38. The effect LCAMPs on FtsZ ring formation in *E.coli ATCC 25922* cells.

A. Percentage of cells showing intact Z-ring after treatment with LCAMPs at their ½ MICs. ** P value < 0.01.
B. Representative image of normal and impaired Z-ring (an arrow pointing Z-ring).

4.2.6. **Interaction of peptides with the plasmid DNA.**

Since recent evidence suggests that LCAMPs have a multi-target mechanism of action [142] and can interact with multiple anionic targets, such as DNA [267,363,393,394], we examined the ability of *de novo* LCAMPs to interact with DNA by employing EMSA. As shown in Table 13 and Fig. 39, peptides 24L, 24D, and L1L completely inhibited the migration of pUC18 plasmid DNA through the agarose gel at a molar ratio of 1:1000, while at a molar ratio of 1:500, these AMPs only partially retarded DNA migration. The majority of the rest of the LCAMPs partially retarded DNA migration through the gel at a molar ratio of 1:1000.

Peptides	**DNA : Peptide**	
	1:1000	**1:500**
24L	**F**	**P**
24D	**F**	**P**
L1L	**F**	**P**
L1D	**P**	**-**

Table 13. Electro-mobility shift assay (EMSA) results of LCAMPs at two different DNA: peptide molar ratios.

F – full retardation; P – partial retardation; N – no retardation.

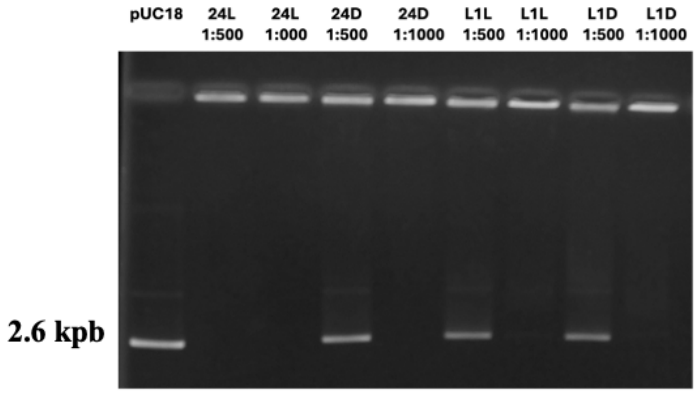


Figure 39. Representative image of EMSA gel for pUC 18 DNA incubated with LCAMPs at different molar ratios.

4.2.7. **LCAMPs induce DSB formation in *E. coli* genomic DNA.**

To study the ability of LCAMPs to induce DSBs in bacterial genomic DNA, we selected LCAMPs with different DNA-binding properties.

Live *E. coli* ATCC 25922 cells encapsulated in 1% LMA blocks were treated with peptides (24L, 24D, L1L, L1D, ST1L, LCAPL2, and LCAPL8) or conventional antibiotics (levofloxacin and ampicillin) as described in the Materials and Methods section. As shown in Fig. 40 A, the pattern of background DNA damage in the cells treated with ampicillin did not differ from that of control samples, however, levofloxacin induced the generation of DNA fragments of approximately 50 kbp. A similar but less intense DNA damage pattern was noticed for peptides 24L, L1D, and ST1L. For 24D, L1L, LCAPL2, and LCAPL8 ~50 kbp fragments were barely detected. To determine whether the generation of ROS was responsible for observed DSB formation, we compared DNA fragmentation patterns of LCAMP-treated samples in the presence or absence of TU. As depicted in Fig. 40 B, TU did not significantly affect the formation of DNA fragments.

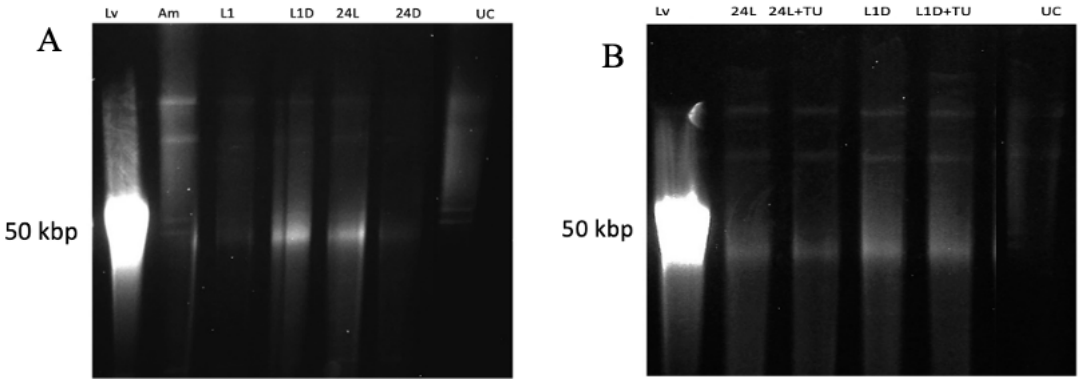


Figure 40.LCAMPs induce ROS-independent DSBs in genomic DNA of *E. coli ATCC 25922.*

