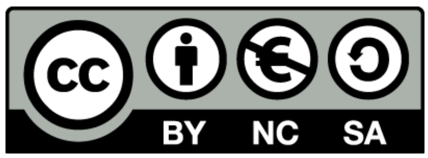
Synthetic Polymyxin-based Peptides Against Multidrug Resistant Bacteria: A Therapeutic Option

Héctor Rudilla Mateo



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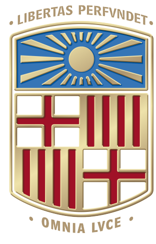

Department of Pathology and Experimental Therapeutics

Laboratory of Molecular Microbiology and Antimicrobials

Faculty of Medicine and Health Sciences

University of Barcelona

PhD Thesis

Synthetic Polymyxin-based Peptides Against Multidrug Resistant Bacteria: A Therapeutic Option

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Doctorate in Medicine

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C E R T I F I C A N,

Que la tesis doctoral firmada por el licenciado **Héctor RUDILLA MATEO**, y titulada "*Synthetic polymyxin-based peptides against multidrug resistant bacteria: a therapeutic option",* ha sido realizada en el laboratorio de microbiología molecular y antimicrobianos del departamento de patología y terapéutica experimental de esta facultad bajo nuestra dirección.

Que se autorizó su presentación a la Comisión del programa de doctorado *"Medicina i Recerca Translacional"* en fecha 21 de octubre de 2018.

Que la tesis cumple a nuestro juicio los requisitos necesarios formales y conceptuales para optar al título de doctor por la Universidad de Barcelona.

Y para que conste, firman el presente en l'Hospitalet de Llobregat a 23 de octubre de 2018.

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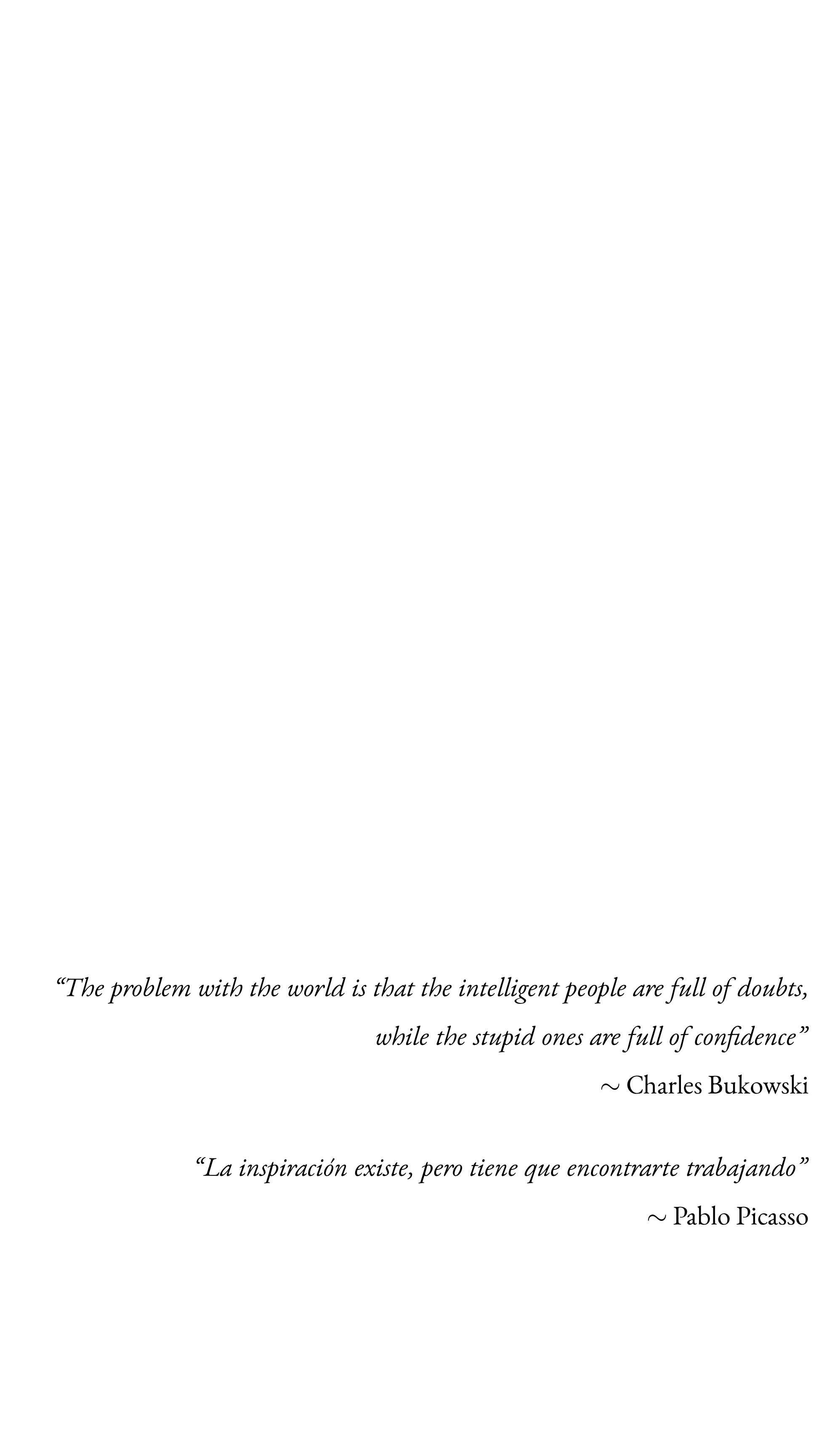

"The problem with the world is that the intelligent people are full of doubts,
while the stupid ones are full of confidence"
∼ Charles Bukowski

"La inspiración existe, pero tiene que encontrarte trabajando"
∼ Pablo Picasso

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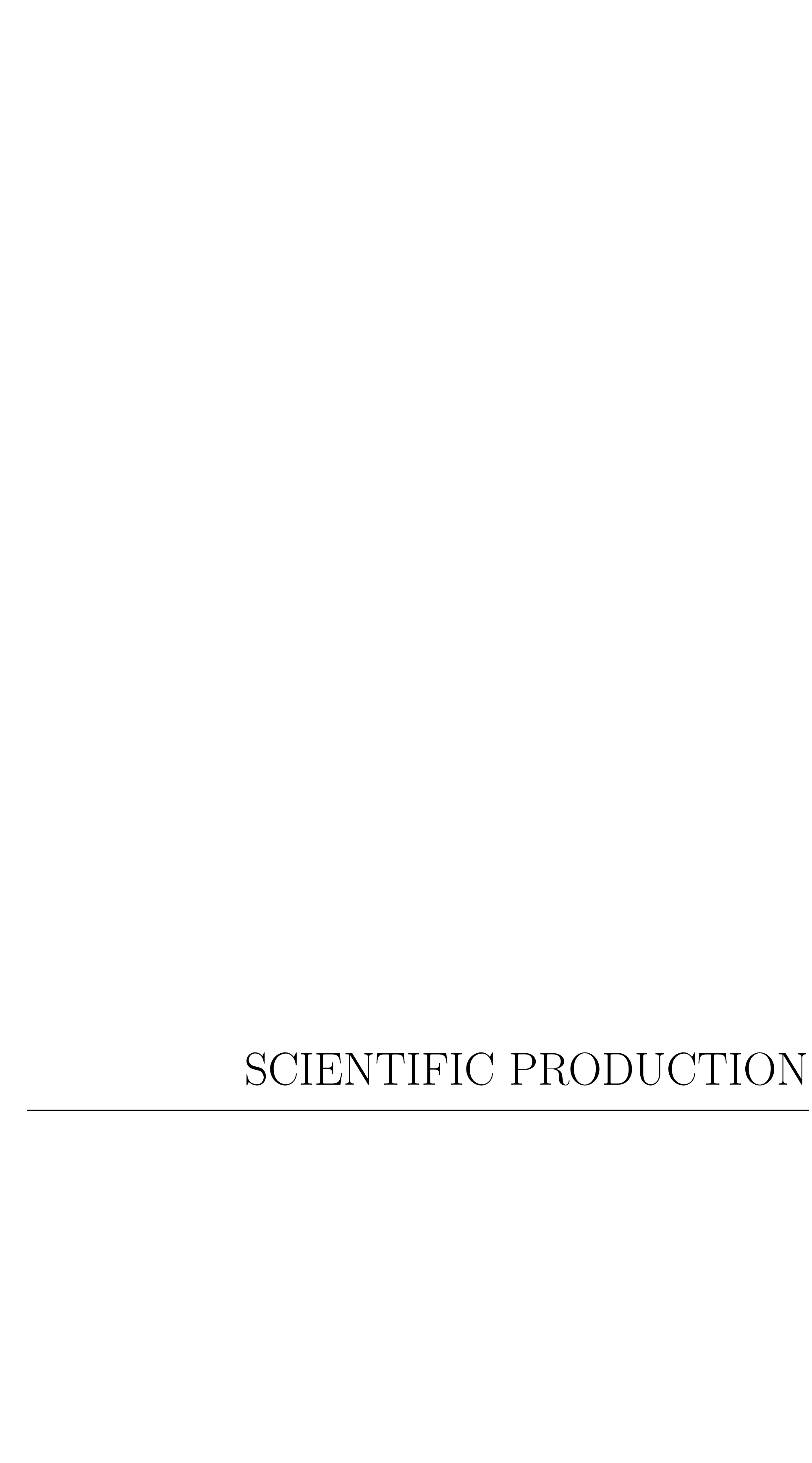


SCIENTIFIC PRODUCTION

The three years period employed in this PhD thesis has allowed the participation in research projects which production is presented in the following list:

Publications in international peer-reviewed journals:

1. **Rudilla H**, Fusté E, Cajal Y, Rabanal F, Vinuesa T, Viñas M. Synergistic Antipseudomonal Effects of Synthetic Peptide AMP38 and Carbapenems. **Molecules** 2016; 21: 1223. **Impact Factor** according to 2017 Journal Citation Reports released by Thomson Reuters (ISI) is **3.098**

2. **Hector Rudilla**, Alexandra Merlos, Eulalia Sans-Serramitjana, Ester Fuste, Josep M. Sierra, Antonio Zalacain, Teresa Vinuesa, and Miguel Vinas. New and old tools to evaluate new antimicrobial peptides. **AIMS Microbiology** 2018, 4(3):522-540.

3. **Rudilla H**, Pérez-Guillén I, Rabanal F, Sierra JM, Vinuesa T, Viñas M. Novel synthetic polymyxins kill Gram-positive bacteria. **Journal of Antimicrobial Chemotherapy** 2018: 6–11. **Impact Factor** according to 2017 Journal Citation Reports released by Thomson Reuters (ISI) is **5.217**

Congress participations:

1. **Héctor Rudilla**, Ester Fusté, Francesc Rabanal, Guadalupe Giménez Galisteo, Teresa Vinuesa Aumedes. Anti-pseudomonal activity of a new antimicrobial peptide. XXV Congreso Nacional de Microbiología. 7-10 July 2015. Logroño, Spain.

2. Miguel Viñas, **Héctor Rudilla**, Francesc Rabanal, Teresa Vinuesa. AMP38 a synthetic peptide promoting the imipenem penetration in imipenem resistant *Pseudomonas aeruginosa*. IMI Translocation meeting 2015 - Molecular basis of antibiotic permeability in Gram-negative bacteria. 11-15 July 2015. Bremen, Germany.

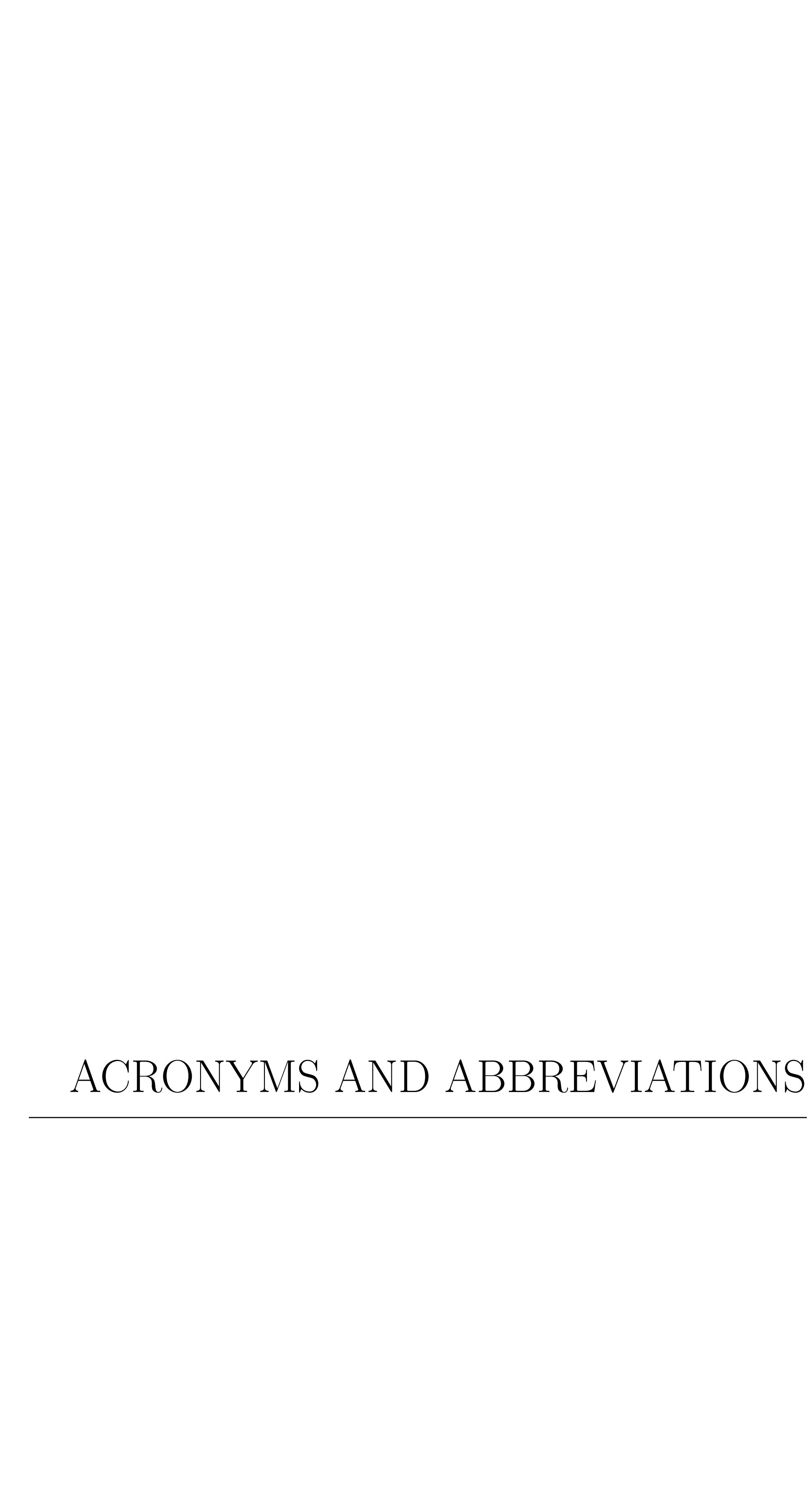
3. Viñas M, **Rudilla H**, Fusté E, Rabanal F, Cajal Y, Vinuesa T. Synergistic effects of synthetic peptides and Carbapenems. IMI Translocation meeting 2016 - Novel approaches to fight bacteria. 10-14 July 2016. Bremen, Germany.

4. Armengol E, Sierra JM, **Rudilla H**, Herráez R, Jiménez G, Betancourt P, Vinuesa T and Viñas M. Antipseudomonal activity of free and nano-encapsulated tobramycin. IMI Translocation meeting 2016 - Novel approaches to fight bacteria. 10-14 July 2016. Bremen, Germany.

5. **H. Rudilla**, E. Fusté, S.S. Eulalia, F. Rabanal, T. Vinuesa, M. Viñas. Antimicrobial synthetic cyclopeptides activity on *Staphylococcus aureus*. Microbiology and Infection 2017 - 5th Joint Conference of the DGHM & VAMM. 5-8 March 2017. Würzburg, Germany.

6. **Héctor Rudilla**. Infections caused by *Pseudomonas aeruginosa*. Antimicrobial peptides: new therapeutic approaches. Eurolife Summer School. "Antimiceobial drug resistance-Research and Innovation". July 2017. Barcelona, Spain.

7. J. Sierra, A. Merlos, E. Sans-Serramitjana, **H. Rudilla**, R. Herraez-Moral, G. Jimenez-Galisteo, E. Fusté. Atomic force microscopy for the study of novel antimicrobial agents in biofilms eradication. 28th ECCMID European Congress Of Clinical Microbiology and Infectious Diseases. 21-24 April 2018. Madrid, Spain.



ACRONYMS AND ABBREVIATIONS

°C:	Celsius degrees
μg:	Microgram (10^{-6} grams)
μm:	Micromole (10^{-6} moles)
ADP:	Adenosine diphosphate
AIDS:	Acquired immune deficiency syndrome
AMEs:	Aminoglycoside-modifying enzymes
ANT:	Aminoglycoside nucleotidyl transferase
APH:	Aminoglycoside phosphoryl transferase
ATCC:	American Type Culture Collection
Bap:	Biofilm-associated protein
BCC:	*Burkholderia cepacia* complex
CAMP:	Cationic antimicrobial peptide
CA-MRSA:	Community-associated methicillin-resistant *Staphylococcus aureus*
CF:	Cystic fibrosis
CFTR:	Cystic fibrosis transmembrane conductance regulator
CFU:	Colony-forming unit
ClfA:	Clumping factor A
ClfB:	Clumping factor B
COPD:	Chronic obstructive pulmonary disease
DMF:	Dimethylformamide
DNA:	Deoxyribonucleic acid

Eap:	Extracellular adherence protein
eDNA:	Extracellular deoxyribonucleic acid
ENaC:	Epithelial sodium channel
ESBL:	Extended-spectrum β-lactamase
ESKAPE pathogens:	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii, Pseudomonas aeruginosa*, *Enterobacter* spp.
et al:	and others
ExoA:	Exotoxin A
ExoS:	Exoenzyme S
ExoT:	Exoenzyme T
ExoU:	Exoenzyme U
ExoY:	Exoenzyme Y
FICi:	Fractional inhibitory concentration index
Fmoc:	Fluorenylmethyloxycarbonyl
FnBPA and FnBPB:	Fibrinogen-binding proteins A and B
HlB:	β-hemolysin
HPLC:	High performance liquid chromatography
hVISA:	Heterogeneous VISA
IC50:	Half maximal inhibitory concentration
IgA:	Immunoglobulin A
IgG:	Immunoglobulin G
ISPa:	Insertion sequence *Pseudomonas aeruginosa*

ITC:	Isothermal titration calorimetry
LPS:	Lipopolysaccharide
MALDI-TOF:	Matrix-assisted laser desorption/ionization time-of-flight
MBEC:	Minimum biofilm eradication concentration
MDR:	Multidrug resistant
MIC:	Minimum inhibitory concentration
mL:	Milliliter
MLST:	Multilocus sequence typing
MoA:	Mechanism of action
MoR:	Mechanism of resistance
MRSA:	Methicillin-resistant *Staphylococcus aureus*
MSSA:	Methicillin-sensitive *Staphylococcus aureus*
OprD:	Outer membrane porin D
P. aeruginosa:	*Pseudomonas aeruginosa*
PBPs:	Penicillin-binding proteins
PFGE:	Pulsed-field gel electrophoresis
PIA:	Polysaccharide intercellular adhesin
PMN:	Polymorphonuclear leukocytes
PSM:	Phenol-soluble modulin
PVL:	Panton–valentine leukocidin
QRDR:	Quinolone resistance determining region
QS:	Quorum sensing

rRNA:	Ribosomal ribonucleic acid
S. aureus:	*Staphylococcus aureus*
SasG:	*S. aureus* surface protein G
SCCmec:	Staphylococcal cassette chromosome mec
SCVs:	Small colony variants
TD-SCV:	Thymidine auxotrophic small-colony variants
TEM:	Transmission electron microscopy
TMP/SMX:	Trimethoprim/sulfamethoxazole
USA:	United States of America
VISA:	Vancomycin intermediate resistance *S. aureus*
VRSA:	Vancomycin resistance *S. aureus*
vWF:	Von Willebrand Factor
WHO:	World Health Organization

ABSTRACT

Pseudomonas aeruginosa and *Staphylococcus aureus* are nosocomial opportunistic pathogens causing a wide variety of both acute and chronic infections, such as pneumonia, bacteraemia, and urinary tract infections. Immunocompromised patients and those suffering cystic fibrosis show a particularly high susceptibility to infection by these microorganisms. Moreover, the increasing frequency of the isolation of multidrug-resistant bacteria (MDR) is a major cause for concern.

Polymyxins are cyclic peptides with antimicrobial action against Gram-negative bacteria that have been available since 1949, although they were left largely unused during the seventies because of their nephrotoxicity and the availability of less toxic antimicrobials to which bacteria had not yet developed resistance. The most known polymyxin is colistin; like other cationic polypeptides, colistin is an amphipathic compound and, it is believed that this amphipathic nature is relevant to its activity against bacteria.

Hence, the aim of this thesis was to synthesize antimicrobial peptides inspired in colistin scaffold and explore their antimicrobial activity against multidrug resistant bacteria such as *P. aeruginosa* and *S. aureus*, determine possible synergistic interactions with commercial

antibiotics and explore their mechanisms of action.

Synthesis The main attempt in this first part was to synthesize peptides in solid phase by the Fmoc/tBu method. After synthesis, peptides were purified by preparative HPLC method and finally, peptides were characterized by MALDI-TOF.

Antimicrobial activity This part focused on the study the antimicrobial capacity of our peptides against multidrug resistant bacteria, specially *P. aeruginosa* and *S. aureus*. First peptide (AMP38) showed an acceptable antimicrobial activity against *P. aureuginosa*. Moreover, several imipenem-resistant *P. aeruginosa* were tested with AMP38 and imipenem showing a quite considerable synergistic action, both with planktonic and sessile bacteria.

In addition, two peptides of the same family (CAMP113 and CAMP207) were tested against *S. aureus* (both planktonic and sessile) showing a surprising antimicrobial action since Gram-positive bacteria are regarding as naturally resistant to polymyxins. Moreover, these peptides showed an inordinately high selectivity index.

Mechanisms of action The final part of this doctoral thesis focused on an initial exploration of mechanisms of action of peptides above mentioned. Transmission electronic microscopy

(TEM) assays were performed in order to elucidate possible interactions between peptides and outer membrane of Gram-negative bacteria. In addition, isothermal titration calorimetry assays were carried out to determine peptide-teichoic acid interactions.

Data obtained from these studies are promising, being able to be a therapeutic alternative for infections produced by multidrug resistant bacteria.

RESUMEN

Pseudomonas aeruginosa y *Staphylococcus aureus* son patógenos nosocomiales oportunistas causantes de una gran variedad de infecciones tanto crónicas como agudas, tales como neumonía, bacteriemia e infecciones del tracto urinario. Los pacientes inmunocomprometidos y aquellos que padecen fibrosis quística muestran una susceptibilidad particularmente alta a infectarse por estos microorganismos. Además, la mayor frecuencia de aislamientos de *P. aeruginosa* y *S. aureus* resistentes a múltiples fármacos (MDR) es una causa importante de preocupación.

Las polimixinas son péptidos cíclicos con capacidad antibiótica contra las bacterias Gram-negativas que han estado disponibles desde 1949, aunque se dejaron en gran parte de usar durante los años setenta debido a su nefrotoxicidad y a la disponibilidad de otros antimicrobianos menos tóxicos a los cuales las bacterias aún no habían desarrollado resistencias. La polimixina más conocida es la colistina e, igual que otros polipéptidos catiónicos es un compuesto anfipático. Se cree que esta naturaleza anfipática es relevante en su actividad contra las bacterias.

Por lo tanto, el objetivo de esta tesis fue sintetizar péptidos antimicrobianos inspirados en el esqueleto molecular de la colistina y explorar su actividad antimicrobiana contra bacterias resistentes

a múltiples fármacos como *P. aeruginosa* y *S. aureus*, determinar posibles interacciones sinérgicas con antibióticos comerciales y realizar un primer acercamiento a sus mecanismos de acción.

Síntesis

El principal objetivo de esta primera parte fue sintetizar los péptidos en fase sólida por el método Fmoc/tBu. Después de la síntesis, los péptidos se purificaron por el método de HPLC preprativo y, finalmente los péptidos se caracterizaron por MALDI-TOF.

Actividad antimicrobiana

Esta parte se centró en el estudio de la capacidad antimicrobiana de nuestros péptidos contra bacterias multirresistentes, especialmente *P. aeruginosa* y *S. aureus*. El primer péptido (AMP38) mostró una actividad antimicrobiana aceptable frente a *P. aeruginosa*. Además, se probaron varias cepas de *P. aeruginosa* resistentes a imipenem con AMP38 mostrando una actividad sinérgica bastante considerable, tanto en bacterias planctónicas como sésiles.

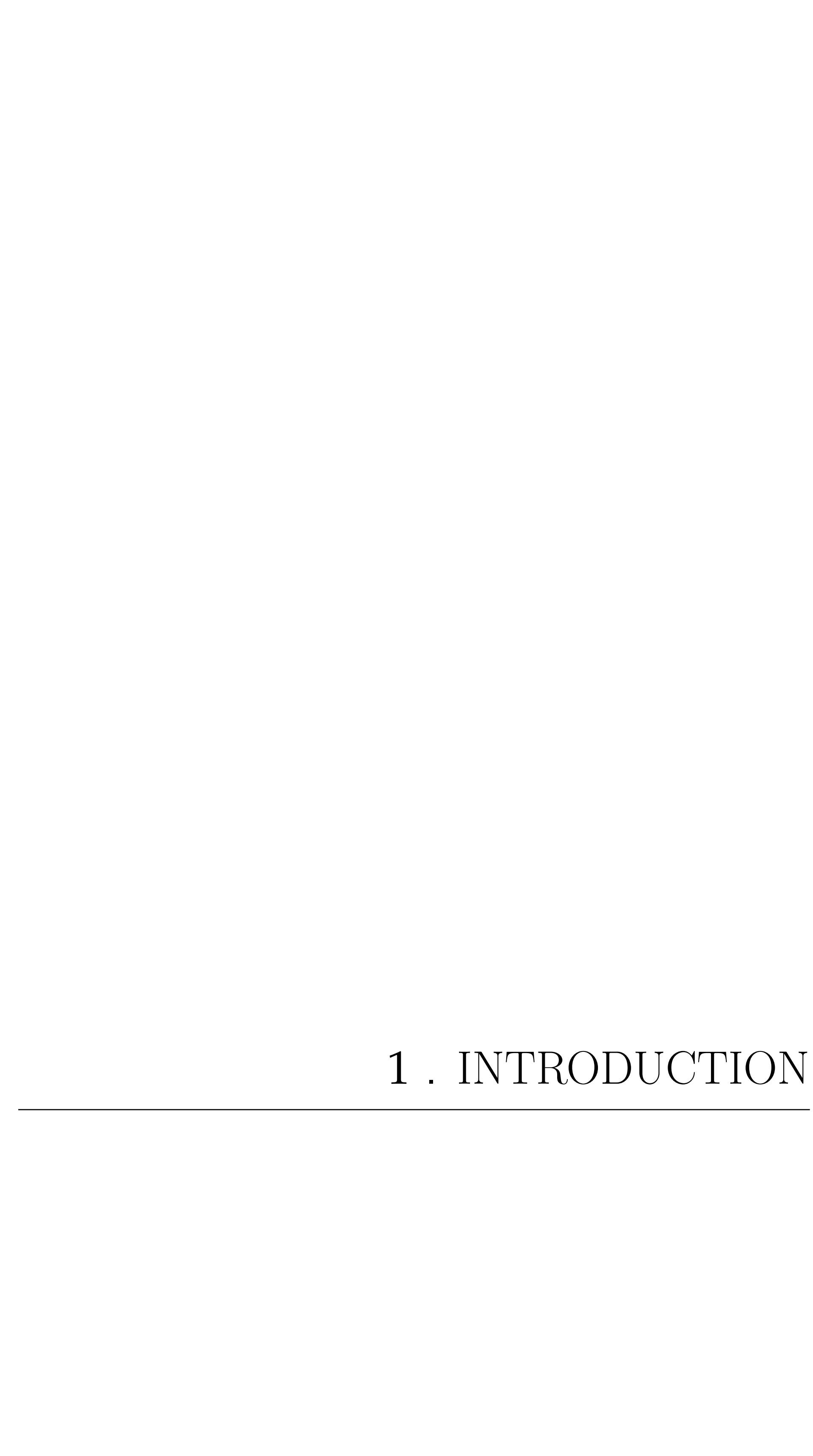
Adicionalmente, se realizaron ensayos con dos péptidos de la misma familia (CAMP113 y CAMP207) frente a *S. aureus* (tanto

planctónicos como sésiles) mostrando una acción antimicrobiana sorprendente, ya que las bacterias Gram-positivas como *S. aureus* se consideran naturalmente resistentes a las polimixinas.

Mecanismos de acción

La parte final de esta tesis doctoral se centró en una exploración inicial de los mecanismos de acción de los péptidos mencionados anteriormente. Se realizaron ensayos de microscopía electrónica de transmisión (TEM) para aclarar las posibles interacciones entre los péptidos y la membrana externa de las bacterias Gram-negativas. Además, se realizaron ensayos de calorimetría de titulación isotérmica para determinar las interacciones péptido-ácido teicoico.

Los datos obtenidos de estos estudios son prometedores, pudiendo ser una alternativa terapéutica para las infecciones producidas por bacterias resistentes a múltiples fármacos.



1 . INTRODUCTION

1.1 Multidrug-resistant bacteria

Bacteria are the leading cause of infections worldwide, although they may affect individuals in different ways particularly depending on social conditions, thus, in developed and third world countries bacterial behaviour is different. Most bacterial infections in the third world are those known as "classical", whereas in developed countries hospitalized patients or individuals who have undergone medical treatments, such as surgery, solid organ transplantation and anticancer treatments are the most vulnerable. AIDS patients are quite similar to the last ones. In recent years, the progressive increase in the incidence of multidrug-resistance bacterial infections has raised concern. In 2014, the office of the President Obama published a detailed report on antimicrobial resistance in the United States. Then, the president published on September 18, an Executive Order entitled "Combating Antibiotic-Resistant Bacteria".[1] This document included sections on new policies, changes in funding for research focusing this relevant public health challenge and recommendations, among others. Following the election of Donald Trump, more than 30 scientific, social alliances and scientific societies signed a document in which they expressed the hope these investments would be

maintained and even expanded. Also in 2014, The World Health Organization (WHO) published an extended report calling attention to this crucial health problem.[2] Bacterial resistance to antimicrobials has likewise attracted the attention of the government of several other countries, particularly the funding agency for European research.

Nevertheless, antibiotics research had an erratic history. The clinical use of antimicrobials started approximately in 1932, with the release of Prontosil (an antibacterial drug discovered at Bayer Laboratories, Germany), a molecule with a lethal effect on gram-positive cocci. Prontosil was the first sulfamide and it ushered in antibiotic era. Thereafter, new antibiotics were rapidly discovered, including penicillin and streptomycin, and became available for clinical use. Immediately research on antimicrobials grew exponentially since both scientific community and pharmaceuticals thought that infectious diseases may be erradicated in a relatively short time.

Recently, due mostly to economic, rather than to medical or scientific reasons, the pipeline of novel antimicrobial molecules under development has mostly closed.[3] Instead, most of the antimicrobial drugs under development are improved derivates of

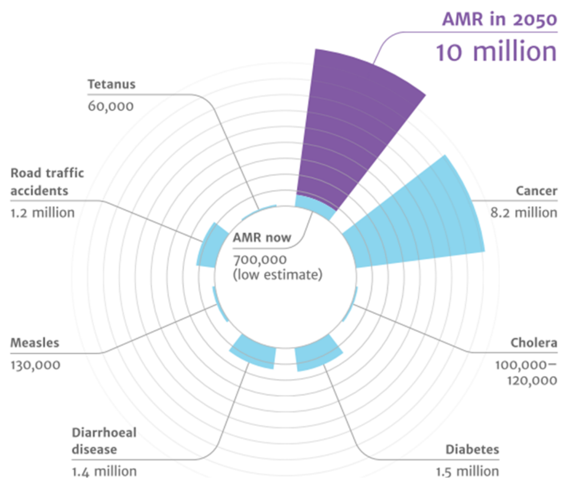


Figure 1.1: Causes of death in the year 2050[4]

those already on the market. This has had several non-negligible consequences. Firstly, modified compounds, while frequently enlarging the spectrum of drug activity or enhancing its antimicrobial effect, do not change the target, including the resistance mechanisms promoted in organisms exposed to these agents. New molecules, acting through newly recognized mechanisms of action and on different targets, are expected to be much more effective, but very few have been developed over the past several years. The recent emergence of new mechanisms of resistance, the increased level of knowledge on these mechanisms, and the scarcity of novel antimicrobial products able to target them account for current growing concern and the revival of research efforts.

The main sources of antibiotics discovered during the golden era of antibiotics were soil bacteria and fungi. In fact, antibiotics were long regarded as defence mechanisms of soil bacteria, although this function has yet to be definitively demonstrated in nature.[5] Although the further exploration of natural products for their antimicrobial activities is expensive and the chance of successes limited, the identification of not previously appreciated delivery methods or products, including those derived from natural molecules, will open up new research perspectives regarding antimicrobials.[6]

On the other hand, the World Health Organization (WHO) released for first time ever a list of the highest priority needs for new antibiotics.[7] This list encloses 12 bacterial species:

Priority 1: Critical

1. *Acinetobacter baumanii*, carbapenem-resistant
2. *Pseudomonas aeruginosa*, carbapenem-resistant
3. *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing

Priority 2: High

4. *Enterococcus faecium*, vancomycin-resistant
5. *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant
6. *Helicobacter pylori*, clarithromycin-resistant
7. *Campylobacter* spp., fluoroquinolone-resistant
8. *Salmonellae*, fluoroquinolone-resistant
9. *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant

Priority 3: Medium

10. *Streptococcus pneumoniae*, penicillin-non-susceptible
11. *Haemophilus influenzae*, ampicillin-resistant
12. *Shigella* spp., fluoroquinolone-resistant

The therapeutic options for these microorganisms are extremely inadequate and the physicians are forced to prescribe expensive drugs with significant side effects.[8]

Moreover, other group of pathogens to take into acount is the so-called ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter species*). The proportion of these bacteria in common urinary pulmonary, skin and bloodstream infections has increased notably in recent years.[9] Moreover, patients with vascular prostheses, indwelling catheters, dialysis shunts, etc. are particularly vulnerable to the virtually untreatable infections resulting from ESKAPE colonization and subsequent biofilm formation.[10]

1.2 Respiratory infectious diseases

1.2.1 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) encompasses various low-airways disorders including emphysema. Periods of acute bronchitis exacerbations with dyspnea, cough and purulent secretions are common in patients. These exacerbations episodes are due to infections in respiratory airways by bacteria, predominating three species: *Haemophilus influenzae, Streptococcus pneumoniae and Moraxella catarrhalis*.[11] Moreover, it is often finding other species such as

Pseudomonas aeruginosa and Staphylococcus aureus. This chronic presence of bacteria in COPD patients leads in a lower lung development in childhood, a chronic inflammatory response with lung damage and a hypersensitivity caused by bacterial antigens.[12]

In the following sections, it will be deepened into bacterial species.

1.2.2 **Cystic Fibrosis**

Cystic Fibrosis (CF) is an autosomal recessive disorder that affects 70,000 individuals worldwide, primarily to Caucasians (high prevalence in Europe, North America and Australia), although it has been reported in all races and ethnicities.[13] It is estimated that 1 out 5000 live births might be affected by CF in Europe, being it the main common autosomal recessive disease in Europe.[14] Lately, median age survival in developed countries has increased until 40 years old, however in third world countries it remains very low.[15]

The first scientist who described CF was Dorothy Andersen in 1938. She described CF as disease that affects pancreas, nevertheless subsequently it was associated with lung infection as

well.[16] The disorder usually appears as an exocrine pancreatic insufficiency, an increase in the Cl^- concentration in sweat, male infertility and airway illness that leads to progressive lung dysfunction, which is the major cause of morbidity and mortality.[17] Lung disease is characterized by chronic lung infection and inflammation, leading to irreversible lung damage and finally, to death.[18] Nowadays, lung transplantation is the lass-resort option treatment in order to cure CF patients.[19]

The malfunction is caused by mutations in a single gene on the long arm of chromosome 7 that encodes a chloride-conducting transmembrane protein (Cystic Fibrosis Transmembrane Regulator, called CFTR).[20] The main defect in CFTR channel involves chloride and bicarbonate transport, besides CFTR interacts with other ion channel such as the epithelial sodium channel (ENaC) and also with another cellular pathways related to inflammation.[21] Airway mucus is also dependant on the presence of bicarbonate for normal function and reduced anion concentrations, increasing viscosity in that mucus.[22] CFTR dysfunction throws numerous consequences to hydration, mucociliary clearance, mucus tethering and innate immunity.[23]

More than 200 gene variants have been identified; these

mutations have different effects on the manufacture of CFTR proteins, its processing function and its stability at the cell membrane.[23] Historically, CFTR variants have been grouped into six functional classes according to their effects on protein function.[13] Classes I-III are related with ineffective CFTR function, patients with these mutations have a severe phenotype. Whereas classes IV-VI have some residual function of CFTR protein and have mild lung phenotype and pancreatic sufficiency.[24] Lately, it has been proposed to be grouped in seven categories according to their functional defect in order to optimize gene therapy.[25] The most common mutation (85% of patients worldwide), F508del (deletion of phenylalanine at 508 residue)17, is a class II trafficking mutation, approximately 3% of protein is trafficked to the cell membrane where it is not functional.

The hypothesis that mutations in CFRT make epithelial cells intrinsically more pro-inflammatory compared with healthy cells is becoming more and more accepted.[23,26] Possibly, this inflammation has a role in early life; when infection becomes regular and it is also a chronic contributor to the airway microenvironment. Thus, inflammation is the main pathology in the lung due to failure to clear microorganisms (such as bacteria, fungi and viruses) and the generation of a local toxic microenvironment.[27]

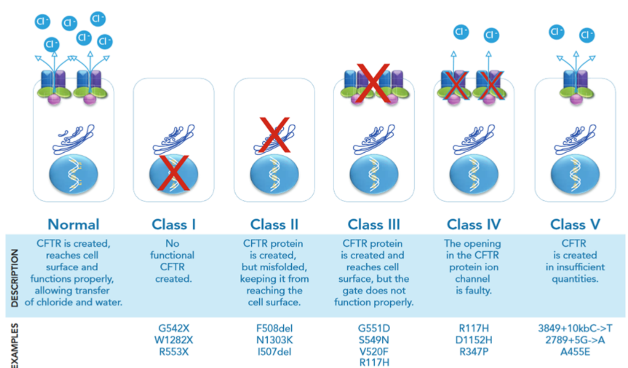


Figure 1.2: Mutations of the CFTR gene. (Adapted from the 2016 Annual Data Report of the Cystic Fibrosis Foundation Patient Registry, Bethesda, MD)[15]

1.2.3 Microbial infections in Cystic Fibrosis

In Children with CF, respiratory tract infections by bacteria such as *Staphylococcus aureus* and *Haemophilus influenzae* result in direct and indirect damage directly caused by the inflammatory response to airway infection.[28] When the disease progresses, bronchiectasis develops, and sick individuals become susceptible to a wide range of Gram-negative bacteria such as *Pseudomonas aeruginosa*, the most predominant lung infectious agent in CF patients. These microorganisms are ubiquitous in the

environment and generally they are only associated with infection in immunocompromised individuals.[29] Other non-fermentative Gram-negative bacteria are increasing their relevance in patients with CF, among them, *Stenotrophomonas maltophilia* and *Achromobacter* spp. All these Gram-negative bacteria have in common that they acquire resistance to antibiotics easily.[30] *Burkholderia cepacia* complex, have been major pathogens in CF and are associated with increased mortality.[29] Moreover, some studies have identified a much greater range of other bacteria in the airway, including obligate anaerobes.[23] These studies suggest that bacterial diversity in lower airways is associated with better lung function compared with a very high abundance of one microorganism such as *P. aeruginosa*.[31] For all this, important questions are arising about use of antibiotics in a prophylactic way, before children with CF are being seen with an increased prevalence of *P. aeruginosa*.

Briefly, the most predominant bacterial species in CF, although *S. aureus* and *P. aeruginosa* that are described extensively in following chapters are:

Haemophilus influenzae: This organism is usually unencapsulated (nontypeable). *H. influenzae* most frequently infects CF patients in childhood persisting for an average of

2 ½ months.[32] Thus, it is one of the first colonizers to infect individuals with CF.

Staphylococcus aureus: Historically was the first bacterium found in CF respiratory disease specially in children. In addition, some authors have proposed that *S. aureus*-infected patients may experience more rapid decline in its pulmonary functions.[33] Prior to the advent of antibiotics, this organism was the major cause of death in infants but with the beginning of the antibiotic era was surpassed in prevalence by *P. aeruginosa*.[16] Nonetheless, from 2000 to 2010 there was a significant increase in CF individuals with methicillin-resistant *S. aureus* (MRSA). After 2010 prevalence have plateaued due to infection prevention and control strategies.[34]

Pseudomonas aeruginosa: The most studied microorganism in the context of CF because of it is the most common identified in respiratory secretions of CF patients. Lately, the prevalence of *P. aeruginosa* continues to decrease, it is believed that is due to the implementation of therapy to eradicate initial acquisition. Concurrently, the prevalence of multidrug-resistant *P. aeruginosa* (MDR-PA) has remained constant.[35]

***Burkholderia cepacia* complex (BCC)**: BCC is a group

of Gram-negative bacteria that is ubiquitous in the natural environment. In recent times, BCC has achieved much notoriety for its propensity to spread rapidly among CF patients. Additionally, interpatient spread was recognized as a clinical trouble in 80's. Unlike *H. influenzae* and *S. aureus*, BCC often infects in more advanced stages of the disease. Studies throw evidences that BCC causes huge morbidity and mortality in CF, even more *P. aeruginosa*.[36]

Other opportunistic pathogens such as ***Stenotrophomonas maltophilia, Achromobacter xylosoxidans*** and Nontuberculosis Mycobacteria have been found with increasing frequency in CF cultures.

Aspergillus* Species**: These species are natural inhabitants of soil, plants or decomposing organic matter.[37] It is not uncommon to find these fungi species in culture from respiratory tracts of individuals with CF, reporting a prevalence of 9 to 57%.[38,39] The most isolated strain is ***A. fumigatus following by ***A. niger, A. terreus, A. versicolor*** and ***A. flavus***.[40]

Bacteria	Percent with Infection	Median Age in Years at First Infection	Distinctive Features In CF
P. aeruginosa	46.4	6	• A leading cause of airway infection • Associated with a decline in lung function • 17.7% of strains are multi-drug resistant
B. cepacia complex	2.7	20	• Small proportion of people with CF infected • Can lead to rapid deterioration • Multi-drug resistant
MRSA	26.0	12	• Prevalent among people with and without CF • Multi-drug resistant • Healthcare and community-associated strains
S. maltophilia	13.1	10	• Found in water, soil, plants, animals and hospital environments • Often multi-drug resistant
Achromobacter xylosoxidans	6.3	14	• Inhabits natural environment, including soil and water • Often multi-drug resistant
Non-tuberculous mycobacteria	12.6	22	• Found in water and soil • Sporadic reports of person-to-person spread • Treatment is rigorous and often poorly tolerated

Figure 1.3: Culture data seen in 2016 (Adapted from the 2016 Annual Data Report of the Cystic Fibrosis Foundation Patient Registry, Bethesda, MD)[15]

1.3 *Staphylococcus aureus*

1.3.1 General points

S. aureus is one of the members of the genus *Staphylococcus* (staphylococci) that tend to be arranged in grape-like clusters (from the Greek "staphyle", bunch of grapes), although it is possible to see some single cells, pairs and short chains. They are nonflagellate, nonmotile, and non-spore forming like all medically important cocci. Staphylococci are facultative anaerobic, but it

grows aerobically faster. Moreover *S. aureus* produces catalase and it is the most virulent staphylococci because it has the ability to form coagulases. Two of up-mentioned, coagulases are called staphylocoagulase and von Willebrand factor (vWF), which are responsible for transforming fibrinogen to fibrin.[41, 42]

S. aureus has a typical Gram-positive cell wall structure, having peptidoglycan interspersed with considerable amounts of teichoic acid and overlaid with polysaccharide and surface proteins. The microorganism grown-up in blood agar produces white colonies that tend to turn a buff-golden colour with time, that is why it is called aureus.[41] Most of the *S. aureus* present when cultured in blood agar, a surrounding area of clear β-haemolysis.

In addition, *S. aureus* expresses a number of toxins (α, β, δ), enzymes, adhesins and several surface proteins which together promote the survival of this bacterium on tissues and other substrates and protect it from elimination by the immune system.[43] Briefly, α-toxin is the most important and the most well studied toxin, being secreted by almost all strains of *S. aureus*.[41] This protein is a pore-forming β-barrel that lyses red blood cell and leukocytes, but not neutrophils.[43] After toxin inserts into the lipid bilayer, critical molecules go out from the cell provoking death.

Besides, *S. aureus* produces a bi-component toxin with similarity to the α-toxin scaffold. It is called Panton-Valentine leucocidin (PVL). Lately, this protein has been associated with infections by community-associated methicillin-resistant *S. aureus* (CA-MRSA) strains.[42, 44] Another toxin is δ-cytotoxin, it has been classified as a phenol-soluble modulin (PSM) that does not require a receptor for its haemolytic activity. It is known that this toxin has multiple function in pathogenesis.[45] Moreover, PSM has showed non-specific cytolytic activity and it may contribute to the allergic skin disease atopic dermatitis by inducing mast cell degranulation.[46] Finally, β-toxin is a sphingomyelinase of type C; this molecule degrades the sphingomyelin of the host cells causing cell lysis. This gene encodes for this toxin is considered pathogenicity island thus it is an important marker.[47]

Almost all enzymes secreted by *S. aureus* are proteases that degrade host molecules provoking interferences in the signalling cascades and leading tissue destruction.[42]

1.3.2 **Epidemiology**

Among ten to thirty percent of the population is colonized by *S. aureus* at any given time; in a hospital environment the proportion increases considerably (hospital personnel and patients).[41] The main habitat in human host is the anterior nares but it is possible to find behind the nails or in the perianal area as well. These bacteria attached to the nose are able to shed from the skin and colonize other hosts or stay in fomites inasmuch as *S. aureus* can stand long times in a very dry environment. Most of the infections are in fact self-infections caused by strains carried in nares or on the skin of the host.

In hospital environments, outbreaks caused by *S. aureus* usually involve patients with wounds, surgical or other invasive procedures or immunocompromised patients.[41] In addition, one of the most vulnerable groups are infants with less than 60 days old because of exposure to pathogens in perinatal period and lack of vaccine immunity.[48]

In the context of CF, *S. aureus* is the primary pathogen colonizing the airways and in some times the host remains colonized until the end of his life.[49] Additionally, there is an

increase in the number of isolates resistant to antimicrobial agents, specially MRSA, in CF patients.[50] Nowadays, the incidence of staphylococcal colonization in CF patients needs more studies since is still poorly recognized.[51]

Typically, *S. aureus* is divided in methicillin resistant (MRSA) and methicillin sensitive (MSSA) this is useful to discriminate and to improve the antimicrobial treatment. Multiple typing systems have been developed for MSSA and MRSA including pulse-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and variable tandem repeat region of staphylococcal protein A (spa typing). Moreover, MRSA isolates are discriminated by the Staphylococcal Casette Chromosome (SCC)mec types which carries the gene for methicillin resistance.[52] Almost 80% of all *S. aureus* isolates are resistant to penicillin and in addition, after the introduction of methicillin in 1959 *S. aureus* developed resistance to this antibiotic in just two years.[52, 53]

MRSA prevalence worldwide is very variable. For instance, in Europe, less than 5% of all *S. aureus* are MRSA in the north (Netherlands, Norway, Sweden and Denmark). On the other hand, in southern Europe, this percentage increases inordinately until 25-50% (Portugal, Spain, Italy and Greece). Similar outcomes may

be found in United States, where 50% of *S. aureus* are MRSA. MRSA is endemic in Asia, it has the highest percentages of MRSA worldwide. Up to 50% of *S. aureus* bloodstream infections in certain regions of Asia are MRSA. Particularly, Korea and Japan have the highest prevalence with >70% of all clinical isolates. Moreover, other Asian nations have no official data but probably prevalence is similarly to Korea or Japan. Since 2011, Australia has accounted for 30-33% prevalence of MRSA from all *S. aureus* isolates. Finally, in Africa we find quite variability; published data are available for South Africa, Nigeria and countries from the Mediterranean coast. Moreover, most of these studies come from one single facility and identification was obtained by phenotypic methods that are less effective. MRSA prevalence in these countries is estimated around <25%. However, this prevalence has been increasing since 2000s, although in South Africa prevalence is has started to decrease due to infection control practices.[54]

1.3.3 **Pathogenesis**

In most cases, colonization precedes the progress of infection. It is less common that infection occurs in the absence of known *S. aureus* colonization. For instance, as a result of open wound or

contamination of catheters.[55]

The leading stages of *S. aureus* attached are mediated by a number of surface proteins that bind to cells by glycoprotein fibronectin. Once *S. aureus* is attached to mammalian cells they begin to produce α-toxin, thus causing injures to host cells. In this moment, activity of host phagocytes is ineffective due to the effect of protein A that binds IgG. Moreover, α-toxin continues to destroy keratinocytes and other host cells favouring the lesion. According the tissue involved, process may be different; in the case of lungs, kidneys and other organs α-toxin action is astoundingly destructive creating cavities and massive necrosis. At worst *S. aureus* are not contained, spreading to the bloodstream, the bacterium detach peptidoglycan activating massive complement response, this response is the cause of leukopenia, thrombocytopenia and septic shock.[41]

S. aureus has a big bunch of virulence factors that are found on mobile genetic elements in most cases. Furthermore, some of these virulence factor cannot be investigated because they are human-specific, making their characterization difficult.[56] Depending on the kind of infection and tissues involved, *S. aureus* expresses and uses different virulence mechanisms. Briefly,

S. aureus surface proteins such as **Clumping factor A** (ClfA), **Cumpling factor B** (ClfB) and **Fibronectin-binding protein A** (FnBPA) are involved in binding extracellular matrix proteins enabling bacteria to attach to wound and multiply.[57] **Coagulase proteins** from *S. aureus* are responsible for the creation of a fibrin capsule surrounding bacteria preventing leukocyte entrance.[58] By the formation of a polysaccharide microcapsule, *S. aureus* is able to impede opsonization.[59] Abscesses of *S. aureus* can be disrupted releasing bacteria into bloodstream causing bacteraemia. These free bacteria are almost impossible to be controlled; they can adhere to endothelial surfaces provoking endocarditis among others.[60]

These are some of the virulence factors involved in the pathogenesis of *S. aureus*.[61]

- **Membrane damaging toxins**
- **Cell wall-anchored proteins**
  - Clumping factor A (ClfA)
  - Clumping factor B (ClfB)
  - Fibronectin-binding protein A (FnBPA)
  - SasX
  - Protein A
  - Iron regulated surface proteins

Since *S. aureus* is one of the earliest bacteria detected in infants and children with CF, studies in this segment of the population (peak prevalence between ages 11-15 years) acquire a profound impact.[62] In the las 10 years, it has been found an overwhelming increase of MRSA isolates, especially in USA;[52] some studies thrown a prevalence of *S. aureus*, both MRSA and MSSA, of 43.2%.[63] Notwithstanding, there is a continuous debate about the significance of *S. aureus* in the pathogenesis

of CF lung infection. It was believed that anti-staphylococcal therapy has improved the longevity of the patients[64] but some recent studies have questioned the prophylactic anti-staphylococcal antibiotics benefits.[65]

1.3.4 Adaptation

S. aureus adaptation, especially in lung, has been studied widely; three of the most common strategies are small colony-variants, hypermutable phenotype and persisters.