(A) LMA-encapsulated cells were treated with LCAMPs (100 µg/mL), levofloxacin (0.5 µg/mL), or ampicillin (50 µg/mL) (see Materials and Methods section). (B) LMA-encapsulated cells were treated with 24L and L1D, in the absence or presence of 100 mM TU. Lv-levofloxacin; Am - ampicillin; TU-thiourea; UC- untreated cells. DSB formation was monitored by PFGE.

Chapter 5 : Discussion.

Antibiotic resistance represents one of the major threats to humanity. Among numerous strategies applied to tackle this problem, AMPs have the leading role due to their rapid and diverse mechanisms of action and low probability of resistance development. However, their broad clinical application is limited due to low stability, toxicity, and high manufacturing costs. With the development of QSAR computational models, it became possible to design novel AMPs with desired/improved biological properties in order to circumvent the above-listed problems. However, there is limited data regarding the mechanisms of action of *in silico*-designed *de novo* AMPs. Extensive *in vitro* testing is needed to explore and understand their mechanisms of action against target bacterial species. This, in turn, will contribute to the optimization of AMP predictive algorithms and force the development of effective AMPs.

In this work, we studied the biological properties of *in silico* generated *de novo* LCAMPs predicted to be active against *E.coli ATCC 25922* strain and to possess low hemolytic activity. At the initial stages of research antibacterial activity (alone or in combination with conventional antibiotics), toxicity, and stability to proteases were evaluated. De novo LCAMPs revealing the most promising therapeutic qualities such as antibacterial activity, low toxicity, and stability towards proteases) were further investigated for the mechanisms of action underlying their antibacterial properties.

Antibacterial susceptibility experiments have shown that all 10 *in silico* generated *de novo* LCAMPs and D-enantiomers of 3 of them, are able to inhibit the growth *E.coli ATCC 25922* strain. Almost all tested *de novo* LCAMPs reveal their full antimicrobial potency after 1 hour of incubation with bacterial cells. This can be explained by the fact that at MIC concentrations, the mechanisms of action of *de novo* LCAMPs mostly involve the direct disruption of bacterial membrane integrity [142,395,396], while for commercial antibiotics it takes time to reach and interact with their targets. As expected, all D-enantiomers of the tested *de novo* LCAMPs have shown lower MICs compared to their L-variants, explained by the protease stability of D-variants.

In our experiments, almost all LCAMPs have shown similar efficacy to inhibit the growth of dividing and non-dividing *E.coli ATCC 25922* cells. These results provide evidence that *de novo*, LCAMPs target cellular structures that are not significantly altered depending on the bacterial cell division state and/or are membrane-active compounds. Previously it was shown that conventional antibiotics are most effective when bacteria are actively dividing [397], while AMPs reveal similar activities against both dividing and non-dividing bacteria [398].

Interestingly, 24D was the only *de novo* LCAMP showing decreased antimicrobial activity against non-dividing bacteria. It was therefore proposed that 24D might be exerting its antimicrobial potential (at least partially) through targets available only in actively dividing cells.

Together with having antibacterial potency *de novo* LCAMPs used in this study were also predicted to be non-toxic against human erythrocytes. However, five (L8, L9, L10, L14, and ST-1L) *de novo* LCAMPs out of 12 appeared to be hemolytic at their MICs (at a standard inoculum density). One possible reason behind the relatively high discrepancy between predicted and experimentally obtained results can be explained by the limited amount of data on hemolytic activity against human erythrocytes. The development of predictive models requires a large collection of data. Since the amount of data for antimicrobial activity of AMPs is prevailing over the amount of data regarding the hemolytic activity of AMPs, the antimicrobial activity of *de novo* LCAMPs predicted by APP was more accurate in comparison to hemolytic activity. Interestingly, most *de novo* LCAMPs (except L8) at their MICs appeared non-cytotoxic against mammalian cell culture. The discrepancies between hemolytic and cytotoxic activities might be explained by the difference in membrane compositions of erythrocytes and murine Hepa 1-6 cells. It is known that, compared to the tissue cells, the erythrocyte membrane is characterized by a higher content of negatively charged glycoproteins [399], which might result in better adhesion of positively charged LCAMPs.