Small-colony variants (SCVs) are very common and observed in CF patients. SCVs have a very slow growth rate, reduced expression of haemolysins and loss of pigmentation, instead they have an increased resistance to aminoglycosides and they are able to persist after non-professional phagocytosis.[66] Due to the number of mutations in metabolic genes SVCs have a deficiency in electron transport and they are auxotrophic for both menadione or haemin.[67] Their molecular characterisation reveals that SVCs are lack in the expression of the virulence factor *agr*.[68] In CF patients, most of the SVCs are auxotrophic for thymidine (TD-SCV), and it is believed that the reason is a long

term trimethoprim-sulphomethoxazol treatment.[69] Nonetheless, it is assumed that thymidine is very abundant in airway secretions of CF patients and can balance this thymidine auxotrophy.[70]

Hypermutable phenotype is one of the key processes in resistance to antibiotics. Those hypermutable bacteria present a mutation ratio much higher than normal counterpart, this mutation lead the adaptation in stressful environments.[66] This phenotype is caused (in the most part) by defect in *mut*S or *mut*L genes that lead in a methyl-directed mismatch repair system malfunction. Some authors throw that this mutation frequency do not contribute significantly to the antibiotic resistance developing in *S. aureus*.[71] On the other hand, another study shows that it has found more *S. aureus* hypermutable strains in CF patients compared to non-CF isolates.[72] In recent past, the up mentioned thymidine-dependant SCV isolates was pointed to be associated with hypermutability.[73]

Persisters were described in 1944 by Joseph Bigger. He found that after treating with penicillin a growing population of *Staphylococcus* spp., a small number of persisters cells survived.[74] Nowadays, persisters are defined as "a small subpopulation of bacteria that survive lethal concentrations of antibiotics without any specific resistance mechanism".[75] These isolates are not drug-resistant

mutants nay; they have a phenotypic switch that can revert to wild-type easily.

Main resistance mechanisms, such as target modification by both mutation or specialized enzymatic changes, target substitution, modification or destruction of antibiotics, restricted permeability to antibiotics, antibiotic efflux, among others, are widely studied and understood, more details will be forthcoming in the next sections. Nonetheless, persisters have *tolerance* to antibiotics. It is believed that its metabolism is dormant thus, there is not interaction between antibiotic and persister cell. Becoming a persister has the handicap to the bacterium that they are not able to proliferate.[75]

1.4 *Pseudomonas aeruginosa*

1.4.1 General points

Pseudomonas aeruginosa is a respiratory, motile, Gram-negative rod and it can produce several different water-soluble pigments. Pyocyanin (blue pigment) is produced only by *P. aeruginosa*; fluorescin (yellow pigment) is shared with other free-living non-pathogenic *Pseudomonas* species. When these pigments are

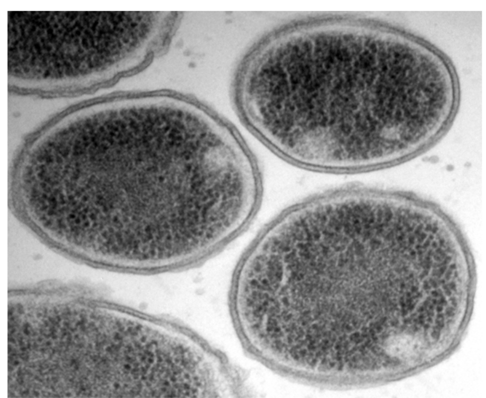


Figure 1.4: Transmission electron microscopy (TEM) image of *P. aeruginosa*

combined produce the characteristic most well-known green pigment. *P. aeruginosa* is able to growth in absence of oxygen if nitrate is present as an electron acceptor.[41] It has a wide temperature range of survival (20 – 42°C) and it can growth in a high salt concentration medium as well. *P. aeruginosa* emits a characteristic intense "fruity" non-disagreeable odour. As all Gram-negative bacteria, *P. aeruginosa* has lipopolysaccharide (LPS) in its outer membrane. Moreover, alginate is a copolymer formed by mannuronic and glucuronic acids which is secreted by some *P. aeruginosa* strains. Mutants that produce a huge amount of alginate are the principal burden in CF patients.

P. aeruginosa secretes multiple extracellular products as well, such as exotoxin A (ExoA), elastase and Exoenzyme S (ExoS).

Exotoxin (ExoA) is the most toxic virulence factor of *P. aeruginosa*, which has enzymatic activity. It belongs to the mono-ADP-ribosyltransferase family and is composed by A domain which is the responsible of the enzymatic activity and a B domain responsible of binding.[76] Invasive and locally destructive lesions are due to the action of ExoA.[77]

Elastase is another major virulence factor in *P. aeruginosa* and it is secreted by non-mucoid strains. This protein acts on biologically important substrates such as elastin, human IgA and IgG, complement and collagen.[78]

Exoenzyme S (ExoS) and other proteins (ExoT, ExoY, ExoU) are injected into host cells directly by type III secretion system. Once inside the host cell, ExoS acts on regulatory G proteins affecting the cytoskeleton, inducing apoptosis.[41,79]

Due to the action of these virulence factors, *P. aeruginosa* is able to cause a wide range of urinary, pulmonary or soft tissue infections. Moreover, once infection is stablished, *P. aeruginosa*

is overwhelming virulent and very difficult to treat. In addition, immunocompromised patients such as CF patients or AIDS patients are more prone to suffer these infections.

1.4.2 **Epidemiology**

P. aeruginosa is known as an opportunistic pathogen which habitat is the environment (water, soil or vegetation). However, 2 to 10% of the humankind are colonized by this bacterium, being throat and stool the main places of isolation. As said above, *P. aeruginosa* rarely infects healthy people but is one of the most important causes of nosocomial infections in non-healthy people i.e. CF patients, extensive burns or leukaemia.[41]

1.4.3 **Pathogenesis**

P. aeruginosa expresses an abundance of virulence factors but against expected strains isolated from CF lungs lack some of them. Firstly *P. aeruginosa* have to invade and/or penetrate host.[80] Flagella and type 4 pili are involved in adherence to epithelial cells which is the first step to initiate infection. However, the most studied virulence factors in *P. aeruginosa* are type 3 secretion system,

quorum sensing (QS) and lipopolysaccharide (LPS). Briefly, type 3 secretion system injects toxins into host cells and its expression is related to acute infections.[81] QS is a poor-known mechanism of bacteria that leads an intercommunication between individuals in order to adapt to environmental changes.[82] Finally, LPS is involved on antigenicity, interaction with antimicrobials and inflammatory response.[80, 83]

1.4.4 **Adaptation**

P. aeruginosa is not static, but it is able to adapt (specially in lung) in order to escape the antimicrobial treatment. Moreover, prolonged infections characteristic of CF help to select resistant or mutant isolates.

Some adaptative traits such as mucoid phenotype, antibiotic resistance, alterations in lipopolysaccharide (LPS), loss of type III secretion and motility, auxotrophy, SCVs, defects in quorum sensing and hypermutability.[33]

Mucoid phenotype is the most studied *P. aeruginosa* adaption in CF patients because it is the most predominant adaption in these patients. This phenotype is due to an overproduction of the

exopolysaccharide alginate, this polymer is based on D-manuronic acid and L-guluronic acid.[84] *P. aeruginosa* has an operon that contents most of the genes responsible for alginate synthesis. Upregulation of the operon throw an overproduction of alginate and the subsequent mucoid phenotype. This overproduction is beneficial to *P. aeruginosa* because it prevents bacterial clearance by both host and antimicrobial therapy growing in biofilm.[85] Moreover, opsonization and phagocytosis are impeded by alginate.[86] Finally, it is suggested that exopolysaccharide has immunomodulatory properties which would be able to dysregulate immune response.[87] In conclusion, some authors postulate that emergence of the mucoid phenotype coincides with the transition from the intermittent to the chronic phase of infection.[88,89]

Antibiotic resistance is another adaptation mechanism which is very common because of patients (especially CF patients) are exposed during long time to antimicrobials.[90] This exposition acts as a selective pressure in *P. aeruginosa*, selecting individuals that are intrinsically resistant to a given antibiotic. The level of resistance tends to increase according to the number of antibiotic courses, therefore is a marker of how the advance the disease is. Panresistant bacteria has the worst survival outcome.

Modification of LPS. LPS consist of three parts: Lipid A which is very toxic and highly acylated, a central core oligosaccharide attached to lipid A and the O antigen which is nonessential polysaccharide.

O-antigen usually is missing in *P. aeruginosa* isolates from CF patients. This absence may contribute to chronic persistence within the CF patients because of O-antigen is highly immunogenic provoking a strong antibody response.[91] Recently, it has been demonstrated that lipid A may be altered in *P. aeruginosa* isolates from CF patients. These alterations consist in different acylation patterns.[92]

Loss of type III secretion. Almost all environmental *P.*

aeruginosa are able to secrete toxic proteins (such as ExoS, ExoT, ExoU, ExoY among others) trough type III secretion, but during infection only 45% of isolates from newly infected children or 12% of chronically infected adults are able to secrete these proteins.[93–95] This adaptation is due to individuals with CF secreting antibodies against proteins secreted by type III secretion, therefore a *P. aeruginosa* isolates defective in type III secretion are more likely to survive in CF patient lung.

Loss of motility. *P. aeruginosa* cultured from CF patients are usually defective in swimming motility since they are unable to produce their flagella.[96] It has been seen that *P. aeruginosa* cultured from early infections patients has their flagella therefore, this loss of flagella occurs during infection. In addition, *P. aeruginosa* has the ability of movement between surfaces through a process called twitching motility. This movement is mediated by the extension and retraction of type IV pili32. CF chronic infections is linked with loss of twitching movement as well.[97]

Auxotrophy and metabolic adaptations. Frequently, *P. aeruginosa* from CF patients growths slowly, this effect suggest the existence of a defective metabolic pathway such as an auxotrophy.[98] The most common auxotrophy involves amino acids methionine,

leucine and arginine i.e. that auxotroph strains are unable to synthesize these amino acids in order to synthesize proteins.[99] Nevertheless, secretions of CF patients have a high concentration of amino acids therefore these auxotrophic strains may survive and expand infection. Some studies thrown that auxotrophic strains are associated with severe infections.[100]

Small-colony variants (SCVs). Like *S. aureus*, *P. aeruginosa* is able to form SCVs. They are present in almost 10% of the respiratory samples of CF patients[69] and have some special characteristics: take more than 48h to appear in culture plates, they autoaggregate in liquid culture, are hyperadherent to surfaces, have reduce motility and enhanced antimicrobial resistance.[33]

These characteristics promote the biofilm mode of growth in respiratory airways. Their phenotype is correlated with changes in expression of chemosensory, exopolysaccharide and two-component system. However, SCVs differs significantly from each other their gene expression. Therefore, SCVs represent a heterogeneous group that have in common just their phenotypes.

Defects in quorum sensing (QS). QS is defined as the mechanism whereby individual bacterium communicates with another

in order to alter gene expression in response to changes in population density or external stimuli.[101] In order to achieve these proposals, bacteria secrete molecules called autoinducers which are detected by other bacteria. The most studied molecules are 3-oxo-dodecanoyl homoserine lactone produced by the LasI/LasR system and butyryl homoserine lactone produced by RhlI/RhlR system.[102] It has been seen that *in vitro* QS systems modulate 6 to 10% of the genes in *P. aeruginosa* genome, determinant virulence factors are included such as elastase, alkaline protease, phospholipase C, pyocyanin and exotoxin A, among others.[103–105] Due to these virulence factors regulation, QS plays a key role in pathogenesis of *P. aeruginosa* infection. Notwithstanding, many *P. aeruginosa* isolates from CF patients fail to produce homoserine lactones.[106] This paradoxical event is explained by the emergence of "social cheaters", bacteria that do not respond to autoinducer signals, but they benefit by autoinducers produced by other bacteria.[107] As occurs with type III secretion, flagella or other virulence systems are lost once chronic infection is established inasmuch as this energy expenditure is only affordable to initiate an infection.

Hypermutable phenotype. *P. aeruginosa* may presents a hypermutable phenotype as occurs with *S. aureus*. Briefly, 37 to 54% isolates from CF patients are hypermutable, while acute infections

has 1% or environmental isolates are 6%.[108,109] This phenotype occurs when *mut*S, *mut*L and *uvr*D genes mutate preventing expression of proof-reading proteins that control correcting errors in DNA replication.[110,111] Mutation ratio in hypermutable phenotype is 20- to 1000-fold than normal phenotype; it is believed that these mutations are responsible for many adaptations such as above mentioned. Moreover, hypermutable strains are more resistant to antibiotics, usually are mucoid and defective in QS, and in general more versatile to adapt in tough environment CF airways.[105,112–114]

1.5 Microbial biofilm

The concept of biofilm is controversial and remains unclear. In 1975, Marshall defined it as "very fine extracellular polymer fibrils".[115] Later, in 2002, Donlan and Costeron amplified the definition to "a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription".[116] Growth in a biofilm provides bacteria a defence against several clearance mechanisms.[117] Extracellular polymeric

matrix is one of these protection mechanisms; composition varies between species and strains but in general contains host factors, polysaccharides and extracellular DNA (eDNA).[118,119] Contrary to was expected, biofilms growth stronger and faster in a very rapid flowing milieus environment. It has been established that bacteria form stronger biofilm the higher Reynolds number (the higher Reynolds number, the more turbulent the flow).[116]

Many infections that affect to human beings are caused by bacterial biofilms such as endocarditis, otitis media, chronic bacterial prostatitis, cystic fibrosis, periodontitis; additionally, biofilms have the ability to colonize abiotic surfaces such as prosthetic heart valves, central venous catheters, urinary catheters, contact lenses, intrauterine devices, dental unit water lines, among others116. Biofilm life cycle has been studied for years and nowadays remains quite unclear. Briefly, biofilm life cycle consists at least three phases: attachment to an abiotic or biotic surface, maturation and dispersal. Some authors add another phase called “microcolony formation” as intermediate between attachment and maturation. Bacterial adhesins are responsible to the attachment, whilst maturation is mediated by cell-cell adhesion. Finally, dispersion is carried out by enzymes that degrade biofilm matrix.[117,120]

1.5.1 *S. aureus* biofilm

Depending on the bacterial species, biofilm will be formed in a different way. Recent studies show that *S. aureus* biofilm formation has another more stage; this stage consists in an early dispersal phase called "exodus". Exodus leave behind small foci of biofilm that grows in tower structures.[121] Another interesting point is biofilm matrix, *S. aureus* biofilm matrix is a cement that encompasses bacteria, host factors, secreted and lysis-derived proteins, polysaccharides and eDNA from lysed bacteria and from host neutrophil cell death. Polysaccharide intercellular adhesin (PIA) is the component with the largest proportion in *S. aureus* biofilm.[122] PIA is built with β-1,6-linked N-acetylglucosamine molecules encoded in the *ica*ADBC locus.[117] Structural integrity of biofilm both *in vivo* and *in vitro* are rule by PIA polymers, notwithstanding some reports have shown that some *S. aureus* strains are able to growth in biofilm while lacking *ica* locus.[123] These PIA-deficient biofilms have a matrix rich in eDNA and proteins that play the role of adhesins in the absence of PIA.[124] Proteins such as protein A, fibrinogen-biding proteins (FnBPA and FnBPB), biofilm-associated protein (Bap), clumping factor B (ClfB) or *S. aureus* surface protein (SasG) are involved in the attachment and biofilm matrix development.[125,126] Moreover, biofilm maturation

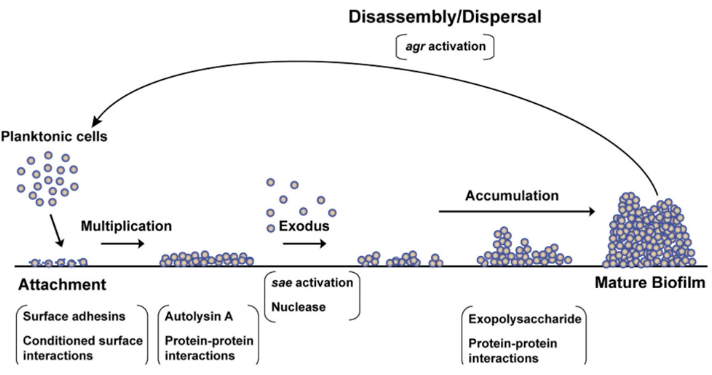


Figure 1.5: *S. aureus* biofilm formation phases[120]

is regulated by extracellular adherence protein (Eap) and beta toxin (HlB).[127] Recently, eDNA is one of the latest identified in biofilm matrix which acts as an electrostatic polymer that anchors biofilm to surfaces. Because of this presence of eDNA, non-mature biofilms are more sensitive to DNases having a key role during attachment to surfaces.[128] Some studies thrown that this eDNA is produced by autolysis of a subpopulation of the bacteria.[129] It is presumed that this altruistic suicide is important in the biofilm dispersal mechanisms117. Additionally, adhesins and a number of cytoplasmic proteins are found in biofilm matrix as well.[130] Interestingly, although teichoic acids have been also found in biofilm matrix, their role in virulence and infection remain not fully stablished.[131–133]

Nowadays, dispersal strategies developed by *S. aureus* are the subject of many studies. Briefly, *S. aureus* uses various exo-enzymes such as DNases and surfactants to degrade the extracellular polymeric matrix.[134] Its accessory gene regulatory (*agr*) system is the responsible to control the production of these degrading enzymes. For instance, an hypothetical inhibition of genes encoding these proteases (*agr*) results in a significant increase in biofilm formation and in an almost inexistent disassembly.[135] Another DNase known as thermonuclease has been also involved in cell detachment from biofilm as well.[128]

Enzymatic dispersal mechanisms developed by *S. aureus* involve protease-mediated dispersal, nuclease-mediated, dispersin B-mediated, among others.[117]

1.5.2 *P. aeruginosa* biofilm

Two stages are differentiated in the formation of *P. aeruginosa* biofilm. First stage occurs in a reversible way, in the case of CF, planktonic *P. aeruginosa* cells adhere to the gel phase of airway lining fluid. Flagella and pili are critical in this stage. Second stage is irreversible, bacterial cells begin to divide and form

clusters giving rise to an early biofilm. In this step intervenes type 4 pili and *las*IR which produces a signal molecule called 3-oxododecanoyl-homoserine lactone.[136] Moreover, alginate is produced due to mutations in *muc*A. This mutation occurs because of the presence of oxygen free radicals that activates polymorphonuclear leukocytes (PMN) in the inflammatory reaction. Finally, autolysis of PMN entails releasing of DNA facilitating biofilm formation as occurs in Gram-positive bacteria.[137]

As it happens with *S. aureus*, a huge number of factors are involved in the formation of *P. aeruginosa* biofilm. One of the main factors is quorum-sensing systems; *P. aeruginosa* has two of them that are key processes in biofilm development, *las* and *rhl*.[138,139] For instance, maturation biofilm entails the formation of tower-like structures within water channels that allow exchange of nutrients and is led by *rhl*R which produce the signal molecule called N-butanoyl-homoserine lactone.

In the deeper layers of biofilm, it can be found metabolically inactive bacteria involved in high resistance to antibiotics. Finally, biofilm lifecycle is completed with the dispersion of planktonic bacteria from the outermost layers of biofilm.[140]

1.6 Antimicrobial treatment and resistance mechanisms

In the context of CF both *S. aureus* and *P. aeruginosa* are major concern in antimicrobial therapy. Since these two microorganisms are morphologically and physiologically different, different family of antimicrobials are used.

1.6.1 *S. aureus*

The prevalence of MRSA in the respiratory tract of CF patients is increasing year by year, becoming the main concern in *S. aureus* antibiotic therapy.

Vancomycin is one of the first elections to treat MRSA in CF paediatric population. Nevertheless, vancomycin has a limited penetration into the lung and biofilm formed by *S. aureus*. Another first-line antibiotic to treat *S. aureus* is linezolid. In opposition to vancomycin, linezolid has a good oral bioavailability.[141] Some cases of resistance to linezolid have been reported , but nowadays resistance remains at very low percentages.[142]

Tetracyclines are in a second antibiotic election. Historically, tetracyclines such as doxycycline are known as effective against MRSA. Resistance to tetracyclines is relatively low but increasing year by year.[143]

Trimethoprim/Sulfamethoxazole (TMP/SMX) is an oral and intravenous antibiotic with broad-spectrum action, but this combination has a worthy lung penetration. It has been reported many resistance rates but in CF population remain low. TMP/SMX has demonstrated a huge activity against *S. aureus* SCVs.[144]

Other antibiotics used to treat *S. aureus* in CF population are ceftaroline, ceftobiprole, clindamycin, telavancin or tigecycline. Ceftaroline and ceftobiprole are cephalosporins that have a promising potential due to their unique antibacterial spectrum and good tolerability. They are used against MRSA, VISA, linezolid-resistant *S. aureus* and even against Gram-negative bacteria such as *P. aeruginosa*. On the other hand, clindamycin use is limited by its high rates of resistance. Telavancin is derivate from vancomycin with a narrow-spectrum against Gram-positive bacteria, including MRSA, hVISA and VRSA. Its use is limited because of a higher frequency of nephrotoxicity, especially in CF patients due to these patients probably are medicated with another nephrotoxicity antibiotic such as colistin. Finally, tigecycline has a broad-spectrum activity and an extensive tissue distribution. In addition, lung penetration is good and resistance rates remains low in MRSA isolates form CF patients. Unfortunately, tigecycline

has demonstrated a limited action against *S. aureus* in CF patients.[141] Although daptomycin is not recommended use for lung MRSA because is inactivated by surfactant,[145] it is very active in combination with other antibiotics such as β-lactams, ceftaroline, TMP/SMX, rifampin, or Fosfomycin.[146]

1.6.2 *S. aureus* antimicrobial resistance mechanisms

S. aureus resistance comes mainly from horizontal DNA transfer of another microorganisms. These mechanisms have the advantage of gaining a pre-assembled packet encoding resistance to multiple antibiotics147. Resistance to β-lactam is produced by an enzyme called penicillinase which hydrolyses ring penicillin amide bond. Practically all *S. aureus* strains have acquired resistance to penicillin family in the first decades of its clinical use. Penicillinase production is regulated by *bla*Z gene, which in turn is under the control of *bla*I and *bla*R1 genes. Usually, these genes are carried in plasmids together with other genes that provides resistance to another antibiotics, giving them a large dispersion.[147]

Methicillin resistance is one of the most relevant resistance in *S. aureus*. Due to this characteristic, *S. aureus* is divided into

Table 1.1: *S. aureus* antimicrobial treatment

ANTIBIOTIC	FAMILY	MoA	MoR
Vancomycin	Glucopeptide	Inhibition cell wall synthesis	Alteration NAM/NAG subunits
Linezolid	Oxazolidinone	Protein synthesis inhibitor	Efflux pumps Mutation genes encoded 23s ribosomal RNA
Daptomycin	Lipopetide	Disrupts bacterial cell membrane	Cell membrane/wall perturbations
TMP/SMX	DFRInh+ Sulfonamide	Inhibits folate synthesis	Stop folate pathway
Doxycycline	Tetracycline	Inhibits protein synthesis	Efflux pump Ribosomal protection protein
Clindamycin	Lincosamide	Inhibits ribosomal translocation	adenine methylation in 23S subunit
Telavancin	Lipoglicopeptide	Inhibits cell wall synthesis	-
Tygecicline	Glycylcycline	Proteins synthesis inhibitor	Efflux pump Mutations in ribosomal genes
Ceftaroline	Cephalosporin	Binds to penicillin-binding proteins (PBPs)	-

MRSA or MSSA. MRSA distinction implies resistance to β-lactam antibiotics including cephalosporins and carbapenems. Frequently, MRSA is usually resistant to other antibiotics such as tobramycin or erythromycin, among others. *S. aureus* mechanism of resistance to methicillin is the production of an additional penicillin-binding protein (PBP2a) with reduced affinity for penicillins.[147] Genes involved are *mec*A, *mec*I and *mec*R1 which have similarities with *bla* genes family.

Vancomycin resistant was practically inexistent until late nineties. Lately, it has been reported an increasing number of Vancomycin-resistant (VRSA) isolates as well as Vancomycin intermediate-resistant (VISA) *S. aureus*. First VISA was found in Japan in 1997[148] and first isolate of VRSA was found in United States in 2002.[149] VISA phenotype is the result of a failure in the treatment of nosocomial infections, nowadays viable alternatives to VISA treatment are combination of high-dose daptomycin with another antibiotic such as gentamicin, rifampicin or linezolid.[150] It is being believed that VISA apparition is due to the existence of a heterogeneous VISA culture (hVISA). This culture has a very low number of colonies (10^{-6} to 10^{-5}) VISA and when the treatment with vancomycin starts only these isolates survive resulting in a fully VISA culture.[151]

Resistance is mediated by the *vanA* operon, probably transferred from *Enterococcus faecalis* on the plasmid-borne transposon Tn1546.[152] *VanA* encodes a modified synthesis of peptidoglycan containing D-Ala-D-Dlac instead of D-Ala-D-Ala. Nowadays, VRSA has remained extremely rare, but the number of these isolates is increasing.

Although linezolid remains highly active against *S. aureus*, resistance was firstly described in a patient with a MRSA peritonitis in 2001.[153] It occurs when there are mutations in the linezolid 23S rRNA binding site ([G2576T]), other ribosomal mutation described is [T2500A] in ribosomal proteins of the peptide translocation centre or by plasmid-borne acquisition with methyltransferase gene (*cfr*).[154]

Daptomycin has a unique mechanism of action consisting in binding and as further insertion into bacterial cytoplasmic membrane in the presence of calcium ions.[147] Due to this uncommon mechanism of action, resistance has no developed swiftly. However, it has been reported some protein mutations involve daptomycin resistance, although the main cause of resistance is due to perturbations in the cell membrane and even in the cell wall of bacteria.[155] Recently, some cross-resistance with VISA

has been reported. Some authors attribute this phenomenon to a thickened cell wall typical of VISA that would prevent daptomycin penetration.[156]

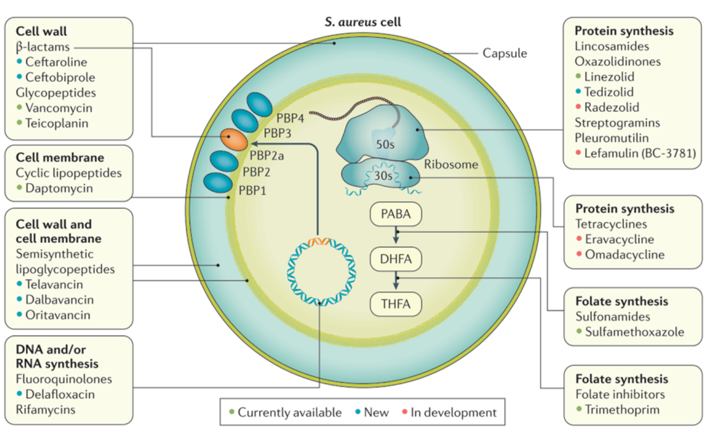


Figure 1.6: Targets of antimicrobials in *S. aureus*[54]

TMP/SMX is usually chosen as treatment in severe lung infections due to its appropriate lung penetration. Nevertheless, several resistances due to the interruption of folate pathway in an easy manner have been reported. Moreover, TMP/SMX selects *S. aureus* SCVs, associated with worse lung prognosis.[141,143]

On the other hand, tetracycline resistance is due to two different mechanisms; ribosome protection encoded by *tet*(M) and

tet(O) genes and drug efflux by *tet*(K) and *tet*(L) genes carried on plasmids.[157] Resistance to clindamycin is rising year by year. Briefly, resistance is due to a family of genes called *erm*, encoding adenine methylation in 23S subunit.[158] Telavancin resistance involve up-mentioned genes plasmid-mediated *vanA*.[159] Bacterial tigecycline resistance involve mutations in efflux pumps.[160]

1.6.3 *P. aeruginosa*

Nowadays, antibiotic therapy against *P. aeruginosa* is focusing in reduce bacterial mass in lung in a fastest manner in order to avoid selection of mutants and prevent phenotypic tolerance. Bacterial drugs such as β-lactams or aminoglycosides are the best choice to face *P. aeruginosa* infections.

Cephalosporins are one of the most studied family of antibiotics. Their mechanism of action consist on the inhibition of the third step of the synthesis of bacterial cell wall by binding to penicillin-binding proteins (PBPs).[161] Carbapenems, specially meropenem and imipenem, are β-lactam a broad-spectrum antimicrobials used for treatment of multidrug-resistant *P. aeruginosa* (MDRPA). The use of imipenem is in principle reserved to

severe infections where microorganisms have acquired resistance to up-mentioned drugs. Monobactam is a subfamily of β-lactam antibiotics. The best known molecule is aztreonam, which binds to the PBP3 disrupting bacteria.[162]

Fluoroquinolones have a slower bacterial activity proportional to the dose but, contrary to expected higher doses are less efficient. Fluoroquinolones, such as ciprofloxacin, levofloxacin, norfloxacin, among others, target type II topoisomerases DNA gyrase (which are encoded by *gyrA* and *gyrB*) and topoisomerase IV as well. The duty of these enzymes consists on modulate DNA supercoiling replication. Fluoroquinolones prevent this modulate provoking cell death.[163] Aminoglycosides, such as amikacin or tobramycin, are another family chosen in order to facing *P. aeruginosa* infections. Their mechanism of action dwells on block the synthesis of ribosome proteins preventing codon-anticodon matching.[164]

The emergence of MDRPA, as well as, the lack of new drugs to combat them, has stimulated the rescue of polymyxins as a therapeutic option. Polymyxins are known since 1949 but they were left largely unused in seventies because of their nephrotoxicity and neurotoxicity and the availability of less toxic drugs which bacteria had not yet developed resistance. Colistin is the most

known polymyxin; its amphipathic character is relevant to its activity against bacteria. Hydrophilic part, positively charged, would interact with the negatively-charged bacterial outer membrane. On the other hand, hydrophobic part would be allowed entry through the bacterial cytoplasmic membrane.

Figure 1.7: Polymyxin B molecule

This mechanism remains quite unclear, some authors propose that divalent cations such as Mg^{2+} and Ca^{2+} have an important role in the interaction with binding sites between colistin and outer membrane, among others.[165] Competitive binding resulting would disturb the properties and stability of outer membrane. Ultimately, death would be due to peptide insertion into the

cytoplasmic membrane, forming channels where small molecules, ions and even proteins would pass.[161]

Table 1.2: *P. aeruginosa* antimicrobial treatment

ANTIBIOTIC	FAMILY	MoA	MoR
Cephalosporin	β-lactam	Bind to PBPs	β-lactamases and Efflux pumps
Tobramycin Amikacin	Aminoglycoside	Block the synthesis of ribosome proteins	AMEs and rRNA methylases Efflux pumps
Ciprofloxacin	Fluoroquinolone	DNA gyrase and Topoisomerase IV	DNA gyrase and Topoisomerase IV mutations and Efflux pumps
Imipenem Meropenem	Carbapenem	Bind to PBPs	β-lactameses, Efflux pumps and Loss OprD
Aztreonam	Monobactam	Bind to PBPs	β-lactamases and Efflux pumps
Colistin	Polymyxin	Disrupt outer membrane	Modification Lipid A

1.6.4 *P. aeruginosa* antimicrobial resistance mechanisms

Resistance to β-lactams (penicillins, cephalosporins, carbapenems and monobactams) is increasing year by year. *P. aeruginosa* has a quite variety of mechanisms to avoid bactericidal effect: β-lactamases, carbapenemases efflux pumps and permeability. In the context of β-lactamases, four molecular families have been described (A-D), class B is a metal dependent enzyme (Zn^{2+}) and

A, C and D are metal independent enzymes.[166,167]

Within β-lactamases it can be differentiate between endogenous or acquired. Mostly *P. aeruginosa* strains have two intrinsic β-lactamases, AmpC (giving resistance to cephalosporines) and PoxB (giving resistance to oxacillinase).[168] Acquired β-lactamases usually are encoded by plasmids and they can hydrolyse a wider range of β-lactams; for instance, extended-spectrum β-lactamase (ESBL) are able to hydrolyse cephalosporins and monobactams.[169] In addition, other family of β-lactamases called carbapenemases leading to hydrolyze carbapenems. There are several families of carbapenemases such as GES, IMI, KPC, NMC-A and SME. One of the most important is KPC which is able to hydrolyse oxy-iminocephalosporins, monobactams and carbapenems.[170] Unfortunately, imipenem resistant isolates have emerged in last years.[161] Principally, mechanism of resistance to carbapenem in *P. aeruginosa* consists of the loss or the inactivation of OprD porin, this porin facilitates amino acids diffusion and allows carbapenems penetrance.[171]

Resistance to aminoglycosides occurs principally by aminoglycoside-modifying enzymes (AMEs) and rRNA methylases. These enzymes have been classified depending on molecular

target. Briefly, aminoglycoside acetyltransferase modifies 3 [3-N-aminoglycoside acetyltransferase] and 6' [6'-N-aminoglycoside acetyltransferases] (AAC family give resistance to gentamicin, tobramycin, netilmicin, amikacin, among others166. Aminoglycoside nucleotidyltransferases (ANT) inactivate gentamicin, tobramycin, amikacin. Finally, aminoglycoside phosphoryltransferases (APH) give resistance to kanamycin, neomycin and streptomycin.[172]

Lately, other mechanism involving 16S rRNA methylation which interferes in the aminoglycoside binding site giving resistance to gentamicin, tobramycin and amikacin have been described.[173]

Resistance to fluoroquinolones is mainly mediated by DNA gyrase and topoisomerase IV mutations.[174] Quinolone resistance determining region (QRDR) is the region where mutations occur with more percentage. This region encompasses GyrA of DNA gyrase and ParC of topoisomerase.[175]

All these antibiotic families (β-lactams, fluoroquinolones, aminoglycosides) are susceptible to be expelled by efflux pumps. *P. aeruginosa* has a wide variety of efflux pump systems. Shortly, resistance to β-lactams is provided by MexAB-OprM, MexCD-OprJ or MexXY-OprM systems. MexAB-OprM, MexCD-OprJ,

MexEF-oprN and MexXY-OprM provide resistance to fluoroquinoles. And finally MexXY-OprM gives resistance to aminoglycosides.[166]

Nowadays, it has been reported some resistances to polymyxins (polymyxin B and colistin) or other polycations. Resistance mechanism(s) remains quite unclear, but it is believed that a reduced negative charge on the bacterial outer membrane leading a lipid A modification is key in developed the resistance.[161]

1.7 Discovery of new compounds

The discovery of new compounds with antimicrobial capacity is expensive (hundreds of millions of euros) and long process (12-15 years). Moreover, there are hard regulatory laws that complicate even more the success. There are not too many strategies in order to develop these possible antimicrobial compounds; they consist on seeking new molecules in nature, laboratory synthesis, in silico studies and modifications of known molecules.

1.7.1 Natural compounds and synthesis

Historically, most of antibiotics used in clinics are natural compounds from environment whether from plants, animals or other microorganisms.[176] Nowadays, there are an overwhelming number of publications seeking for new molecules in nature with antimicrobial capacity and low toxicity. One of the main handicaps lies isolating these molecules from natural extracts. For this reason, laboratory synthesis mimicking natural molecules is envisaged as a promising strategy.

In conclusion, these two methods are the most extended to develop new antimicrobial molecules.

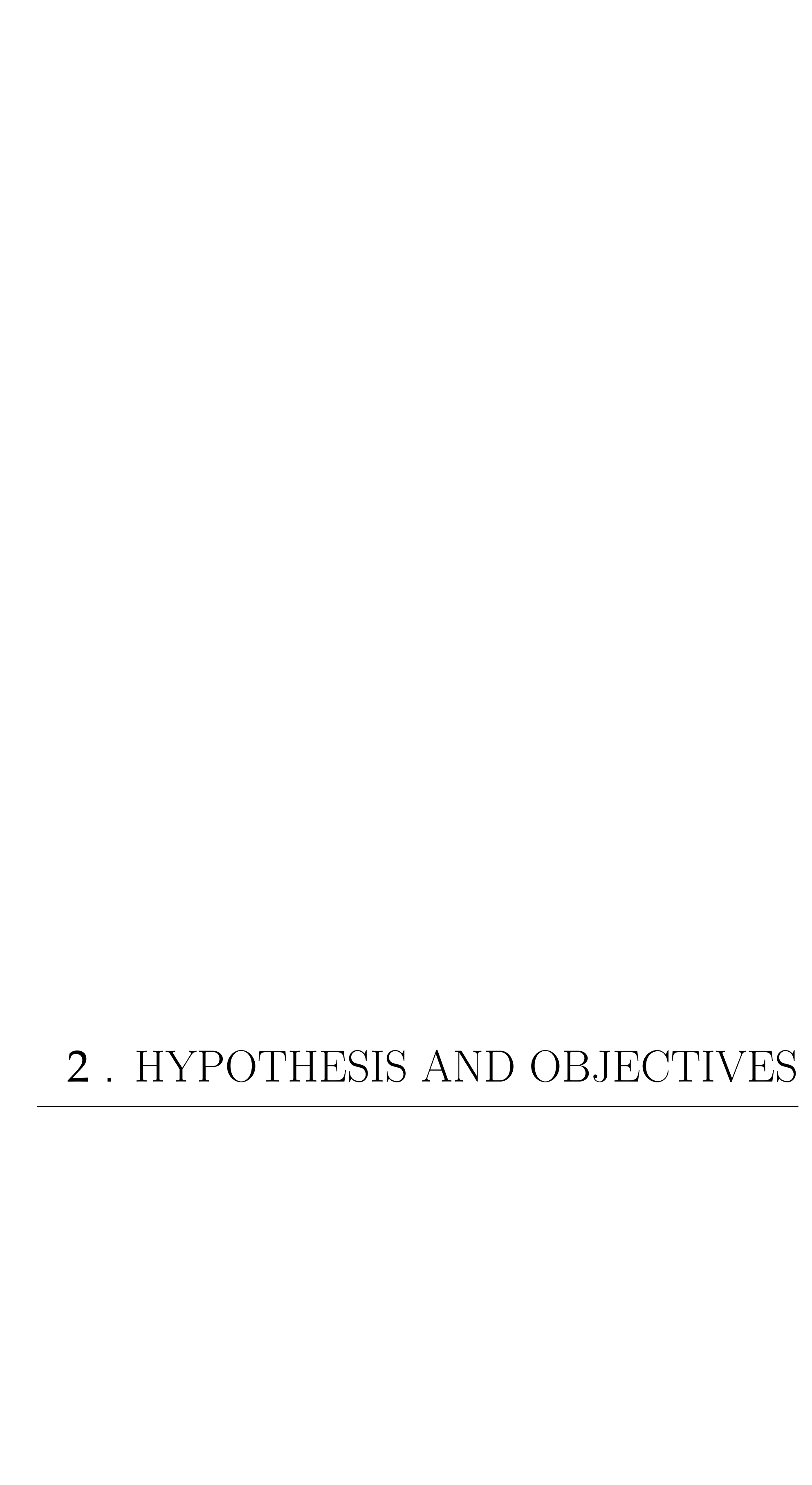
1.7.2 *In silico*

In silico is a contemporaneous term that refers experimentation performed by computer. Briefly, software is used in order to analyse and integrate biological and medical data creating models that simulate and predict hypotheses results. One of the most important kind of *in silico* experiments is to predict possible interactions between studied molecule and its target with the final

purpose determine an eventual antimicrobial capacity.[177]

1.7.3 **"Old compounds" modifications**

One of the strategies which nowadays are most studied is scaffold modifications of known drugs. As mentioned in previous sections, polymyxins were abandoned due to toxicity concerns in seventies but nowadays is one of the last-line antibiotics to tackle multidrug-resistant bacteria. In parallel, there are several both academic and industrial groups working on reducing toxicity and improving antimicrobial capacity of polymyxin-based new molecules[161,178,179]or even granting new therapeutic possibilities extending its spectrum of action[180]For all of this, we have focused on synthesizing and exploring polymyxin-based peptides against multidrug resistant bacteria.



2 . HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

The Synthetic Peptides based on Polymyxin structure may constitute a therapeutic option in infections caused by Multidrug Resistant Bacteria.

2.2 Justification of the study and objectives

Multidrug-resistant bacteria is one of the major concerns in public health. Nowadays, the number of isolates resistant to almost all known antibiotics has increased in an overwhelming way. It is worrisome in the context of cystic fibrosis; colistin has been rescued, despite of its toxicity, in order to treat strains which have developed resistance to antimicrobials frequently used against this disease such as carbapenems or aminoglycosides. Unfortunately, colistin-resistant strains are increasing as well year by year. The goal standard of our studies was the synthesis, antimicrobial determination and exploration of mechanisms of action of new compounds mimicking colistin scaffold with some chemical modifications as a new therapeutic option to multidrug-resistant bacteria specially in the context of cystic fibrosis.

Main objectives:

1. **Synthesis and characterization of polymyxin-based peptides**
   (a) Peptide synthesis following standard Fmoc/tBu methods
   (b) Purification and characterization of the peptides
2. **Antimicrobial activity of peptides against MDR clinical isolates**
   (a) Determination of the antimicrobial effect on planktonic bacteria
   (b) Determination of the synergistic antimicrobial effect on planktonic cultures with commercial antibiotics
   (c) Determination of the antimicrobial effect on biofilm formation
   (d) Determination of the synergistic antimicrobial effect on biofilm formation with commercial antibiotics
3. **Study of the toxicity of the peptides**
4. **Exploration of the mechanisms of action of the peptides**
   (a) Study outer membrane disruption using Transmission Electronic Microscopy
   (b) Study peptide interaction with teichoic acids from *S. aureus*

3 . PAPER 1

Article

Synergistic Antipseudomonal Effects of Synthetic Peptide AMP38 and Carbapenems

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Abstract: The aim was to explore the antimicrobial activity of a synthetic peptide (AMP38) and its synergy with imipenem against imipenem-resistant *Pseudomonas aeruginosa*. The main mechanism of imipenem resistance is the loss or alteration of protein OprD. Time-kill and minimal biofilm eradication concentration (MBEC) determinations were carried out by using clinical imipenem-resistant strains. AMP38 was markedly synergistic with imipenem when determined in imipenem-resistant *P. aeruginosa*. MBEC obtained for the combination of AMP38 and imipenem was of 62.5 µg/mL, whereas the MBEC of each antimicrobial separately was 500 µg/mL. AMP38 should be regarded as a promising antimicrobial to fight MDR *P. aeruginosa* infections. Moreover, killing effect and antibiofilm activity of AMP38 plus imipenem was much higher than that of colistin plus imipenem.

Keywords: *Pseudomonas*; antimicrobial peptides; synergism; biofilm eradication

1. Introduction

Pseudomonas aeruginosa is a nosocomial opportunistic pathogen causing a wide variety of both acute and chronic infections, such as pneumonia, bacteraemia, and urinary tract infections. Immunocompromised patients and those suffering cystic fibrosis show a particularly high susceptibility to infection by this microorganism [1].

Moreover, the increasing frequency of the isolation of multidrug-resistant *Pseudomonas aeruginosa* (MDRPA) is a major cause for concern. Antimicrobial resistance in *P. aeruginosa* is caused by three well-known basic mechanisms: uptake and efflux balance, target modifications, and inactivation of the drug [2,3].

Carbapenems—particularly imipenem—are broad-spectrum antimicrobials commonly used for the treatment of MDRPA infections. Their mechanism of action is based in the inhibition of the third step of the synthesis of bacterial cell wall by binding to certain penicillin-binding proteins (PBPs). Imipenem is active against a wide variety of microorganisms, both Gram-negative (*P. aeruginosa*, *Acinetobacter* spp.) and Gram-positive (*Streptococcus pneumoniae* and *Enterococcus faecalis*) [4,5]. The use of this antimicrobial is in principle restricted to severe infections where microorganisms have acquired

resistance to other drugs. Unfortunately, imipenem-resistant isolates have emerged in the last few years. The main mechanisms of resistance to carbapenems in *P. aeruginosa* are the loss or the alteration of the OprD porin, an outer membrane protein that facilitates the diffusion of basic amino acids and allows penetration of carbapenems (and particularly imipenem) into the bacterium, the production of β-lactamases, and the overexpression of efflux pumps [6,7]. The loss of porin OprD and the overproduction of extended-spectrum cephalosporinases (ESACs) that weakly hydrolyze carbapenems has been observed in 100% and 92% of the meropenem-resistant isolates, respectively. *P. aeruginosa* can very often accumulate different resistance mechanisms, including ESAC production, leading to carbapenem resistance [8]. The emergence of multidrug-resistant Gram-negative bacteria, as well as the lack of new drugs to combat them, has stimulated the rescue of polymyxins as therapeutic options. Polymyxins are cyclic peptides with antimicrobial action that have been available since 1949, although they were left largely unused during the seventies because of their nephrotoxicity and the availability of less toxic antimicrobials to which bacteria had not yet developed resistance [9].

The most known polymyxin is colistin; like other cationic polypeptides, colistin is an amphipathic compound. It is believed that this amphipathic nature is relevant to its activity against bacteria. The hydrophilic part (positively charged) would interact with the negatively-charged bacterial outer membrane. In this way, the hydrophobic part of the polypeptide would be allowed entry through the bacterial cytoplasmic membrane. Another mechanism proposed is the interaction with binding sites of divalent cations such as Mg^{2+} and Ca^{2+}; this competitive binding would disturb the properties and stability of the outer membrane. Ultimately, bacterial death would be due to the insertion of the peptide into the cytoplasmic membrane, forming channels where small molecules, ions, and even proteins would pass—and that could also eventually be used as a way for other antimicrobial agents to penetrate the bacteria [10,11].

The exploration of antimicrobial peptides (AMP) mimicking the structure and mechanism of action of polymyxins can be regarded as a main goal of this field of research. Such synthetic AMPs would allow opening perspectives in order to reduce secondary effects and enhance antimicrobial action. Moreover, there are several experimental and clinical studies regarding the synergistic activity of colistin with other antimicrobial agents against MDRPA (azlocillin, aztreonam, ceftazidime, or ciprofloxacin, among others) [12–14]. Carbapenems are the most commonly used antibiotics to test these eventual synergies, although there is a huge heterogeneity in the published results [15]. The efficacy of colistin in monotherapy against beta-lactam-susceptible bacteria is lower than that of β-lactams, but when used combined with other antimicrobials, they reach higher effectiveness [16].

The increase in the use of polymyxins has resulted in the emergence of worldwide polymyxin-resistant *P. aeruginosa* isolates. This resistance lies behind a reduced negative charge on the bacterial outer membrane which has been shown to be a specific modification of lipid A in the LPS [17]. The synthesis of safe and effective antimicrobial peptides in the laboratory could open new frontiers to combating multidrug resistance—an unlimited number of new molecules with antimicrobial activity could be designed. The challenge lies in finding new molecules with higher activity and lower toxicity than conventional known drugs.

The aim of this study was to explore the antimicrobial activity of a novel synthetic cyclolipopeptide analog of polymyxin (AMP38) and its synergy with carbapenems in order to contribute to the improvement of treatments for infections caused by carbapenem-resistant *P. aeruginosa*. AMP38 was previously described as one of the most effective peptides of a series synthesized by Rabanal et al. [18].

2. Results

Clinically used polymyxins are cyclic lipopeptides with a tail-to-side-chain amide bond (Figure 1). The fatty acid tail typically contains a stereocenter. Although their synthesis is accessible using chemical methods, a first goal was to reduce the complexity of the macrocyclic backbone scaffold to facilitate the generation of analogues and future preparation at high scale. A plausible approach would consist of substituting the amide bond by an isosteric and more chemically-accessible link,

such as a disulfide bond. Disulfides are also chemical linkages present in some cyclic peptide drugs, as discussed below. Loop structures linked by disulfide bonds are not uncommon in cyclic AMPs. Examples are found in bactenicin (from cattle), lactoferricin, brevinins (from frog). The similarity between the macrocyclic heptapeptide structure in ranalexin and polymyxin was reported by Zasloff [19]. Similarly, Porro et al. [20] described all-L-amino acid polymyxin-derived cyclic heptapeptides capable of binding to lipid A having the capability to detoxify bacterial endotoxin (LPS) in vitro, but lacking antimicrobial activity. Altogether, this background and these structural data supported the theoretical feasibility of substituting the tail-to-side-chain amide bond with a disulfide bond. This modification would imply changing both Thr10 and Dab4 for cysteines. In addition, the C-terminal cysteine would need to be derivatized as a carboxamide to mimic the neutral hydroxyethyl threonine moiety being substituted, as shown in Figure 1. Furthermore, Cys10 was introduced with the opposite configuration (D-Cys) to maintain the relative orientation of the moiety.

We also observed in a previous structure–activity study that a norleucine amino acid in position seven yielded more active analogues than the natural leucine in polymyxin. Finally, the branched natural fatty acid tail of polymyxin was substituted by a linear tail.

Figure 1. Chemical structure of the cyclolipopeptide analogue of polymyxin AMP38 (**below**) and of natural polymyxin B (**above**). The structural and chemical features modified in the analogue with respect to polymyxin are highlighted in blue.

The minimum inhibitory concentration (MIC) values of several antimicrobials against control and clinical strains are given in Table 1. All clinical isolates were imipenem resistant, and some of them also presented resistance to tobramycin, aztreonam, amikacin, ciprofloxacin, and meropenem.