Synergy of AMPs with commercially available antibiotics represents a promising approach to lower AMPs' toxicity and reduce effective concentrations of antibiotics. Although numerous papers have described the ability of AMPs to synergize with conventional antibiotics [277,400,401], to the best of our knowledge, there is no data describing the effect of bacterial inoculum density on the combinatorial activity of both types of these antimicrobials. In our experiments, bacterial inoculum density had little effect on the activity of *de novo* LCAMPs, however we observed a more prominent effect of bacterial cell density on the ability of LCAMPs to synergize with antibiotics. Most synergistic interactions in this study were observed at a standard inoculum density of $5x10^5$ CFU/mL, while at low inoculum, synergy was only observed for ampicillin in combination with LCAPL2 or ST1L. The mechanisms underlying this phenomenon are yet to be uncovered. Overall, three *de novo* LCAMPs (24L, L1, and L1D) revealed synergy with ampicillin, gentamicin, and levofloxacin, suggesting that these LCAMPs might possess similar modes of action, most probably involving the distortion of the bacterial cell envelope, making it easier for antibiotics to reach their targets.

This assumption is in line with our finding that, out of 22 peptide-antibiotic combinations that showed a potentiation effect, in about 82% of cases, the activity of the antibiotic was potentiated

by the peptide. Interestingly, all tested LCAMPs showed the ability to potentiate an antibiotic in at least one combination. Earlier the potentiating properties of antimicrobial peptides were reported for several peptide/ peptidomimetic-antibiotic combinations (e.g. a combination of daptomycin with ampicillin) [401,402]. It can be concluded that *de novo* peptides used in this work have the potential to enhance the activity of ampicillin, gentamicin, and levofloxacin against *E. coli ATCC 25922* strain depending on particular favorable conditions, such as bacterial cell density.

In order to investigate the mechanism of action of LCAMPs on the bacterial envelope, we selected 3 LCAMPs (24L, L1L, L1D), revealing synergy with commercial antibiotics and D-enantiomer of 24L (showing low activity against non-dividing bacteria as discussed above).

FITC-labeled variants of all selected *de novo* LCAMPs were used to study their localization in bacterial cells. All *de novo* LCAMPs were shown to preferentially localize along bacterial cell surface, however the presence of IBs substantially changes this localization pattern. Since IBs are localized in the proximity of the regions with altered membrane curvature [59,60], the uptake of LCAMPs might be easier at these specific sites, allowing IBs to absorb harmful substances (peptides, in this case) and lower their overall concentration for the rest of the cells thus promoting their survival. Indeed, recent reports suggest that protein aggregates help bacteria to cope with proteotoxic stresses and improve survival during antibiotic exposure [383,403]. Further research is needed to understand the mechanisms and outcomes of IB-LCAMP interactions. Also, all the selected *de novo* LCAMPs were able to induce bacterial cell blebbing. The highest percentage of blebbed cells was observed for L1D-treated samples. It should be noted that bacterial blebbing is often the result of structural changes in a bacterial envelope and does not necessarily always lead to bacterial death but helps bacteria to cope with stressful conditions [251]. Bacterial cell blebbing was previously described for several natural antimicrobial peptides [251,260]. For example, synthetic cathelicidin BM 22 was reported to induce the generation of reactive oxygen species by disrupting the membrane-bound aerobic respiratory electron transport chain, leading to the accumulation of ROS and bleb formation. ROS scavenger TU reduced cellular ROS levels and overcame these bactericidal effects [260]. In our experiments, treatment with TU indeed resulted in a partially decreased percentage of blebbed cells indicating that mechanisms (other than ROS) might also be involved in the generation of blebbs.

Although all four selected *de novo* LCAMPs have shown the same pattern of interaction with bacterial membranes, differences in their abilities to induce anionic phospholipid redistributions in bacterial envelope were observed. Among four *de novo* LCAMPs, the lowest percentage of

cells with phospholipid redistribution was observed for 24D (6%), indicating that these *de novo* LCAMP is less membrane active compared to 24L (~25%) L1L (~76%) and L1D (~80%).

Apart from bacterial envelope destabilization, the selected *de novo* LCAMPs were shown to induce changes in bacterial cell shapes. Elongated phenotypes were observed for 24 L and 24D but not for L1L and L1D.