Table 1. Antimicrobial susceptibility (minimum inhibitory concentration, MIC) (μg/mL). R: resistant; S: susceptible.

Antimicrobial Agent	ATCC	PA116136	023VH	481SJD	536SJD	846VH
IMIPENEM	4 (S)	16 (R)	16 (R)	32/16 (R)	16 (R)	>32 (R)
COLISTIN	2 (S)	1 (S)	1/2 (S)	1/2 (S)	1/2 (S)	4 (S)
AMP38	4	32	4/2	16	8/16	0.5/1
TOBRAMICIN	1 (S)	1 (S)	16 (R)	2 (S)	2 (S)	2 (S)
AZTREONAM	8 (S)	2 (S)	2 (S)	>32 (R)	>32 (R)	16 (S)
AMIKACIN	0.5 (S)	1 (S)	>32 (R)	4 (S)	2/4 (S)	32 (R)
CIPROFLOXACIN	0.5 (S)	0.25 (S)	0.5 (S)	2/4 (R)	4 (R)	16 (R)
MEROPENEM	0.5 (S)	4 (I)	4 (I)	2 (S)	4/8 (I)	32 (R)

Complementary studies, such as time-kill kinetics or effect on the growth curve have to be performed to accurately evaluate antimicrobial combinations, mostly to investigate the events occurring during the period of first hours. As seen in Figure 2A, combinations of sublethal concentrations of colistin with 4 µg/mL of imipenem did not increase bacterial death. However, when the concentration of colistin was raised to 4 µg/mL and combined with 0.5 or 4 µg/mL of imipenem, bacterial death was markedly enhanced and similar for both imipenem concentrations.

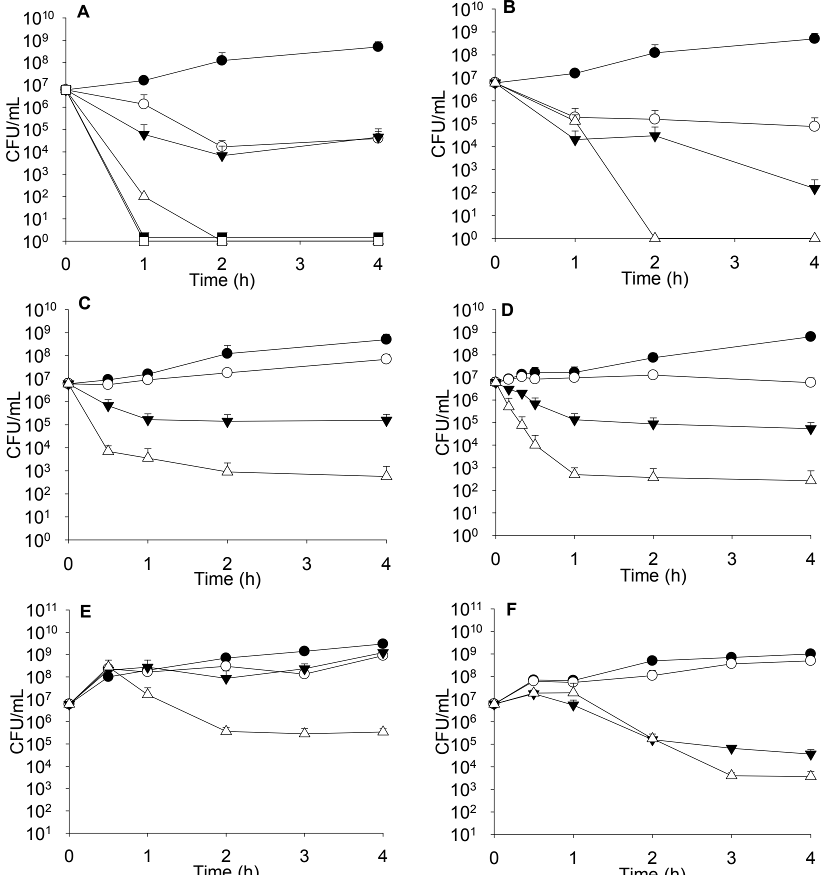

Figure 2. Antibacterial activity of colistin sulphate (COL), imipenem (IMI), meropenem (MER) and AMP38 in different combinations. (**A**) ATCC strain; Control (filled circles), IMI 4 µg/mL (open circles), COL 0.5 µg/mL and IMI 4 µg/mL (filled triangles), COL 4 µg/mL (open triangles), COL 4 µg/mL and IMI 0.5 µg/mL (filled squares), COL 4 µg/mL and IMI 4 µg/mL (open squares); (**B**) PA116136 strain; Control (filled circles), COL 0.25 µg/mL and IMI 32 µg/mL (open circles), COL 2 µg/mL and IMI 4 µg/mL (filled triangles), COL 2 µg/mL and IMI 32 µg/mL (open triangles); (**C**) PA116136 strain; Control (filled circles), IMI 4 µg/mL (open circles), AMP38 8 µg/mL (filled triangles), AMP38 8 µg/mL and IMI 4 µg/mL (open triangles); (**D**) PA116136 strain; Control (filled circles), MER 1 µg/mL (open circles), AMP38 8 µg/mL (filled triangles), AMP38 8 µg/mL and MER 1 µg/mL (open triangles); (**E**) 536SJD strain; Control (filled circles), IMI 4 µg/mL (open circles), AMP38 2 µg/mL (filled triangles), IMI 4 µg/mL and AMP38 2 µg/mL (open triangles); (**F**) 481SJD strain; Control (filled circles), IMI 4 µg/mL (open circles), AMP38 4 µg/mL (filled triangles), IMI 4 µg/mL and AMP38 4 µg/mL (open triangles).

When determining the killing effect on strain PA116136, we found that colistin at 0.25 µg/mL had no effect, but when combined with 4 µg/mL imipenem, a synergistic effect was observed (data not shown). Moreover, higher colistin concentrations (2 µg/mL) exerted a clear concentration-dependent synergistic effect with imipenem, as can be seen in Figure 2B (with 4 and 32 µg/mL imipenem).

Time kill-kinetics with sub-lethal amounts of imipenem and AMP38 are shown in Figure 2C. Assays performed with meropenem are summarized in Figure 2D. Other imipenem-resistant isolates were studied at sublethal concentrations of both AMP38 and imipenem, and with combinations of both (Figure 2E,F). Similar results to those seen in PA116136 were obtained.

Moreover, the combination of both drugs was also assayed for their effect on the growth curve. Figure 3 shows one example where a full inhibition of growth occurred when AMP38 and imipenem were combined at 4 µg/mL of each.

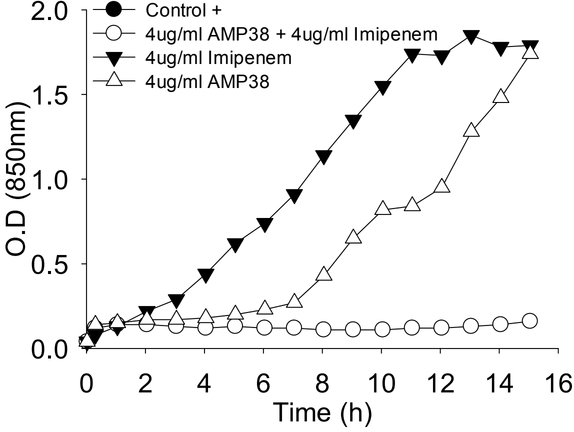

Figure 3. Growth inhibition by the combination of imipenem and AMP38 at 4 µg/mL of each.

To quantitatively determine the interaction between APMP38 and imipenem, FIC (fractional inhibitory concentration) values were calculated. This index is calculated according to the following formula: FIC of drug A (FIC A) = (MIC of drug A in combination)/(MIC of A); FIC of drug B (FIC B) = (MIC of drug B in combination)/(MIC of B). The FIC index (FICi) is calculated by adding FIC A and FIC B. Table 2 shows FICi values of different bacterial strains when tested with combinations of AMP38 and imipenem.

Table 2. Fractional inhibitory concentration index (FICi) values of imipenem and AMP38 combination.

STRAINS	FICi
ATCC	0.62
PA116136	0.14
023VH	0.18
481SJD	0.12
536SJD	0.07

A biofilm of PA116136 was eradicated by the addition of 62.5 µg/mL of both imipenem and AMP38, whereas imipenem alone failed in eradicating the biofilm at concentrations below 500 µg/mL, and AMP38's minimal biofilm eradication concentration (MBEC) was higher than 500 µg/mL (Table 3).

Table 3. Minimal biofilm eradication concentration (MBEC) of imipenem, AMP38, and the combination of both antimicrobials (μg/mL).

Antimicrobial Agent	PA116136
IMIPENEM	>500
AMP38	500
IMIPENEM + AMP38	62.5

TEM observations of ultrathin sections of both *P. aeruginosa* and *Serratia marcescens* are shown in Figure 4. The effect on the bacterial envelopes is in *S. marcescens* is at least apparently identical to the one produced by colistin (formation of blebs), whereas in *P. aeruginosa*, injuries had a different aspect, lacking blebs and appearing as a rough surface.

Acute toxicity was assessed by an in vivo acute toxicity test on CD-1 mice. The lethal dose (LD50) was determined according to the up-and-down procedure. Compound AMP38 was administered subcutaneously at designated doses (100, 200, and 400 mg/kg). Mice treated with 100 and 200 mg/kg of compound survived with no signs of toxicity; whereas mice administered 400 mg/kg died. After 14 days, necropsy of the surviving mice (dosed at 200 mg/kg) showed no signs of pathology in vital organs. LD50 was determined using the maximum likelihood method, and a value of 283 mg/kg was obtained. This value is significantly higher (almost five times) than the LD50 reported for subcutaneous administration of colistin (59.5 mg/kg). Moreover, preliminary cytotoxicity determination in L-929 and HepG2 cells showed no differences between controls and peptide wells up to 100 μg/mL (data not shown).

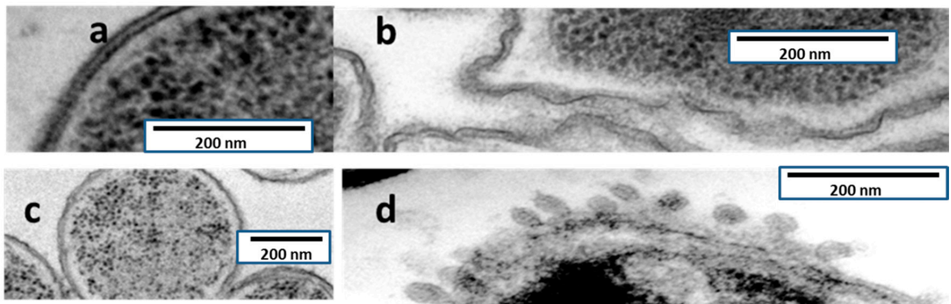

Figure 4. TEM electromicrographs of *P. aeruginosa* PA116136 (**a**) untreated and (**b**) AMP38-treated; and *Serratia marcescens* NIMA strain (**c**) untreated and (**d**) AMP38-treated.

3. Discussion

Colistin has been reported as synergistic with carbapenems and other antimicrobials in *Acinetobacter baumanii*, as well as in *Pseudomonas* and a few other Gram-negative bacteria [15].

Combinations of colistin and imipenem gave a FICi of 0.625 for ATCC 27853; such a value has to be considered as indifferent. Moreover, when assays were performed with AMP38, the FICi value was 0.625—a value identical to the one determined with colistin and imipenem. This suggests that both imipenem and colistin act in a similar manner on this imipenem-susceptible strain. It should be noted that, in principle, the entry of imipenem in fully susceptible bacteria is not prevented by the outer membrane, since OprD is functional and can allow the penetration of imipenem to the periplasmic space, and subsequently, the antibiotic can reach its target. On the contrary, when acting on imipenem-resistant strains, the combination of imipenem and AMP38 gave FICi values below 0.5; this value has to be considered as strongly synergistic (Table 2). PA116136 is imipenem-resistant, since the OprD gene is knocked out by the presence of an insertion sequence [21]; the rest of the resistant

strains are OprD defective. Subsequently, the entry of imipenem is strongly limited in standard culture conditions, and this is the main (if not unique) reason why these bacteria are resistant to imipenem. Thus, the synergism between the peptide and the carbapenem should be interpreted as a consequence of the ability of peptides to open ways by which imipenem can reach PBPs. Similar scenarios were seen in the other resistant strains lacking OprD. Moreover, these results pointed out that the injuries in the outer membrane caused by AMP38 seem to facilitate the entry of imipenem.

FICi values have certain limitations, since they do not give information about the kinetics of bacterial killing. The results are determined after 24 h exposure to antimicrobials, and thus represent a fixed picture of the state after the incubation period. Consequently, with this type of assay, one could disregard the eventual usefulness of antimicrobial combinations for the treatment of complicated microbial infections. Thus, we have performed a series of experiments in order to explore the antimicrobial actions during the first steps of the growth cycle. Combinations of sublethal concentrations of colistin with imipenem failed in increasing bacterial death. On the contrary, the 4 μg/mL concentration of colistin combined with either 0.5 or 4 μg/mL of imipenem markedly enhanced death, irrespective of imipenem concentration; this is consistent with the hypothesis that colistin facilitates the entry of carbapenem, and that eventual resistance would be due to restrictions in the access of imipenem to PBPs (Figure 2A).

Combinations of AMP38 and imipenem enhanced bacterial death. Whereas 8 μg/mL of AMP38 alone had a bacteriostatic effect, the combination of AMP38 (also at 8 μg/mL) plus imipenem (4 μg/mL) resulted in a bactericidal effect—despite the fact that the MIC of imipenem is 16 and that of AMP38 is 32—although the combination failed to completely eradicate the bacterium (Figure 2C). Meropenem at 1 μg/mL had a bacteriostatic effect, keeping bacteria at 10^7 CFU/mL. Nonetheless, the combination of 4 μg/mL of AMP38 plus 1 μg/mL of meropenem completely killed bacteria after 6 h of contact.

Time kill-kinetics with sub-lethal amounts of imipenem and AMP38 and assays performed with meropenem (Figure 2D), as well as those at sublethal concentrations of both AMP38 and imipenem and combinations of both (Figure 2E,F) confirmed that AMP38 facilitates the entry of imipenem by disturbing the outer membrane. Figure 3 shows the effect on the mucous 023VH strain growth curve to be very similar to the results obtained with the rest of strains studied (data not shown).

One of the main goals of antimicrobial chemotherapy is the eradication of biofilms. Stable biofilms can play a key role in pathogenesis, and this is particularly a matter of concern in respiratory infections produced by *P. aeruginosa*. This species is able to produce stable biofilms in many situations, particularly when causing lung infections. Sessile bacteria living in a biofilm state are generally more resistant to antimicrobial agents. The ability of combinations of AMP38 and imipenem to eradicate biofilms was explored. In our experience (data not shown,) the use of colistin to eradicate biofilms needs concentrations higher than 1000 μg/mL; imipenem's MBEC is 500 μg/mL, and that of imipenem is higher than 500 μg/mL, whereas the combination of AMP38 and imipenem was able to completely eradicate the biofilm at 62.5 μg/mL. Thus, a true synergism of imipenem and AMP38 was again observed.

TEM has allowed significant advances in the understanding of bacterial structure and eventually physiology [22], including antibiotic tolerance and biofilm formation. The mechanism of action of AMP38 in *P. aeruginosa* and *S. marcescens* was confirmed by examining untreated and treated bacteria with transmission electron microscopy. Untreated *P. aeruginosa* cells were examined by TEM. Electron micrographs of AMP38-treated *P. aeruginosa* (Figure 4) show a disorganized outer and inner membrane, as compared to the smoothness of untreated membranes. As a control, *S. marcescens* was used. This bacterium is intrinsically resistant to colistin, because although colistin disorganizes its outer membrane, it is not capable of altering the inner membrane [23], and the bacterium remains viable. Typically, the effect of colistin on *S. marcescens* cells is visualized as the production of blebs. We performed an ultrastructural analysis via TEM in order to explore whether colistin and AMP38 share mechanisms of action.

When *S. marcescens* was exposed to colistin, blebs appeared on the outer layer, but no changes were visualized in the cytoplasmic membrane (Figure 4). Figure 4 shows *S. marcescens* exposed to the AMP38, where the same blebs were observed in the outer membrane. Although further research has to be done, the microscopically visualized effects of AMP38 and colistin on Gram-negative bacteria seem to be similar, if not identical.

In conclusion, our data pointed out that the use of synthetic AMPs inspired by natural products that are potentially less toxic [18] than the natural parent compound may contribute to the rescue of antimicrobial agents to which some pathogens have become resistant. In this case, it seems feasible to kill imipenem-resistant *Pseudomonas aeruginosa* with imipenem when combined with other molecules (such as AMP38) which are able to sensitize the bacterium to the antibiotic. Since carbapenems remain the main antimicrobials for treating multidrug-resistant *P. aeruginosa* infections, and the development of carbapenem resistance may significantly compromise their efficacy, the use of peptides such as AMP38 could serve to rescue the use of carbapenems for these purposes. Their use should give better results than the use of colistin and polymyxins, which have already demonstrated synergistic effects [24].

4. Experimental Section

4.1. Bacterial Strains, Media, and Antimicrobial Substances

The imipenem-resistant *P. aeruginosa* PA116136 isolate was obtained from a patient with chronic pulmonary disease at the Servei de Microbiologia of the Hospital Universitari de Bellvitge (L'Hospitalet de Llobregat, Barcelona, Spain). It has an insertion sequence (ISPa133) sited just before nucleotide 697. This interrupts the coding region, producing a loss of OprD expression and consequently contributes to the resistance to carbapenems [25]. *P. aeruginosa* 536SJD and 481SJD isolates were obtained from patients with chronic pulmonary disease at the Servei de Microbiologia of the Hospital Sant Joan de Déu (Esplugues de Llobregat, Barcelona, Spain). *P. aeruginosa* 023VH and 846VH isolates were obtained from cystic fibrosis patients at the servei de Microbiologia of the Hospital Universitari Vall d'Hebron (Barcelona, Spain). *P. aeruginosa* ATCC 27853 served as a control strain in susceptibility tests. Cation-adjusted Mueller–Hinton (CAMHB, Becton, Dickinson and Company, San Agustin de Guadalix, Madrid, Spain) was used to determine minimum inhibitory concentrations (MICs) and minimal biofilm eradication concentration (MBEC). Tryptic soy agar (TSA, Scharlau Microbiology, Sentmenat, Spain) was used for the determination of colony counts in time-kill assays. *Serratia marcescens* strain nima [26] was used as a control in electron microscopy. Imipenem monohydrate, tobramycin sulfate, amikacin sulfate, ciprofloxacin, and aztreonam were obtained from Sigma-Aldrich (Madrid, Spain); colistin sulphate was kindly supplied from Zhejiang Shenghua Biok Biology Co., Ltd., (Shanghai, China), and the cyclolipopeptide analog of polymyxin AMP38 was synthetized by us.

4.2. Peptide Synthesis and Purification

The synthesis of peptide AMP38 was performed manually following standard Fmoc/tBu procedures using DIPCDI/HOBt activation on Rink amide resin. Once the sequence was assembled, cleavage of the peptide from the resin was carried out by acidolysis with Trifluoroacetic acid /triisopropylsilane/water (95:3:2, *v*/*v*) for 90 min. TFA was removed with a stream of nitrogen gas. The oily residue was treated with dry diethyl ether, and the precipitated peptide was isolated by centrifugation. The homogeneity of the crude peptide was assessed by analytical HPLC on Nucleosil C18 reverse-phase columns (4 mm× 250 mm, 5 µm particle diameter, and 120 Å porous size). Elution was carried out at 1 mL·min^{-1} flow with mixtures of H_2O containing 0.045% TFA and acetonitrile containing 0.036% TFA, with UV detection at 220 nm. Cyclization of the peptide was carried out in 5% dimethylsulphoxide aqueous solution for 24 h and lyophilized twice. The peptide was subsequently purified by preparative HPLC on a Waters DeltaPrep 3000 system with

a Phenomenex C18 [18,27–29] column (250 mm × 10 mm, 5 μm) eluted with H_2O/acetonitrile/0.1% TFA gradient mixtures and UV detection at 220 nm. Final purity was greater >99% according to analytical HPLC. The peptide was characterized by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry with a PerSeptive Biosystems Voyager-DE instrument. MALDI-TOF MS, m/z ($C_{55}H_{96}N_{16}O_{12}S_2$): 1237.9 $[M + H]^+$, 1259.7 $[M + Na]^+$, 1275.7 $[M + K]^+$, 1219.9 $[M - H_2O]^+$ (Figure 1).

4.3. Determination of Minimum Inhibitory Concentrations

MIC values were determined by broth microdilution method and interpreted according to European Committee on Antimicrobial Susceptibility Testing EUCAST guidelines [30].

4.4. Synergy Study

A checkerboard test was used to determine the fractional inhibitory concentrations (FICs) of colistin in combination with the peptide AMP38. Each well in a 96-well plate was inoculated with 100 μL of a bacterial inoculum of 1×10^5 CFU/mL, and the plates were incubated at 37 °C for 24 h. The FIC was calculated after identifying the first well in each row without growth (MIC), according the following formula: FIC of drug A (FIC A) = (MIC of drug A in combination)/(MIC of A); FIC of drug B (FIC B) = (MIC of drug B in combination)/(MIC of B). The FIC index (FICi) values were calculated by adding the FIC of imipenem to the FIC of AMP 38. FICi values were interpreted as follows [21]: FICi < 0.5, synergistic; FICi ≥ 0.5 and < 4, no interaction; FICi > 4, antagonistic [31].

4.5. Time–Kill Curves

Killing curve assays were performed with a starting inoculum of 6×10^6 CFU/mL. Strains were tested against colistin, imipenem, meropenem, tobramycin, amikacin, ciprofloxacin, aztreonam, and AMP38 alone and in all possible combinations at concentrations above and below the MICs. Antimicrobials were added to 10 mL of bacteria in the exponential phase of growth and incubated at 37 °C with shaking. Samples were obtained aseptically at 0.5, 1, 2, and 4, diluted in Ringer 1/4 and plated on TSA for colony counting. The response of microbial strains to a single antimicrobial and to the combinations of pairs of antimicrobials was determined by lowering logarithms of viable bacteria.

In accordance with Lora-Tamayo et al. [26], an antimicrobial was considered active when a reduction of ≥1 log10 relative to the initial inoculum was observed. A combination of antimicrobials was considered synergistic when a reduction of ≥2 log10 was observed with respect to the most active antimicrobial. A combination of two antibiotics was considered additive when the logarithm of reduction was between 1 and 2.

4.6. Transmission Electron Microscopy

Both *P. aeruginosa* PA116136 and *S. marcescens* nima strains were observed by TEM after treatment with AMP38 or colistin, using untreated bacteria as controls. Bacteria in the exponential phase of growth were centrifuged at 8000× *g* for 10 min to obtain a concentration of 10^8 CFU/mL. Pellets were then suspended in Tripticase Soy Broth (TSB) and antimicrobials were added (300 mg/L for *S. marcescens* and 100 mg/L for *P. aeruginosa* for both colistin and AMP38). Bacteria were incubated for 20 min at 37 °C and harvested by centrifugation. Bacteria were cryo-immobilized by using a Leica HPM100 high-pressure freezer (Leica, Vienna, Austria). Frozen samples were freeze-substituted (Leica Microsystems, AFS2) for 72 h at −90 °C. Temperature was gradually increased to 4 °C, and then samples were stored at room temperature. Substitutions were performed in pure acetone containing 2% (*w/v*) osmium tetroxide and 0.1% (*w/v*) uranyl acetate. Afterwards, samples were washed with acetone and gradually infiltrated in a resin Epon. Samples were embedded in fresh Epon and polymerized at 60 °C for 48 h. Ultrathin sections (50–70 nm) were made with the Leica Ultracut UC6 (Leica, Vienna, Austria). Epon-embedded thin sections were examined in a JEM1010 JEOL transmission electron

microscope (JEOL Ltd., Tokyo, Japan) by working with a tungsten filament at 80 kV. Images were acquired with the software Analysis (version 3.2, Soft Imaging System GmbH, Münster, Germany).

4.7. MBEC Determination

Minimal biofilm eradication concentration (MBEC) determinations were conducted as described by Moskowitz et al. [32] with a few modifications. Briefly, bacterial biofilms were formed by immersing the pegs of a modified polystyrene microtiter lid (catalog No. 445497; Nunc TSP system) into 96-well microtiter plates containing 200 µL of CAMHB each, followed by incubation at 37 °C for 24 h in static conditions. Pegs were then gently rinsed in 0.9% NaCl solution, and biofilms were exposed to different concentrations of antimicrobials for 24 h at 37 °C. Pegs were again rinsed with 0.9% NaCl solution and biofilms were removed by 10 min sonication. Recovered bacteria were incubated for 24 h at 37 °C. Optical densities at 620 nm were measured in order to determine MBEC values, defined as the lowest concentration of antimicrobial that prevented bacterial regrowth from the treated biofilm. All experiments were performed in triplicate.

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Author Contributions: M.V. and F.R. conceived and designed the experiments; H.R. and E.F. performed the microbiology experiments; Y.C. and F.R. performed the chemistry experiments, M.V., T.V. and F.R. analyzed the data; M.V. wrote the paper. All authors significantly contributed substantially to the work reported.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compound AMP38 are available from the authors.

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4 . PAPER 2

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Novel synthetic polymyxins kill Gram-positive bacteria

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Background: *Staphylococcus aureus*, including 'superbug' MRSA, is a major cause of nosocomial infections. In the European Union, up to 171 200 new nosocomial MRSA infections are acquired annually, and in the USA *S. aureus* causes more deaths than HIV/AIDS and tuberculosis combined. MRSA is also the first group of pathogens that infect the pulmonary tract in young patients with cystic fibrosis.

Objectives: We describe two newly developed and synthesized colistin (polymyxin E)-inspired molecules.

Methods: A collection of several isolates of *S. aureus* [including MRSA and vancomycin-resistant *S. aureus* (VRSA)] was tested. To check the antimicrobial activity, we performed time–kill curves, growth curves, biofilm eradication, toxicity and isothermal titration calorimetry.

Results: Both peptides showed high antimicrobial activities (MIC 4 mg/L) and low relative toxicities (selectivity index close to 23).

Conclusions: Successful production of polymyxin-scaffold molecules active against *S. aureus*, both MRSA and VRSA, opens up new approaches to the treatment of these complicated infections.

Introduction

Among the world's most dangerous 'superbugs' are MDR bacteria of the so-called ESKAPE group.[1,2] The proportion of these bacteria in common infections has increased recently.[3] Moreover, patients with vascular prostheses, indwelling catheters, dialysis shunts, etc., are particularly vulnerable to the virtually untreatable infections resulting from ESKAPE colonization and subsequent biofilm formation.

The indiscriminate use of penicillin has increased the percentage of penicillin-resistant *Staphylococcus aureus* strains to nearly 100%.[4] The same is true for several other antibiotics.

Overexpression of penicillin-binding protein 2A in MRSA enables survival in the presence of high concentrations of β-lactams, including methicillin.[5] Isolates with intermediate or full resistance to vancomycin emerge.[4] Owing to this, antimicrobial peptides (AMPs) have become very important drugs in the treatment of Gram-positive infections. None the less, strains resistant to AMPs have been recently identified.

Amphipathic polymyxins are cationic non-ribosomal antimicrobial peptides (CAMPs) produced by *Paenibacillus polymyxa* that became available as antimicrobials in 1949 and were subsequently abandoned because of their nephrotoxicity and neurotoxicity.[6] However, colistin and polymyxin B have recently been rehabilitated for the treatment of infections caused by MDR Gram-negative organisms, mainly *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.[7] Although polymyxins interact with the negatively charged outer membrane of Gram-negative bacteria,[8] their mechanisms of action may additionally include alterations in ribosome binding, respiration, cell division, cell structure and the production of reactive oxygen species.[9] Gram-positive organisms lack an outer membrane and are thus naturally resistant to polymyxins.

The synthesis of new molecules that are less toxic and have greater activity than conventional antibiotics is challenging. One strategy is to make small changes in known molecules, such as polymyxin, to generate derivatives effective against Gram-positive bacteria. The antimicrobial activity of two novel synthetic cyclolipopeptide analogues of polymyxin (CAMP113 and CAMP207) against *S. aureus* and other Gram-positive bacteria is reported here.

Materials and methods

Bacterial strains and growth conditions

The clinical *S. aureus* strains used are listed in Table S1, available as Supplementary data at *JAC* Online. *S. aureus* ATCC 29213 and ATCC MRSA

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Figure 1. Chemical structure of the cyclopolypeptides CAMP113 and CAMP207 (below) and polymyxin B (above). Common structural and chemical features that were modified are highlighted in grey. Differences in the structural features of the two synthetic peptides are indicated in the boxes R and Y.

700698 served as controls. Mueller–Hinton (Becton, Dickinson and Company, Madrid, Spain) broth was used to determine MICs and minimal biofilm eradication concentrations (MBECs), tryptic soy agar for colony counting and tryptic soy broth (Scharlau Microbiology, Sentmenat, Spain) with 0.25% glucose for biofilms. Colistin sulphate was supplied by Zhejiang Shenghua Biok Biology Co., Ltd (Shanghai, China). Other antibiotics were from Sigma–Aldrich (Madrid, Spain). The cyclopolypeptides CAMP207 and CAMP113 were synthesized in our laboratory as described below.

Peptide synthesis and purification

Peptides CAMP113 and CAMP207 were synthesized manually following standard Fmoc/tBu methods and characterized by MALDI-TOF MS using a PerSeptive Biosystems Voyager-DE instrument [MALDI-TOF MS: *m/z* ($C_{55}H_{96}N_{16}O_{12}S_2$): 1237.9 (M + H)+, 1259.7 (M + Na)+, 1275.7 (M + K)+, 1219.9 (M − H_2O)+] (Figure 1).[10]

MIC determination

Broth microdilution was used and interpreted according to EUCAST guidelines.[11]

Time–kill curves and growth curves

Killing curves were obtained with a starting inoculum of 5×10^5 cfu/mL against CAMP113 and CAMP207. Antimicrobials were added to exponentially growing bacteria at 37°C with shaking. Samples were retrieved aseptically at 1, 2, 4 and 6 h, diluted in ¼× Ringer and plated on tryptic soy agar. The response to a single antimicrobial was determined based on a logarithmic decrease in viable bacteria. The effect of CAMPs on the growth of clinical *S. aureus* was assayed with starting inocula of 10^6 and 10^8 cfu/mL.

MBEC determination

Bacterial biofilms were formed by immersing the pegs of a modified polystyrene microtitre lid (catalogue no. 445497; Nunc TSP system) into 96-well microtitre plates containing 200 μL of medium and incubated 37°C for 24 h. Pegs were gently rinsed in 0.9% NaCl solution, and biofilms exposed to CAMPs. Pegs were again rinsed with 0.9% NaCl solution and biofilms removed by sonication. Recovered bacteria were incubated for 24 h at 37°C. Optical densities at 620 nm were determined. MBEC is defined as the lowest concentration of antimicrobial that prevented bacterial regrowth from the treated biofilm.

Toxicity of peptides in human hepatocytes

Colistin, CAMP113 and CAMP207 were assayed for *in vitro* toxicity against human hepatocytes (HepG2) as previously described.[12]

Confocal laser scanning microscopy imaging

Biofilms were washed with ¼× Ringer and treated with antimicrobials. After 4 h of incubation (37°C), biofilms were rinsed with ¼× Ringer and stained using the Live/Dead BacLight kit (Thermo Fisher Scientific, Eugene, OR, USA). Fluorescence was observed using a Leica TCS-SL filter-free spectral confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with a 488 nm argon laser and 543 and 633 nm He/Ne lasers. All experiments were

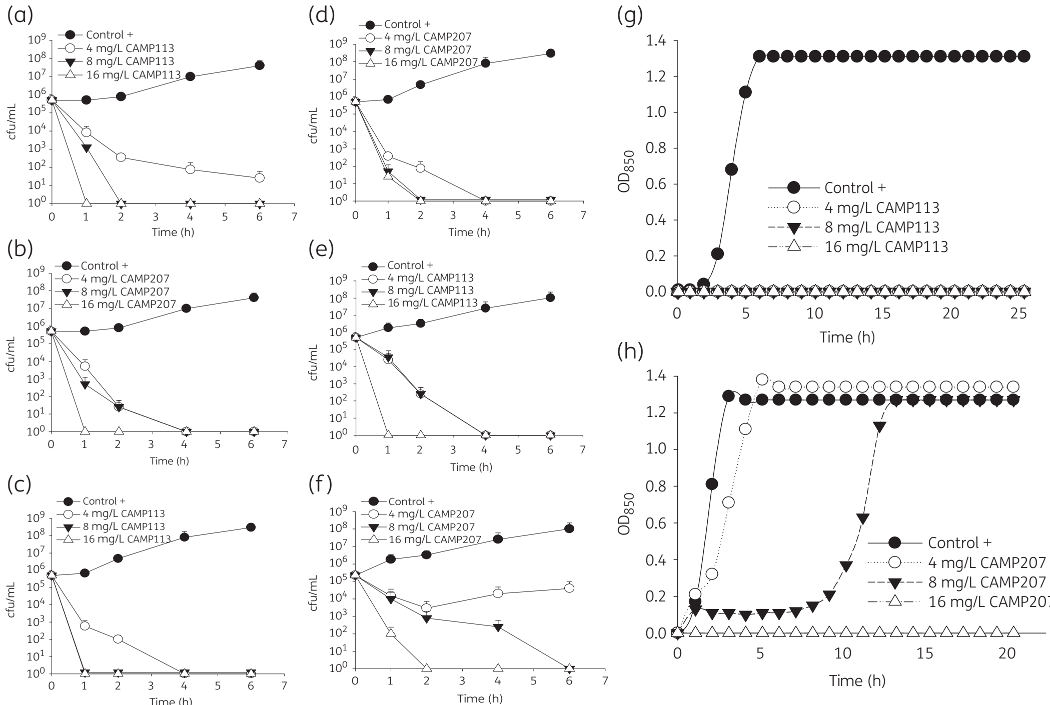

Figure 2. Antimicrobial activity of CAMP113 and CAMP207. (a) Strain SaS06 incubated with CAMP113. (b) Strain SaS06 incubated with CAMP207. (c) Strain SaS16 incubated with CAMP113. (d) Strain Sas16 incubated with CAMP207. (e) Strain Sas18 incubated with CAMP113. (f) Strain Sas18 incubated with CAMP207. (g) Growth curve of strain SaS16 (inoculum of 10^6 cfu/mL) in the presence of CAMP113. (h) Growth curve of strain SaS06 (inoculum of 10^8 cfu/mL) in the presence of CAMP207.

performed in duplicate. The images were analysed using ImageJ software (NIH, Bethesda, MD, USA). The percentages of live and dead bacterial cells were calculated from the total cell number.

Results and discussion

Peptides CAMP113 and CAMP207 have a disulphide bond between two cysteine residues. Moreover, they contain a dodecanoyl chain instead of (*S*)-6-methyloctanoyl, have a Cys residue at position 4, norleucine (NLe) at position 7 and (D)-Cys at position 11, instead of 2,4-diaminobutyric acid, Leu and Thr of polymyxin. CAMP113 was further modified with (D)-Phe and CAMP207 with (D)-(4F)-Phe instead of Leu at position 6. In addition, an Arg was substituted at position 8 of CAMP113 whereas colistin contains 2,4-diaminobutyric acid (Figure 1).

Polymyxin action involves interaction with lipid A and the displacement of Mg^{2+} and Ca^{2+} bridging adjacent LPS molecules.[13] The MIC of both peptides was 4 mg/L. Tested strains were resistant to colistin (>512 mg/L) and susceptible to vancomycin (1–0.5 mg/L), except for SasVR (Table S1). The activity of the two CAMPs was high, their MICs being within the clinical use range. MIC values represent only the cumulative effect at 18 h, rather than the time course of activity, whereas time–kill kinetics or growth curves describe the antimicrobial action, providing insights into the events during its first hours and depicting the biological behaviour over time. As expected based on their chemical similarities both peptides killed the bacteria in a concentration-dependent manner (Figure 2), their behaviour being very similar irrespective of the strain tested.

Two types of responses were observed in the presence of CAMPs: either the bacterium was completely inhibited at all concentrations tested or inhibition was achieved only at high concentrations (Figure 2). Neither peptide was able to eradicate fully the bacteria solely as a function of its concentration; rather, the activities of the two CAMPs strongly depended on the bacterial strain. For example, CAMP113 fully eradicated strain SaS06 (Figure 2a) in 1 h at 16 mg/L and in 2 h at 8 mg/L, but a few individual bacteria survived at 4 mg/L whereas strain SaS16 was completely eradicated at this latter concentration (Figure 2). At a concentration of 8 mg/L or higher, CAMP113 produced the rapid death of strain SaS16 (Figure 2c) during the first hour whereas at an equal concentration of CAMP207 (Figure 2) 2 h was required. At 4 mg/L, both peptides required 4 h to kill fully the strain SaS16. In tests of strain SaS18, complete eradication was achieved within 4 h using 8 mg/L CAMP113 but within 6 h using the same concentration of CAMP207 (Figure 2f). However, even at 4 mg/mL, CAMP207 completely failed to eradicate SaS18. Similar outcomes were obtained with the remainder of the tested strains.

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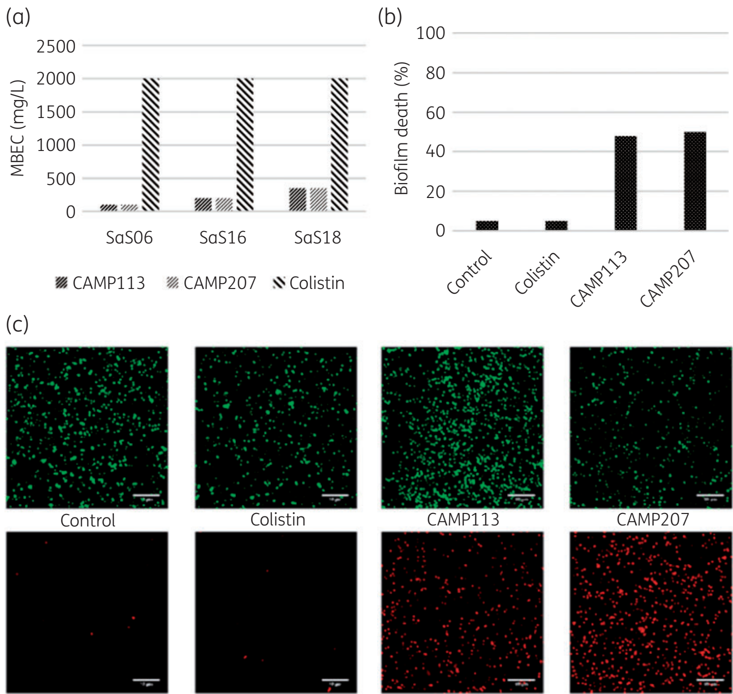

Figure 3. (a) Minimal biofilm eradication concentration assays. Strain SaS06 (CAMP113, 128 mg/L; CAMP207, 128 mg/L; colistin >2048 mg/L). Strain SaS16 (CAMP113, 32 mg/L; CAMP207, 32 mg/L; colistin >2048 mg/L). Strain SaS18 (CAMP113, 256 mg/L; CAMP207, 256 mg/L; colistin, >2048 mg/L); (b) Percentage of biofilm death after 4 h of exposure; (c) Confocal laser scanning microscopy assays. Images (1024×1024 pixels) were taken after 4 h of exposure to: control, colistin, CAMP113 or CAMP207. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Figure 2 also shows representative growth curves. With inocula up to 2×10^6 cfu/mL, growth was fully prevented irrespective of the CAMP and the concentration tested (4, 8 or 16 mg/L). Higher inocula (up to 2×10^8 cfu/mL) gave initial growth delay at 4 mg/L, and a longer delay at 8 mg/L. Finally, both CAMPs were able to inhibit fully the growth at concentrations of 16 mg/L, demonstrating the concentration dependence.

S. aureus have evolved mechanisms to evade innate host defences including proteolysis of natural peptides, secretion of lytic enzymes, modulation of the electrical charge of the cell surface to enhance repulsion, and biofilm formation.[14] Biofilms have been the focus of considerable research attention and their destruction the main goal of current antibacterial therapy. The MBECs of both CAMPs indicated the ability of these compounds to eradicate fully the *S. aureus* biofilms at relatively low concentrations, in contrast to colistin, which had no effect. Confocal imaging (Figure 3) revealed the effects of the CAMPs (at 128 mg/L) on *S. aureus* biofilm during short exposure periods (4 h). No significant death occurred in either the controls or colistin-treated biofilms, whereas CAMP113 and CAMP207 were able to kill roughly one-half the bacteria within the biofilm (47.27% and 50.46%, respectively). Given the current interest in CAMPs in the search for new approaches in anti-biofilm treatment,[15] our study demonstrates the potency of these peptides in targeting biofilms of several clinical isolates of *S. aureus*. Under conditions in which a colistin dose of 2048 mg/L was required, CAMP113 and CAMP207 eradicated the biofilms of all strains tested at much lower concentrations (Figure 3).

The CAMPs were also tested for their cytotoxicity in the human hepatocellular cell line HepG2 (ATCC HB8065). As shown in Figure 4, colistin had relative low cytotoxicity *in vitro* as its mechanism of toxicity involves the accumulation of molecules in organs such as the kidney. At 5 mg/L, a slightly higher concentration than the MICs of the CAMPs, none of the tested compounds had detectable toxicity. A concentration of 50 mg/L yielded 89.49% HepG2 cell viability for colistin and 67.03% and 62.62% for CAMP113 and CAMP207, respectively. At 100 mg/L, colistin resulted in 68.75% HepG2 viability, CAMP113 53.2% and CAMP207 57.3%. At an antimicrobial concentration of 250 mg/L, HepG2 viability was 51.05%, 16.01% and 8.29%, respectively. At 500 mg/L, whereas 38.39% of the cells survived in the presence of colistin both CAMP113 and CAMP207 were highly toxic (7.74% and 7.4%, respectively) (Figure 4). None the less, the selectivity index (the relationship between the IC_{50} and the MIC) in *S. aureus* strains[16] was inordinately high for the peptides (21.25 for CAMP113 and 23.75 for CAMP207 compared with 0.36 for colistin), i.e. CAMP113 and CAMP207 are much more selective than colistin because the concentrations needed to kill the bacteria are much lower. Moreover, colistin is nephrotoxic, as it increases tubular epithelial cell membrane

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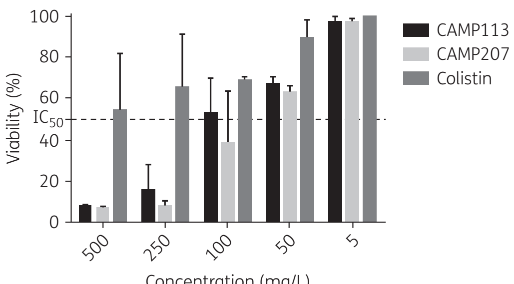

Figure 4. Toxicity of CAMP113 and CAMP207 towards HepG2 human hepatocyte cells.

permeability to cations, anions and water, leading to cell swelling and cell lysis.[17] The presence in the newly synthesized CAMPs of a disulphide bond facilitates their biodegradation and thus avoids their accumulation in the kidney. This is in agreement with previous studies, in which the LD_{50} for peptides of this family in CD-1 mice was 283 mg/kg, compared with the 59.5 mg/kg determined for polymyxin.[10]

The emergence of MDR strains of several species has posed a tremendous challenge in clinical practice and renewed interest in polymyxins, whose activity against Gram-negatives is well established. Colistin is considered the drug of last resort in the treatment of Gram-negative MDR bacteria such as *P. aeruginosa*, *A. baumannii* and *Klebsiella pneumoniae*.[18,19] None the less polymyxins are intrinsically inactive against Gram-positive bacteria because they lack lipid A, the primary binding site of polymyxins.[18,20]

Novel peptides synthesized in an attempt to evade the increasing polymyxin resistance of Gram-negative bacteria have low MIC values in Gram-positive organisms.[8] Similarly, our peptides showed activity against *S. aureus*. Teichoic acids have a chemical structure similar to that of the LPS regions to which polymyxins bind.[15] To determine whether teichoic acids were the targets of CAMP113 and CAMP207, we performed isothermal titration calorimetry experiments. The results showed a three-step reaction between the peptides and teichoic acid, two of which were exergonic and the third endergonic (Figure S1).

To examine the spectrum of action of the CAMPs studied, we explored their action against Gram-positive *Streptococcus pneumoniae*. Our results showed that in this species the MIC of colistin was consistently >32 mg/L, whereas the MICs of both our peptides were between 2 and 4 mg/L depending on the isolate.

Polymyxins, regarded as inactive against Gram-positive organisms, after some small changes, have activity against *S. aureus* and *S. pneumoniae*; subject to more detailed determination of *in vitro* and *in vivo* toxicity as well as the development issues, peptides of this type may open up new frontiers in the treatment of 'untreatable' infections.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online.

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Supplementary material:

Supplementary data

Table S1. Antimicrobial susceptibility (minimum inhibitory concentration, MIC; mg/L).

	Origin	CAMP113	CAMP207	CST	VAN	LZD	ERY	TET	CIP
ATCC 700698*	Sputum	4	4	>512	1(S)	4(S)	>32(R)	>32(R)	>32(R)
ATCC 29213	Wound Smear	4	4	>512	1(S)	4(S)	1-2(S)	1(S)	1(S)
SaSVR	*[1]Conj. exd*	2-4	4-8	>512	>32(R)	>32(R)	>32(R)	1-2	4(R)
SaSVI	*[2]Spleen nec.*	16	4-8	>512	2-4	4(S)	>32(R)	>32(R)	0,25-0,5(S)
SaS01	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	1(S)	2-4(R)
SaS02	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	1(S)	1(S)
SaS03	Wound Smear	4	4	>512	1(S)	4(S)	>32(R)	0,5-1(S)	1(S)
*SaS04**	Wound Smear	4	4	>512	0,5(S)	4(S)	>32(R)	0,125(S)	>32(R)
*SaS05**	Wound Smear	4	4	>512	0,5(S)	2(S)	>32(R)	>32(R)	>32(R)
*SaS06**	Sputum	4	4	>512	1(S)	4(S)	0,5(S)	0,125(S)	>32(R)
*SaS07**	Nasal Smear	4	4	>512	0,5	8(R)	0,25(S)	4(R)	>32(R)
*SaS08**	Wound Smear	4	4	>512	1(S)	4(S)	>32(R)	0,5 - 1(S)	>32(R)
SaS09	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	1(S)	>32(R)
SaS10	Urine	4	4	>512	1(S)	4(S)	1(S)	1(S)	0,5-1(S)
*SaS11**	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	2(R)	>32(R)
*SaS12**	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	2(R)	>32(R)
*SaS13**	Total Blood	2	2-4	>512	0,5(S)	4(S)	1(S)	8(R)	0,5(S)
*SaS14**	Wound Smear	4	4	>512	0,5(S)	4(S)	>32(R)	0,5(S)	>32(R)
*SaS15**	Total Blood	4	4	>512	1(S)	4(S)	1(S)	>32(R)	>32(R)
SaS16	Skin Smear	4	4	>512	1(S)	4(S)	1(S)	0,5(S)	>32(R)
SaS17	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	8(R)	4(R)
*SaS18**	Wound Smear	4	4	>512	1(S)	4(S)	>32(R)	4(R)	4(R)

R: resistant; S: susceptible; CST, colistin; VAN, vancomycin; LZD, linezolid; ERY, erythromycin; TET, tetracycline; CIP, ciprofloxacin.

* MRSA. *[1]Conjunctival exudate. *[2]Sample from a spleen necropsy.

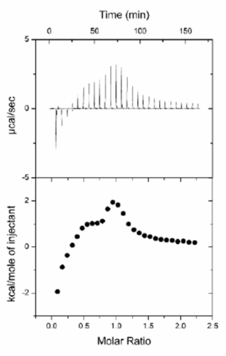

Figure 4.1: Isothermal titration calorimetry curve for CAMP207 (5290 µM) vs. teichoic acids (500 µM)

5 . REVIEW

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Review

New and old tools to evaluate new antimicrobial peptides

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Abstract: The emergence of antimicrobial resistance due to the overuse of antimicrobials together with the existence of naturally untreatable infections well demonstrates the need for new instruments to fight microbes. Antimicrobial peptides (AMPs) are a promising family of molecules in this regard, because they abundantly occur in nature and the results of preliminary studies of their clinical potential have been encouraging. However, further progress will benefit from the standardization of research methods to assess the antimicrobial properties of AMPs. Here we review the diverse methods used to study the antimicrobial power of AMPs and recommend a pathway to explore new molecules. The use of new methodologies to quantitatively evaluate the physical effect on bacterial biofilms such as force spectroscopy and surface cell damage evaluation, constitute novel approaches to study new AMPs.

Keywords: antimicrobial peptides; bacteria; protozoan; fungi; atomic force microscopy; confocal microscopy; growth curves; death kinetics

1. Introduction

Bacteria are the leading cause of infections worldwide but they affect individuals in developed and third world countries in different ways. In the latter, most bacterial infections are those known as classical, whereas in developed areas hospitalized patients or individuals who have undergone medical treatments, such as surgery, solid organ transplantation, and anticancer treatments, are the most vulnerable. In recent years, the progressive increase in the incidence of multidrug-resistant

bacterial infections has raised concern. In 2014, the office of the President Obama published a detailed report on antimicrobial resistance, leading to the publication, on September 18, 2014, of an Executive Order entitled "Combating Antibiotic-Resistant Bacteria" (https://obamawhitehouse.archives.gov/the-press-office/2014/09/18/executive-order-combating-antibiotic-resistant-bacteria). This document included sections on new policies, changes in funding, and recommendations, among others. Following the election of Donald Trump, more than 30 scientific and social alliances and societies signed a document in which they expressed the hope these investments would be maintained and even expanded. Also in 2014, the World Health Organization (WHO) published, an extended report calling attention to this crucial health problem (www.who.int/drugresistance/documents/surveillancereport/en/). Bacterial resistance to antimicrobials has likewise attracted the attention of the governments of several other countries.

However, antibiotics research had an erratic history. The clinical use of antimicrobials started approximately in 1932, with the release of Prontosil (an antibacterial drug discovered at Bayer Laboratories, Germany), a molecule with a lethal effect on gram-positive cocci. Prontosil was the first sulphonamide and it ushered in the antibiotic era. Thereafter, new antibiotics were rapidly discovered, including penicillin and streptomycin, and became available for clinical use.