Elongated phenotypes often indicating on the disruption of cell division were previously reported for several antimicrobials (including AMPs) [284], therefore it was decided to study the ability of *de novo* LCAMPs to impair this process. In our immunochemistry experiments we investigated the effect of *de novo* LCAMPs on the formation of Z-ring -a key structure for bacterial cytokinesis [284]. It was shown that 24 D was the only peptide impacting the formation of Z-ring in *E.coli ATCC 25922* pointing to its alternative mechanisms of inhibiting bacterial cell growth compared to 24L, L1L and L1D. Several other observations confirm this suggestion. Firstly, 24 D has revealed the lowest MIC (3.125μg/mL) among all tested *de novo* LCAMPs. It is possible that such low concentration may not be enough to induce dramatic membrane disruption but rather be enough to allow the peptide pass through the bacterial membrane, inducing minimal perturbations and then interact with inner targets. Secondly, (in contrast to the rest tested de no LCAMPs) 24D showed reduced antimicrobial activity against non-dividing cells – indicating the absence of its target. Thirdly, 24D does not reveal synergy with commercial antibiotics, which is in agreement with the hypothesis that preferentially membrane-active AMPs are able to potentiate antibiotics by enhancing their uptake through bacterial membrane perturbations. Indeed, synergy was observed only for 24L, L1L, and L1D, showing more prominent membrane perturbations compared to 24D. Finally, in contrast to L1D, 24D didn't show hemolytic activity, (at any tested concentrations) which is often directly linked to membrane perturbing properties of AMPs [404].

Altogether, these observations suggest that 24D, is a less membrane-active peptide and most likely exerts its antimicrobial activity through the disruption of inner bacterial targets (such as Z-ring), while 24L, L1L and L1D being membrane-active, reveal antimicrobial activity through the direct distortions in bacterial membrane resulting in synergistic interactions with commercial antibiotics (Table 14).

The reasons underlying changes in the mode of action of 24D compared to its L-variant require further investigations. However, it can be proposed that stability to proteases and stereochemistry are among the factors affecting the mechanism of action of this peptide. Previously, an

observation that AMPs containing D-amino acids may act on bacterial cells differently from their L-forms was reported [405,406].

In the subsequent experiments, it was shown that 24L, 24D, L1L, and L1D form complexes with plasmid DNA however only 24L and L1D induce DSBs in bacterial chromosomal DNA, resulting in the generation of DNA fragments of a similar length as those produced by gyrase inhibitor levofloxacin [407]. However, the formation of ~50 kbp DNA fragments was not affected by the presence of TU and, also, it did not show any correlation with the ability of LCAMPs to form complexes with DNA. Our results propose that the DSBs are neither a result of a direct peptide-DNA interaction nor the consequence of ROS. Instead, the observed DSB formation may be due to either the direct inhibition of DNA replication machinery leading to the formation of DSBs or the impaired osmoregulatory capacity of *E. coli* cells, resulting in structural changes to the genome that hinder DNA replication [408]in a manner similar to the mechanism of action of the replication-targeting antibiotic levofloxacin [407].

Overall, all four *de novo* LCAMPs, with their differences and similarities, undoubtedly show remarkable antibacterial properties that require further research in order to fully evaluate their antibacterial potential.

Effect	24L	24D	L1L	L1D
Reduced activity against dividing cells	-	+	-	-
Ability to act synergistically with antibiotics	+	-	+	+
Phospholipid redistribution	++	+	+++	+++
Elongation	++	+	-	-
Circularization	-	-	+	-
Bleb formation	+	+	+	+++
FtsZ ring assembly impairment	-	++	-	-

- No effect
+ Weak effect
++ Moderate effect
+++ Strong effect

Table 14. Summary of *de novo* LCAMP-induced effects on bacterial cell.

Chapter 6 : Conclusion.

The present study aimed to investigate the biological properties of *in silico* generated *de novo* LCAMPs in the context of exploring potential alternatives to commercial antibiotics to fight antimicrobial resistance.

All *de novo* LCAMPs used in this work are fast-acting antimicrobial agents with low cytotoxicity and moderate hemolytic activity. Their antimicrobial potency is not dramatically affected by a 100-fold change in bacterial cell densities or the ability of bacteria to divide. The latter represents a promising advantage of *de novo* LCAPMs over commercially available antibiotics, becoming less effective against slow-growing/dormant bacterial cells present during persistent infections.

De novo LCAMPs are able to synergize with commercial antibiotics, thus showing a promising strategy to reduce effective concentrations of both antimicrobials. However, we have also shown that this ability is strongly affected by bacterial inoculum density. More studies are needed to shed light on this phenomenon.

The substitution of L-amino acids with D-variants not only leads to increased protease stability but might also impact the ability of *de novo* LCAMPs to synergize with antibiotics and also affect their mechanisms of action. This work represents a comprehensive analysis of the biological properties of *de novo* LCAMPs, however each aspect of their properties needs further detailed investigation.

Altogether these findings will contribute to a better understanding of the unique properties of LCAMPs and help the research community in this field to optimize future investigations of antibacterial activity of LCAMPs alone or in combination with commercially available antibiotics and thus develop optimal strategies to combat antibiotic resistance.

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