Recently, due mostly to economic, rather than to medical or scientific reasons, the pipeline of novel antimicrobial molecules under development has mostly closed [1]. Instead, most of the drugs under development are improved derivatives of those already on the market. This has had several non-negligible consequences. Firstly, modified compounds, while frequently enlarging the spectrum of drug activity or enhancing its antimicrobial effect, do not change the target, including the resistance mechanisms promoted in organisms exposed to these agents. New molecules, acting through newly recognized mechanisms of action and on different targets, are expected to be much more effective, but very few have been developed over the past several years. The recent emergence of new mechanisms of resistance and the scarcity of novel antimicrobial products able to target them account for the current growing concern and the revival of research efforts.

The main sources of the thousands of antibiotics discovered during the golden era of antibiotics were soil bacteria and fungi. In fact, antibiotics were long regarded as defense mechanisms of soil microbes, although this function has yet to be definitively demonstrated in nature [2]. Although the further exploration of natural products for their antibiotic activities is expensive and the chance of successes limited, the identification of not previously appreciated delivery methods or products, including those derived from natural molecules, will open up new research perspectives regarding antimicrobials [3–7].

Once a candidate molecule has been synthesized and purified, its properties, activities, and efficacy, but also its toxicity, must be investigated at the biological level. Clearly, a drug should exhibit greater toxicity towards microorganisms than on its human hosts. Moreover, not only bacteria but also fungi and protozoa produce infections. Therefore, in the process of exploring the antimicrobial action of a new molecule, the possible effect on other infectious microbes should be investigated as well.

This review examines the main methods used to investigate new antimicrobials and, in the form of a flow diagram presented at the end, describes the main criteria for their development.

2. Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is a parameter widely used to assess the

susceptibility of microbes. It is defined as the lowest antimicrobial drug concentration that prevents visible growth of the microorganism after an overnight incubation. An advantage of the MIC is that it is quantitative and, if standardized procedures are used, the values obtained by different laboratories can be compared. Moreover, national agencies, mainly the USA's Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have published cut-off points based on criteria that are relatively homogeneous worldwide.

Either solid (incorporating antimicrobials into the agar medium) or liquid medium can be used to determine MIC values. Liquid medium tests are conducted using either the microdilution (96-well microtiter plates) or the macrodilution (test tubes) method. However, while an excellent parameter for clinical purposes, the MIC has serious limitations in research. First, the inoculum density strongly influences the MIC value. Second, the definition of the MIC is vague, as, for example, neither "visible growth" nor "overnight" (typically 18 h) is well-defined. Moreover, visible growth is determined with the naked eye, and the MIC is a fixed value determined after 18 h of incubation. However, while the presence of visible growth after 18 h should be interpreted as a lack of antimicrobial action, this may not precisely be the case, as illustrated by a simple theoretical example: Microbe A is susceptible to 4 μg of drug X/mL, and a fully resistant isolate (B) is resistant to 200 μg of drug X/mL. After incubation of microbes A and B in medium containing 4 μg X/mL, the growth was visible in both tubes (Figure 1), but the history of the cultures is completely different. Whereas the growth of culture B started immediately, as in the control experiment, culture A was inhibited for the first 12 h, during which time the antibiotic concentration was >3 μg/mL, but began to grow thereafter. This is frequently referred to as "regrowth" and is discussed controversially, although its existence demonstrates the weakness of MIC as a parameter. Moreover, antimicrobial action may be bactericidal, in which the peptide kills the bacterium, or bacteriostatic, in which bacterial growth is completely inhibited (Figure 2). These two mechanisms can be distinguished by plotting a growth curve of the bacterium in the presence and absence of antimicrobials.

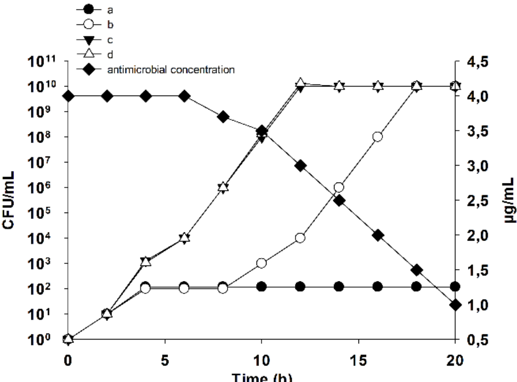

Figure 1. Comparison of the growth curves of different bacteria in the presence of an antibacterial agent added at 4 h. The growth kinetics of a control culture (open triangles), a resistant microbe (filled triangles), a moderately susceptible microbe (open circles), and a fully susceptible microbe (filled circles) are shown. Filled squares represent antimicrobial concentration.

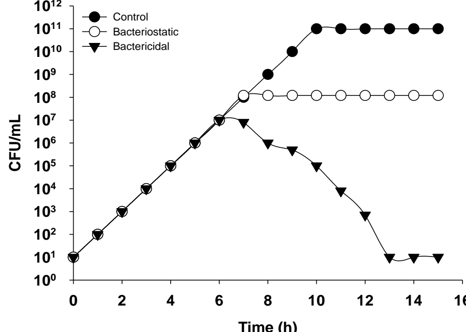

Figure 2. Growth kinetics of a bacterium in the presence of a bacteriostatic (open circles) and a bactericidal (filled triangles) agent.

3. Minimal biocidal concentration

A much more easily defined and informative parameter is the minimal biocidal concentration (MBC): the lowest concentration of an antimicrobial agent required to kill a particular bacterium. Bacterial killing is confirmed when the test culture contains no growing cells. However, the MBC also depends on the chosen methodology. For instance, growth is a function not only of the experimental conditions but also the metabolic state of the bacterium. Thus, bacteria may be viable but non-cultivable (VBNC) due to their poor metabolic activity. Nonetheless, while unable to divide, they are still alive because under the appropriate conditions allowing their "resuscitation" they are "re-cultivable". VBNC unable to grow in standard medium are smaller and have lower levels of nutrient transport, ATP production, and macromolecular biosynthesis but they may still be able to survive for as long as one year [8]. Clinically, VBNC may be the source of recurrent infections but they are not taken into account in MBC determinations. Furthermore, experimental MBC values are often beyond the limits that are pharmacologically achievable in a therapeutic setting; thus, the MBC is mostly valuable in theoretical studies, not in clinical microbiology. Furthermore, whether an antimicrobial is biostatic or biocidal in most cases strongly depends on its concentration.

4. Growth curves

In the search for new antimicrobials, an understanding of the dynamic interactions between the putative drug and the microorganism is clearly important. The activities of natural and synthetic peptides can be easily followed by plotting a growth curve, that is, a graphical representation of the growth of the bacterium of interest in a freshly inoculated culture. During the exponential phase, growth proceeds at a maximal rate (μmax), which depends upon the characteristics of the bacterium and the environmental conditions, including temperature, oxygen availability (for some bacteria), light, medium composition, etc. The growth rate (μ) can be calculated as $N_t = N_0 \times e^{\mu t}$ and thus as $\mu = (\log_n N_t - \log_n N_0)/t$, where N_t is the number of individuals at time t and N_0 the number of individuals at time 0. The exponential phase of growth is followed by a transition phase in which μ decreases until it reaches 0, marking the stationary phase. For a given microbe cultured under

standardized conditions, the growth curve is highly reproducible whereas the addition of an antimicrobial disrupts the growth curve. When added during the exponential phase, antimicrobials can be evaluated as bacteriostatic or bactericidal (Figure 2). Although, in principle, the desired effect of an antimicrobial is bactericidal rather than bacteriostatic, this is not universally true. Many bacteriostatic antimicrobials are excellent therapeutic agents because they promote host defense mechanisms, which help to eradicate the bacterium. Furthermore, bacteriostatic agents can also be used to prevent nosocomial infections as well as infections in indwelling medical devices.

5. Death kinetics

The best way to accurately determine the pharmacodynamics of drug is to plot a so-called time-kill curve. The drug under study is added to a starting bacterial inoculum of 10^6 CFU/mL in MHBCA (Muller-Hinton broth cation adjusted) medium, and parallel experiments adding 25% mammal serum; at different times, aliquots are retrieved aseptically for bacterial counting.

The advantage of a time-kill curve is that provides a dynamic picture and thereby avoids the limitations of fixed time-point studies (Figure 3). Information on the behavior of a drug during its first hours of bacterial contact is extremely important, if only the initial and final time-points are analyzed, the different intervening processes will be missed, as described for the MIC.

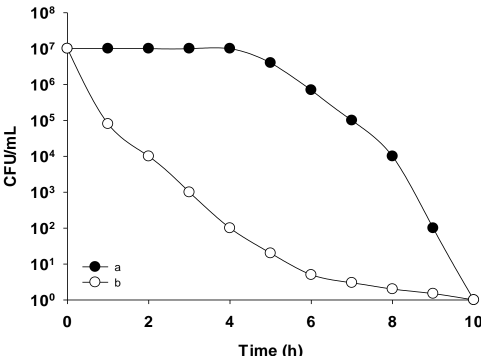

Figure 3. Death kinetics of two antimicrobials: one slow-acting (open circles) and the other fast-acting (filled circles).

Death kinetics can also be used to test for possible synergies between two or more compounds. Synergism occurs when a drug combination results in a reduction of the bacterial counts by two or more logarithms (decimal) compared to the most active drug alone. A reduction of less than two logarithms indicates an additive relationship. A null logarithmic reduction is called indifference, whereas antagonistic effects are reflected by an increase of the bacterial counts by two or more logarithms compared to the counts obtained with each drug separately (Figure 4). In many cases, these data reinforce those obtained by checkerboard (see Section 6).

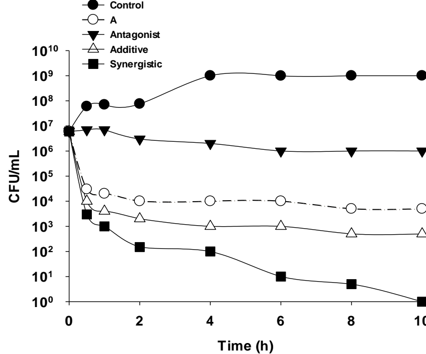

Figure 4. Comparison of the time-kill curves of bacteria incubated in the presence of various antimicrobials (A means the antimicrobial alone).

6. Antimicrobial peptide interactions

The interaction between peptides and other antimicrobials, or between two antimicrobial peptides (AMPs) can be determined quantitatively from the fractional inhibitory concentration (FIC) using the checkerboard technique. The FIC of drug A can be calculated as: (FIC A) = (MIC of drug A in combination)/(MIC of A), and the FIC of drug B as: (FIC B) = (MIC of drug B in combination)/(MIC of B). The FIC index (FICi) is calculated by adding FIC A and FIC B. FICi < 0.5 indicates a synergistic interaction, FICi values between 0.5 and 4 no interaction, and FICi > 4 an antagonistic interaction. Together with calculation of the FICi, determinations of death kinetics provide a useful tool to explore drug interactions whereas growth curves reveal the interactions between two or more antimicrobials. Figure 5 shows an example in which the synergistic effect of imipenem and a newly synthesized AMP (amp38) was determined [9].

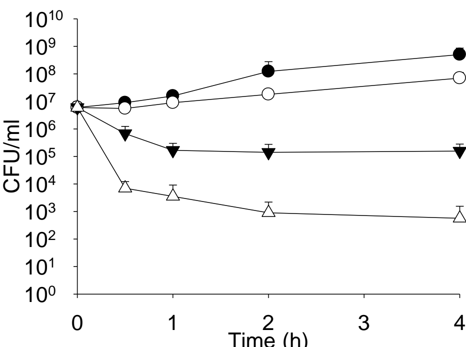

Figure 5. Effect of the control (filled circles), 4 μg imipenem (IMI)/mL (open circles), 8 μg AMP38/mL (filled triangles), and 8 μg AMP38/mL + 4 μg IMI/mL (open triangles) on *Pseudomonas aeruginosa* strain PA116136 [9].

In fact, AMPs have been described as synergistic agents with a wide variety of antimicrobials. The inhalation therapy of respiratory infections suffered by cystic fibrosis patients demonstrate strong antimicrobial synergy of Polymyxin B in combination with silver nanoparticles [10]. Moreover, in fungal infections treatment, AMPs are also regarded as candidates to act through synergistic effect with already known antifungals. A good example is Ctn (15–34), a carboxyl-terminal fragment of crotalicidin (a cathelicidin from the venom gland of a South American rattlesnake), the molecule has *per se* a certain antifungal activity, but an enhancement of antifungal properties was found when combined with amphotericin B [11]. Even looking at viruses different examples of synergism have been reported. It has been shown that synergy emerges when Env (a protein involved in HIV penetration) engages multiple co-receptors prior to inducing fusion and when high-affinity inhibitory peptides are present [12]. Finally, it is worthy to mention that such synergistic effects have been demonstrated even between AMPs and inorganic molecules. This is the case of carbon monoxide-releasing molecules (CORMs): a novel class of compounds (for example the light-activated metal complex [Mn(CO)3(tpa-κ3N)]Br) they have shown high synergism with some AMPs such as colistin [13]. This strongly suggests the need to explore interactions when exploring new AMPs, since even when antimicrobial activity may be weak, their use as enhancers of antimicrobial action of others may restitute their interest. One more question should be addressed when taking into account that, at least in one case, AMPs have been found to be potentially useful to eradicate biofilms and to increase susceptibility to already known antimicrobials [14].

7. Measuring the efficacy of AMPs against biofilms

Traditional antimicrobial susceptibility tests (from disk diffusion to broth microdilution methods) used to calculate the MIC and thus to define the susceptibility breakpoints predicting therapeutic success are performed mostly using planktonic bacteria. However, planktonic microbes are very infrequent in infections. Rather, most infectious diseases are caused by bacteria living in a biofilm, usually attached to a surface or an interface. Within the biofilm, the bacteria are embedded within a slimy extracellular matrix of bacterial origin and they adhere to each other [15]. Biofilm bacteria have a much greater resistance to antimicrobials than their planktonic counterparts [16] and the corresponding susceptibility breakpoints have not been established [17]. This difference in antimicrobial susceptibility between planktonic and biofilm populations of the same organism is due to differences in the diffusion of antimicrobials and to complex physiological changes in biofilm microbes [18,19]. Thus, for biofilm infections, using the MIC to predict the success of antimicrobial treatment is often ineffective, such that susceptibility tests for biofilm-growing bacteria are needed. The most relevant parameters currently used to evaluate the *in vitro* antimicrobial activity of antimicrobial compounds in biofilm-growing bacteria are the minimal biofilm eradication concentration (MBEC) and the biofilm prevention concentration (BPC) [20]. The effect of antimicrobial compounds on the viability and physical stability of the biofilm can be studied with confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM), respectively [21,22].

8. Minimal biofilm eradication concentration

Given the serious challenges associated with infections caused by biofilm microbes and the role of biofilms in promoting recurring infections [23], the development of accurate methods to evaluate

the efficacy of new antimicrobial compounds against biofilms is crucial.

The MBEC is defined as the lowest concentration of antibiotic required to eradicate the biofilm [24], in other words, the lowest concentration preventing bacterial regrowth from the treated biofilm.

The MBEC assay uses the Calgary biofilm device (CBD), a 96-well plate with pegs or projections built into the lid [24]. Each peg provides a surface for bacterial adhesion, colonization, and the formation of a uniform biofilm. The lid is used in conjunction with special troughs for growing, washing, and incubating the bacteria. The growing microorganism are cultured in an opportune growth medium and allowed to form biofilms on the pegs for 4 to 24 h (depending on the specific bacterial growth rate). Bacterial motility greatly influences biofilm formation on the pegs, such that more motile microorganisms, which have a greater tendency to aggregate, form robust biofilms on the pegs [25]. For example, microorganisms such as *Pseudomonas aeruginosa* and *Escherichia coli* are flagellated and motile and form high-density biofilms on CBD pegs [26,27] whereas non-motile *Staphylococcus aureus* is much less effective in biofilm formation [28].

Once the biofilms are formed, the pegs are rinsed and placed onto flat-bottom microtiter plates, where they are incubated for 18–20 h at 37 ℃ in the presence of different concentrations of antimicrobials. Then, the pegs are again rinsed and transferred to antimicrobial-free medium in a biofilm recovery plate. Gentle centrifugation (805 g for 20 min) or a 5-min sonication at room temperature is used to transfer biofilms from the pegs to the plate. The MBEC values are then determined spectrophotometrically, by measuring the optical density at 620 nm. This method permits incubation of the biofilm with antimicrobials at different time-points, with daily rinsing and antimicrobial renewal [29].

It is important to point out that the minimal concentration of an antimicrobial required to eradicate a biofilm (MBEC) may be higher than the MIC determined against planktonic bacteria of the same species or isolate [3,9].

9. Biofilm prevention concentration

Considerable efforts have also been devoted to prevent biofilm formation during the early stages of the colonization process [30,31], by effectively eradicating bacteria with the appropriate antimicrobial therapy [32]. The biofilm prevention concentration (BPC), defined as the antibiotic concentration required to prevent biofilm formation during the early stages of colonization, can be used to correlate *in vitro* measurements with the therapeutic results and may be a better indicator than other concentration-dependent parameters [21].

A modification of the MBEC assay can be applied to determine BPC values. A planktonic bacterial suspension is incubated in the CBD plates in growth medium containing different concentrations of antimicrobials [33]. After 4–24 h (depending on the specific bacterial growth rate) incubation, the pegs are rinsed, placed in antimicrobial-free medium in a biofilm recovery plate, and sonicated for 5 min. The detached bacteria are incubated for 4–24 h and the optical density (620 nm) is measured to determine the minimal concentration of antimicrobial preventing biofilm formation.

Its well-established equivalence or similarity with the MIC, demonstrates the utility of the BPC in assessing antimicrobial-mediated reductions in bacterial density to prevent biofilm formation. Use of the BPC could also improve treatment strategies aimed at eradicating biofilm-producing bacteria already during early biofilm formation rather than at the mature biofilm stage.

10. Determination of bacterial viability in biofilms using confocal laser scanning microscopy

A biofilm is a complex and multicellular structure that harbors physiologically distinct subpopulations of bacteria that together from a community able to adapt to rapidly changing environmental conditions [34]. The efficacy of an antibiotic with respect to biofilm viability, both over time and across the different layers of the biofilm, can be studied using CLSM [35,36]. In biofilms exposed to antimicrobial agents, CLSM reveals the effectiveness of the drug against the (metabolically active) outer layers and (metabolically attenuated) inner layers, and thus the time-dependent destruction of the biofilm. In an analysis of antimicrobial activity on *P. aeruginosa* biofilms, CLSM showed that some agents (such as ciprofloxacin) act preferentially on bacteria with high metabolic rates, whereas others (such as colistin) are effective only against bacteria with low rates of metabolism (Figure 6) [37,38,40].

To prepare the biofilm for CLSM, it is stained with a mixture of SYTO 9 and propidium iodide prepared at a dilution ratio of 1:2 (LIVE/DEAD Bac Light bacterial viability kit; Figure 6). Live bacteria stain green and dead bacteria red [41]. The CLSM images are then analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) [33] or other software tools.

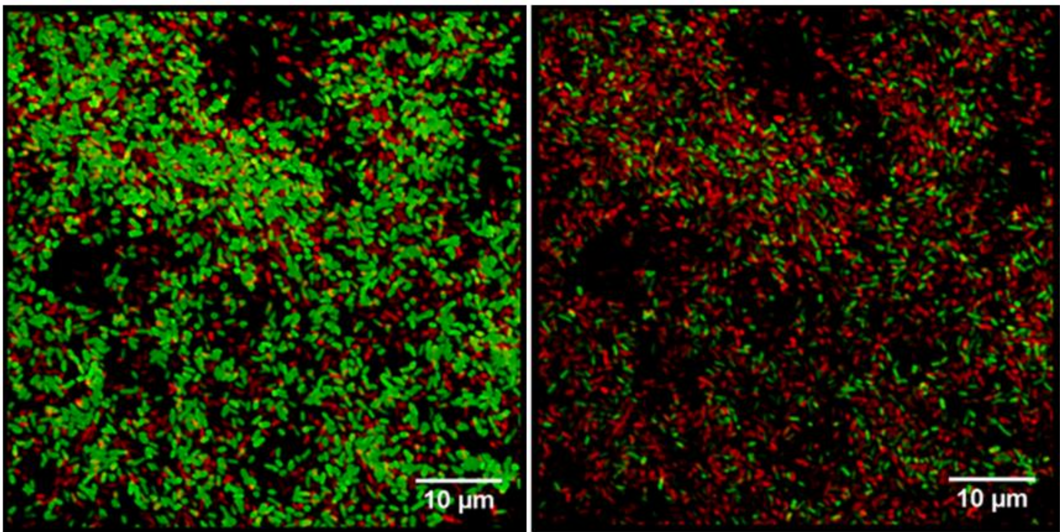

Figure 6. Confocal laser scanning microscopy images of *P. aeruginosa* biofilms stained using the LIVE/DEAD Bac Light bacterial viability kit. Green indicates viable bacteria, and red dead bacteria. (A) Most of the cells stain green, indicating a high level of bacterial viability. (B) Most of the cells are dead, indicated by their red fluorescence. Scale bar = 10 µm.

11. Visualization of the effects of antimicrobials by atomic force microscopy

Invented over three decades ago [42], AFM not only reveals details of the cell surface, but also allows biological samples to be mechanically mapped, touched, dragged, pulled, pushed, or indented

at the single molecular level. The resulting information provides valuable insights into the nanomechanical properties of living cells in their physiological environment and supports the information gained from other methodologies commonly used to assess antimicrobial activity, such as the MIC, minimal eradication assays, CLSM, and flow cytometry.

AMPs alter the physical properties of the cell, specifically, their morphology, volume, surface roughness, and stiffness [43,44]. Data on roughness, obtained by software analysis, are complementary to viability assays. AFM analyses of the integrity of the cell surface and of membrane disruption can reveal whether treated cells display altered cell membrane characteristics, a loss of turgor, and a roughened surface, including bleb formation, with the eventual loss of the membrane permeability barrier and leakage of the intracellular content [45]. Imaging and the subsequent analysis of cell integrity have been used to evaluate alternative or complementary treatments.

Gonçalves et al. [46] evaluated the antifungal activity of AMP Psd1, isolated from a defensin secreted by *Pisum sativum*, against three *Candida albicans* variants, one of them a mutant deficient in glucosylceramide synthase, conferring resistance to the peptide. AMP Psd1 significantly increased surface roughness, an indicator of relevant wall disorganization, resulting in a loss of stiffness of the treated cells of both the wild-type and the clinical isolate. Mularski et al. [47] used time-resolved AFM to study the pore-forming activity of the AMP Caerin 1.1 and its target. Adhesion forces, adhesion energy, the cell's Young modulus and other physic-mechanical parameters can be extracted using AFM-force spectroscopy, through force-distance (F/D) curves. These describe the deflection of a cantilever when approaching and contacting the sample and the displacement along the z axis (Figure 7).

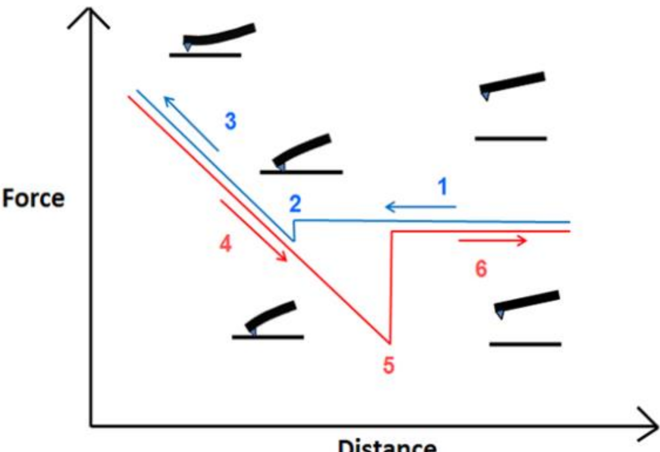

Figure 7. Schematic representation of the different phases in a force-distance curve obtained from AFM force spectroscopy. The blue line corresponds to the approach trace, and the red line to the retraction trace.

As the cantilever approaches the cell (red trace in graphics), there are no changes in its deflection due to the absence of interaction forces at that point; however, when the tip reaches the sample, attractive forces such as Van der Waals forces, overcome the cantilever spring constant, forcing the tip to "jump to contact" the sample. Variation during the retraction regime (blue trace) is often characterized by a prolonged extension due to the tip remaining in contact with the sample, such that the cantilever is reflected downwards. A "jump" of the cantilever indicates its return to its final resting position. In terms of their nanomechanical properties, AMP-treated cells are less stiff than untreated cells [48], as shown by the final linear portion of the respective F/D curve. Moreover, the spring constant of the cell decreases in response to increasing AMP exposure, presumably due to the disintegration of the cell wall. Conversely, adhesion forces, generated between the tip and the cell surface, are considerably higher in AMP-treated cells. These forces represent the maximum external pulling force needed to separate the tip from the bacterial surface and the force needed to undo the recently formed bonds. Here again, degradation of the cell wall eases the penetration of the tip through the peptidoglycan layer and generates a higher number of contact points between them, finally increasing the force necessary to detach the cantilever from the cell [3]. As see in Figure 8, this force can either be continuous until detachment occurs or it can develop as several peaks, or jumps, of the pulling force towards zero when detachment is partial and bonds remain, a process referred to as sequential detachment. Finally, the adhesion energy is the sum of the amounts of energy needed to detach the AFM tip from the cell surface and to deform the cell close to its surface [49,50]. It is calculated by integrating the area under the detachment force curve over the z displacement.

Pan et al. evaluated the interaction between the cell membrane and an AMP by studying the membrane lytic activity of a prion peptide (106–126). They reported decreases in both the Young modulus E of the bilayer and the bilayer puncture force, regardless of the cholesterol concentration of the supported lipid bilayers. In fact, a recent study by Henderson et al. showed that several AMPs reduce the edge stability of lipid membranes, thus altering the porosity of the cell membrane and causing the appearance of worm-like structures at high peptide concentrations. The specific interaction forces between AMPs and membrane components can be explored using single molecule force spectroscopy. This method was used by Oh et al., who characterized the nanoscale effects of the polycationic peptide polymyxin B (PMB) on bacterial membranes by determining the short-range interaction regime mediated by electrostatic forces between lipopolysaccharides and PMB, with 30 pN determined as the average unbinding force.

These examples demonstrate the versatility of AFM as a tool for biomedical research that provides information on many topographic and nanomechanical parameters of microbial and non-microbial cells.

12. Measuring the efficacy of antimicrobials against protozoa

Protozoan parasites include the well-known genera *Trypanosoma*, *Plasmodium*, and *Leishmania*. These organisms cause several serious human diseases that hamper the lives of people mostly in developing countries. Thus far, the control and treatment of protozoan diseases has depended on a rather small number of antiparasite drugs, which are frequently highly toxic and of low efficiency. Moreover, resistance of the parasites to these drugs is becoming increasingly common [51]. Thus, novel compounds and/or strategies are needed, including delivery systems [52] and new molecules, such as AMPs [53]. Membranes and DNA topoisomerases have attracted considerable interest as

potential targets for novel antiparasitic therapeutics [54,55].

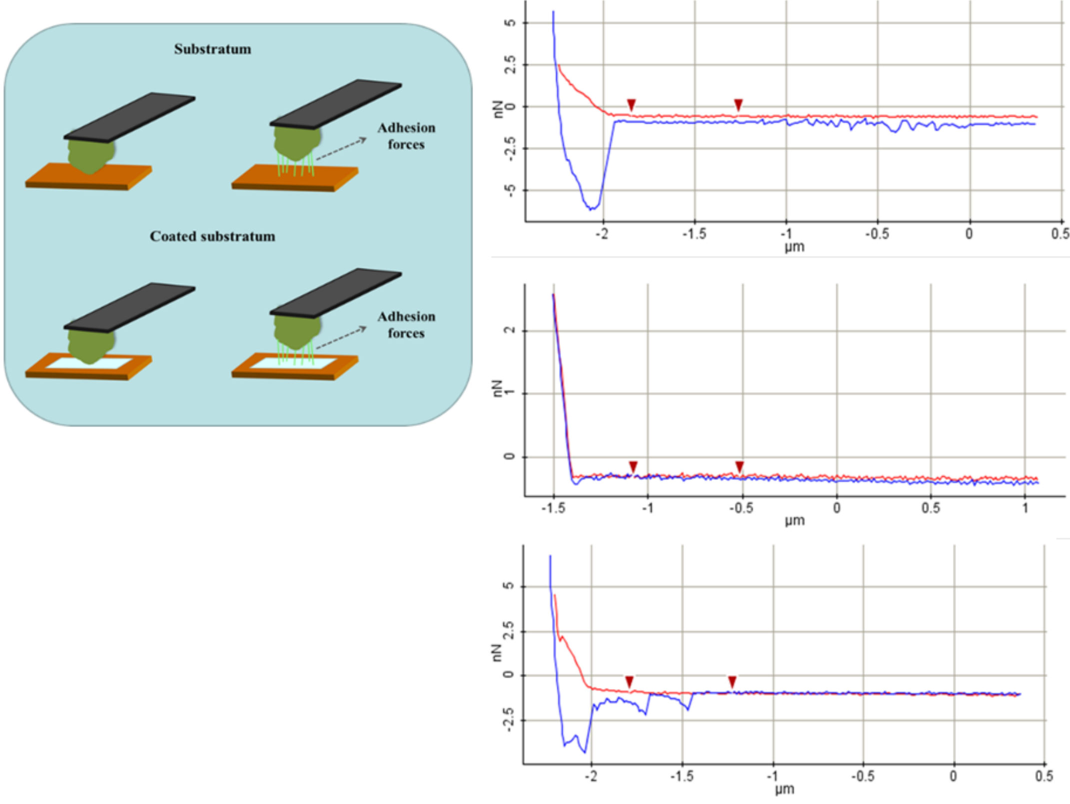

Figure 8. Representation of the retraction of the cantilever and the associated adhesion forces. As the cantilever moves away from the sample, the tip and surface molecules can detach in different ways. The adhesion force corresponds to the maximum external pulling force necessary for detachment to occur. On the right: Schematic representation of the different phases in a force-distance curve obtained from AFM force spectroscopy. The blue line corresponds to the approach trace, and the red line to the retraction trace.

With respect to AMPs, protozoa have received less attention than other microorganisms; however, they may be readily accessible targets because, except in the resistant cyst stage, they do not present structured external barriers, such as the bacterial capsule, the outer membrane of gram-negative bacteria, the thick peptidoglycan layer of gram-positive bacteria, or the compact fungal wall. The absence of these barriers allows direct interactions between the parasite and the AMP, which could facilitate an interpretation of the results. Nonetheless, in addition to determining the biocidal power and therapeutic index of an anti-protozoal therapeutic, its mechanism of action, lethal activity, and other effects, such as membrane permeation or modification of the energetic parameters of the parasite, must be elucidated to obtain valuable information on other potential targets as well as further drug development.

How AMPs perturb and destroy parasites can be studied using AFM, to examine morphological and structural damage of the cell surface, and transmission electron microscopy, to assess structural modifications to the parasite. The topographical relationship of the parasite with the AMP and the identity of intracellular targets, including the induction of apoptosis, can be investigated using confocal microscopy (see below). However, most protozoa have complex life cycles involving intracellular stages. Thus, as a first step, AMP activity should be assessed in extracellular parasites, before time-consuming and labor-intensive intracellular tests are performed.

Methods used to examine the efficacy of an AMP against a parasite include the following:

Cell proliferation measurements: As cell counting is tedious and subject to bias arising from reader expertise, fatigue, etc., the staining of viable cells using chromophores such as MTT, XTT, or resazurin allows an automated, colorimetric, and quantitative measurements of viable cells.

Cytotoxicity against intracellular parasites: The direct examination of mammalian cells stained with Giemsa's azur-eosin methylene blue reveals those infected with the parasite. A minimum count of 200 cells from different fields is required; the results are expressed as parasites per cell.

Assessment of plasma membrane permeation: The interaction between AMPs and the plasma membrane phospholipids of the target cells has been well-documented. For many AMPs, the peptide inserts into the membrane, disrupting its integrity and therefore its function as a permeation barrier, resulting in a lethal loss of internal homeostasis. For other AMPs, lethality is produced intracellularly, after their translocation across the membrane. Plasma membrane integrity can be assessed using fluorescence techniques, including cationic stains such as Syto9 or propidium iodide. Both of these vital dyes bind intracellular nucleic acids such that their entrance into the cell implies severe membrane damage, as also described in bacteria. Small or transient membrane damage results in plasma membrane depolarization, which can be followed using the sensitive probe bisoxonol, an anionic fluorescent molecule that reveals the discrete permeation of ions able to dissipate ion gradients. Mitochondrial membrane depolarization can be investigated based on the differential accumulation of rhodamine 123, a cationic fluorescence dye that enters metabolically active mitochondria.

Confocal microscopy: Using this technique, cells stained with different simultaneous labels can be examined and the intracellular target of a fluorescently labeled peptide identified. For example, organelles can be selectively stained and their distribution pattern compared with that of the tagged peptide. Among the fluorophores used for confocal microscopy are AMPs labeled with fluorescein, MitoTracker red (mitochondrial staining), and DAPI (nuclear and kinetoplast dye).

Measurement of the oxygen consumption rate: If oxidative phosphorylation, rather than glycolysis, is the main source of energy for the parasite, then dissolved oxygen, as an indicator of oxygen consumption, can be measured, typically using a Clark electrode or, as done traditionally, Warburg's instrument [56].

Microscopy. As in bacteria, the visualization of morphological alterations in parasites confirms antimicrobial action. Ultrastructural alterations of parasites can be viewed using transmission electron microscopy, and the three-dimensional surface morphology of the organisms using AFM. Sample treatment form AFM is minimal and the cells remain viable so that they can be evaluated physiologically in parallel.

Assessment of apoptosis by flow cytometry. Sublethal concentrations of peptides tend to cause apoptosis rather than necrosis, induced by higher doses. Apoptosis also occurs in response to slow permeation of the AMP or when the target is not the membrane but an intracellular organelle. Cell

cycle analysis by flow cytometry, based on separation of the cells according to their DNA content, is a fast and easy method to study apoptosis. Ethanol-fixed, permeabilized protozoa are stained using propidium iodide and apoptotic cells then identified based on the appearance of characteristic peaks on the resulting histogram.

13. Evaluation of the antifungal activity of AMPs

Procedures to evaluate novel antifungal peptides are similar to those described above for antimicrobial peptides. However, as yeast and molds are eukaryotes, important differences involving nutritional requirements, optimal temperature, and duplication rate must be considered.

Standardized protocols for both yeasts and molds have been developed by the CLSI [57,58] and EUCAST [59,60]. In the case of the microdilution method, the main difference between the protocols of the two institutions involves the end point, based on the extended duplication time of these microorganisms. The CLSI use the same protocol developed for prokaryotic cells but with a 48-h incubation time, while in the EUCATS protocol results are obtained spectrophotometrically at 24 h. The MIC is defined as the lowest concentration resulting in a 50% (90% in some cases) inhibition of growth compared with the control.

Dynamic studies on the interaction between drugs and fungi should be performed as described for bacterial strains, albeit with differences in terms of the appropriate medium (RPMI), the inoculum size, and an extension of the incubation time up to 48 h.

The method to determine the antibiofilm activity of an antifungal agent differs from the bacterial protocol mainly in how the results are read. Similar to BPC and MBEC determinations for bacteria, the metabolic activity of fungi is measured using XTT or resarzurin [61] and the resulting tetrazolium compound monitored spectrophotometrically. However, for yeasts and molds, the BPC and MBEC are defined as the lowest concentration of the drug that yields a 90% reduction in metabolic activity vs. the untreated control.

Other techniques to study antifungal activity are being introduced. Of particular interest is flow cytometry [62]. The cells are stained with two different fluorescent dyes, one of which penetrates normal or intact cells, and the other only cells with a disrupted membrane, i.e., dead cells. Flow cytometry allows for single-cell fluorescence investigations. Hence, after the analysis of a suspension of cells treated with the antifungal peptide, a count of live and/or dead cells is obtained. The major advantages of this technique are the ability to study a large population of cells and the speed of the cell-by-cell analysis.

14. Remarks

In summary the antimicrobial effect of a new molecule should be assessed in several steps, which are summarized in the flow chart shown in Figure 9.

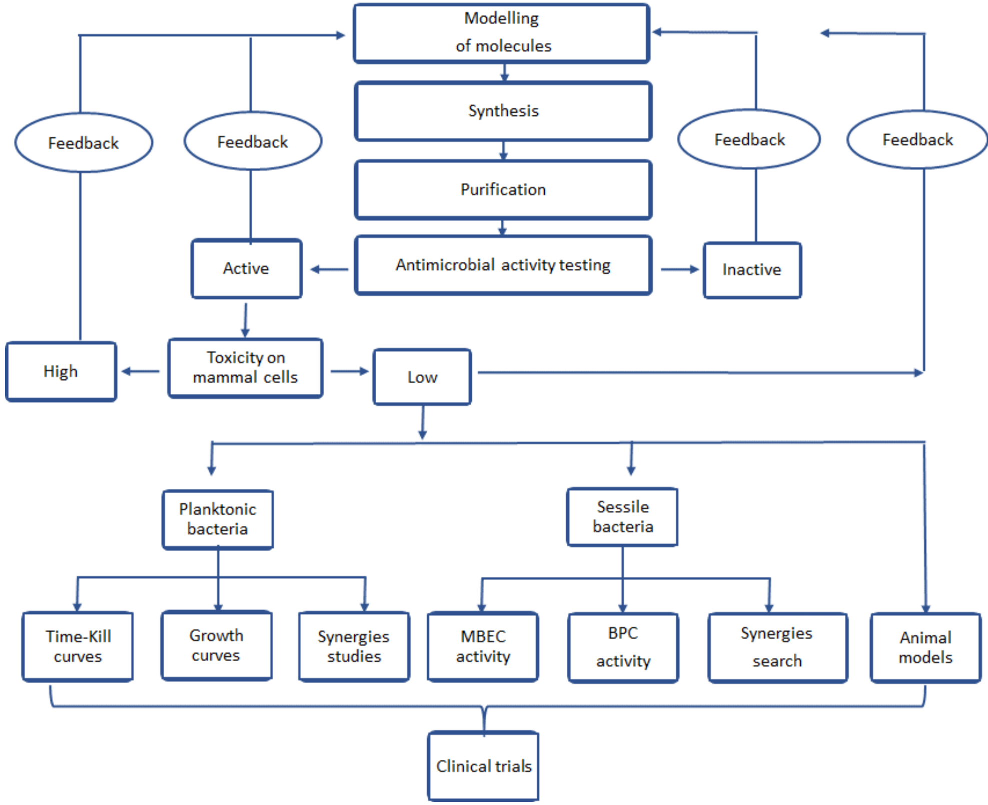

Figure 9. Sequence of techniques to be done at the initial search of new AMPs.

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Conflicts of interest

The authors declare no conflict of interest in this paper.

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6 . DISCUSSION

Colistin and related molecules have been reported to be synergistic with other antimicrobials, particularly with carbapenems in *Acinetobacter baumanii*, as well as in *Pseudomonas* and a few other Gram-negative bacteria. In principle resistance to carbapenems is mostly caused by impermeabilization of outer layers in microbial envelopes to the antibiotic molecule. Imipenem used the porin OprD to penetrate *Pseudomonas*.

As pointed out, when used together, combinations of colistin and imipenem gave a FICi of 0.625 for *P. aeruginosa* ATCC 27853; such a value is close to 0.5 which is the value considered as indifferent when using the recommendations of Journal of Antimicrobial Chemotherapy (JAC). Using other criteria such a value may be interpreted as additive. Despite the recommendations of JAC are the most restrictive ones, in our work we have used them. Moreover, when such combinations were assayed in other strains and isolates, particularly when experiments were performed with AMP38, the FICi value reached values as low as 0.625 a value identical to the one determined with colistin and imipenem. This suggests that both AMP38 and colistin act in a similar manner on this imipenem-susceptible strains. It should be emphasized that, in principle, the entry of imipenem in fully susceptible bacteria is not prevented by the outer membrane, since

OprD is functional and can allow the penetration of imipenem to the periplasmic space, and subsequently, the antibiotic can reach its target easily. On the contrary, when acting on imipenem-resistant bacteria (in which the permeability to carbapenems is altered due to the loss or alteration in the expression of proteins (porins) towards which ones carbapenems may penetrate outer membrane to reach periplasmic space, the combination of imipenem and AMP38 gave FICi values below 0.5; this is to say they act in a synergistic way. In other words, when imipenem may not entry the bacterium, colistin and analogues will facilitate such an entry. These values indicate and should be regarded as strongly synergistic (paper 1 table 2). For instance, strain PA116136 is imipenem-resistant, since its OprD gene is knocked out by the presence of an insertion sequence ISPa133 we described a few years ago (Ruiz-Martinez *et al*).[181] Other resistant strains used in this work, are OprD defective. That is to say they have lost OprD gene probably as a result of selective processes in patients treated with imipenem. Subsequently, the entry of imipenem is strongly limited in standard culture conditions for all these bacterial strains. This is the main (if not the unique) reason why these bacteria are fully resistant to imipenem.

In that way, the synergism between the colistin-inspired

peptide and the carbapenems should only be interpreted as a consequence of the ability of peptides to overtake the limitations in the imipenem entry, since colistin-derived molecules have not enough antibacterial power as to eradicate these bacteria. It seems feasible that they may open ways by which imipenem is going to reach their target molecules (PBPs) this is the reason why we call such molecules "door openers". Similar results were obtained when identical experiments were performed with the rest of resistant strains lacking OprD. Moreover, these results pointed out that the disorganization and injuries in the outer membrane caused by AMP38 (and other polycationic peptides inspired in colistin) seem to facilitate the entry of imipenem to the periplasmic space.

As a parameter, FICi values have certain limitations. In one side the value is simplified; they do not give information about the kinetics of bacterial killing. The results are determined after 24 h exposure to antimicrobials, and thus represent a fixed picture of the state after the incubation period. This is a limitation widely extended in parameters measuring the antimicrobial action. For instance, MIC values have an actually similar limitation. Consequently, with this type of assay, one could disregard the eventual usefulness of antimicrobial combinations for the treatment of complicated microbial infections. If a given combination may

eradicate bacteria during the first four hours incubation, allowing to survive a small proportion of the bacterial population which, when selective antibiotic pressure finish, will generate a new population (re-growth phenomenon) will never be considered as presumably useful if information comes exclusively from FICi or MIC. This make necessary to explore the process along the incubation period. In consequence, we have performed series of experiments in order to explore the antimicrobial actions during the first steps of the growth cycle. Combinations of sublethal concentrations of colistin combined with the appropriate concentrations of imipenem failed in increasing bacterial death. On the contrary, the 4 μg/mL concentration of colistin combined with either 0.5 or 4 μg/mL of imipenem markedly enhanced death. These results resulted to be imipenem concentration independent; this is consistent with the hypothesis that colistin facilitates the entry of carbapenem, and that eventual resistance would be due to restrictions in the access of imipenem to PBPs (Paper 1, Figure 2A). The reason is not new, but the proposal to use colistin analogues to facilitate the imipenem penetration is one of the main proposals of this work.

Combinations of AMP38 behavior changes depend on concentrations assayed. Thus, in general it may be stated that imipenem enhances bacterial death. Nevertheless, whereas 8 μg/mL

of AMP38 alone had a **bacteriostatic** effect, the combination of AMP38 (also at 8 μg/mL) plus imipenem (4 μg/mL) resulted in a **bactericidal** effect despite the fact that the MIC of imipenem is 16 μg/mL and that of AMP38 is 32 μg/mL. That is to say, when acting together MIC decreases up to six-fold. It should be stated that the combination failed to completely eradicate the bacterium, and regrowth appear after long periods of incubation (Paper 1, Figure 2C). Meropenem at 1 μg/mL had a bacteriostatic effect, keeping bacteria at 107 CFU/mL. Nonetheless, the combination of 4 μg/mL of AMP38 plus 1 μg/mL of meropenem completely killed bacteria after 6 h of contact.

On the basis of the initial assumption that the mechanism of cooperation between the different antimicrobials used in this thesis, recommended the performance of some more experiments. Time kill-kinetics with sub-lethal concentrations of imipenem and AMP38 and assays performed with meropenem (Paper 1 Figure 2D), as well as those carried out at low (sublethal) concentrations of both AMP38 and imipenem and several combinations of both (Figure 2 E,F) confirmed that AMP38 facilitates the entry of imipenem by disturbing the outer membrane, confirming the previous hypothesis. In the Figure 3 of paper 1 the effect on the mucous 023VH strain growth curve is shown. It may be seen to

be very similar to the results obtained with the rest of strains studied.

As pointed out for many authors, biofilm constitutes one of the main challenges of modern microbiology. One of the main goals of antimicrobial chemotherapy is the eradication of biofilms. Stable biofilms can play a key role in pathogenesis, and this is particularly a matter of concern in respiratory infections caused by *P. aeruginosa* and some other respiratory pathogens such as *Klebsiella pneumoniae*.

P. aeruginosa is able to produce stable biofilms in many environmental conditions, this is particularly apparent when the bacterium is causing lung infections. Sessile bacteria living in a biofilm state are regarded as generally more resistant to antimicrobial agents. Nevertheless, in some species it has been impossible to demonstrate differences in antimicrobial susceptibility between sessile and planktonic individuals. Since biofilm is so relevant in *Pseudomonas* pathogenicity, the exploration of ways to remove biofilm acquire a central interest. In consequence, the ability of combinations of AMP38 and imipenem to eradicate biofilms was explored. In our experience the use of colistin to eradicate biofilms needs concentrations higher than 1000 μg/mL;

imipenem's MBEC is higher than 500 μg/mL, whereas the combination of AMP38 and imipenem was able to completely eradicate the biofilm at 62.5 μg/mL. Thus, a true synergism of imipenem and AMP38 was again observed when the parameter measured was the ability to remove biofilm.

Electron microscopy and particularly TEM has greatly contribute to decipher structural details of microorganisms allowing significant advances in the understanding of bacterial structure and eventually their physiology, including antibiotic tolerance and biofilm formation. The mechanism of action of AMP38 in *P. aeruginosa* and *S. marcescens* was confirmed by examining untreated and treated bacteria with transmission electron microscopy. Untreated *P. aeruginosa* cells were examined by TEM. Electron micrographs of AMP38-treated *P. aeruginosa* (Paper 1, Figure 4) show a disorganized outer and inner membrane, as compared to the smoothness of untreated membranes. As a control, *S. marcescens* was used, since our group has a long expertise in using colistin in the naturally-resistant species such as *S. marcescens* (Lauferska *et al.*)[182] This bacterium is intrinsically resistant to colistin since no alterations in viability may be seen when it is incubated in colistin presence, although colistin disorganizes its outer membrane, but it seems that it is not capable of altering the inner membrane,

and subsequently the bacterium remains viable. Typically, the effect of colistin on *S. marcescens* cells is visualized as the production of blebs. We performed an ultrastructural analysis via TEM in order to explore whether colistin and AMP38 share mechanisms of action.

When *S. marcescens* is exposed to colistin, blebs appeared on the outer layer, but no changes were visualized in the cytoplasmic membrane. Paper 1, Figure 4 shows *S. marcescens* exposed to the AMP38, where blebs were observed in the outer membrane being, at least morphologically, identical to the ones caused by colistin. Although further research has to be done, the microscopically visualized effects of AMP38 and colistin on Gram-negative bacteria seem to be similar, if not identical. This is also consistent with the phenomenon of synergism described in this work.

In conclusion, our data pointed out that the use of synthetic AMPs inspired by natural products that are potentially less toxic than the natural parent compounds, may contribute to the rescue of antimicrobial agents to which some pathogens have become resistant. If combinations allow the use of toxic antimicrobials at much lower concentrations, taking into account that toxicity is in all cases dose-dependent and in some cases

(as it happens with colistin) a result of the accumulation of antimicrobial molecule in organs along the treatment, will allow a much safer use of old and "toxic" antimicrobial agents. In this case, it seems feasible to kill imipenem-resistant *Pseudomonas aeruginosa* with imipenem (apparently a paradox) when combined with other molecules (such as AMP38) which are able to sensitize the bacterium to the antibiotic. Since carbapenems remain the main antimicrobials for treating multidrug-resistant *P. aeruginosa* infections, and the development of carbapenem resistance may significantly compromise their efficacy, the use of peptides such as AMP38 could serve to rescue the use of carbapenems for these purposes. Their use should give better results than the use of colistin and polymyxins, which have already demonstrated synergistic effects.

During the development of this PhD thesis series of AMPs were synthesized. Two of them were selected due to their antimicrobial activity. Peptides CAMP113 and CAMP207 have also a disulphide bonds between the two cysteine residues. They are similar to colistin although with some significant differences. Moreover, they contain a dodecanoyl chain instead of (S)-6-methyloctanoyl, have a Cys residue at position 4, norleucine (NLe) at position 7 and (D)-Cys at position 11, instead of 2,4-diaminobutyric acid, Leu and Thr of polymyxin.

CAMP113 was further modified with (D)-Phe and CAMP207 with (D)-(4F)-Phe instead of Leu at position 6. In addition, an Arg was substituted at position 8 of CAMP113 whereas colistin contains 2,4-diaminobutyric acid (Paper 2, Figure 1). As it has been stated, Polymyxin action involves interaction with lipid A and the displacement of Mg^{2+} and Ca^{2+} bridging adjacent LPS molecules. In routine experiments we tested a few Gram-positive strains. Surprisingly, both AMPs resulted to be active on *Staphylococcus* tested strains. The MIC of both peptides was of 4 µg/mL. Tested strains were as expected resistant to colistin (higher than 512 µg/mL), this is generally accepted since Gram-positive bacteria are regarded as fully resistant to polymyxins. On the other hand, all tested Gram-positive strains were susceptible to vancomycin (1–0.5 µg/mL). Then, a vancomycin-resistant strain was obtained from the "Centro Nacional de Microbiología, Majadahonda", Madrid Spain, Lab of Dr. Sáez-Nieto; this is the SasVR strain (paper 2, Table S1). The activity of the two CAMPs was actually high, their MICs being more probably within the clinical use range. As we pointed out before, MIC values represent only the cumulative effect after 18 h incubation, rather than the time course of activity, whereas time–kill kinetics or growth curves describe the antimicrobial action, providing insights into the events during its first hours and depicting the biological behavior over time.

As expected, based on their chemical similarities both peptides killed the bacteria in a concentration-dependent manner (Paper 2, Figure 2), their behaviour being very similar irrespective of the strain tested. Two types of responses were observed in the presence of CAMPs: either the bacterium was completely inhibited at all concentrations tested or inhibition was achieved only at high concentrations (Figure 2). Neither peptide was able to fully eradicate the bacteria solely as a function of its concentration; rather, the activities of the two CAMPs strongly depended on the bacterial strain. For example, CAMP113 was able to fully eradicate the strain SaS06 (paper 2 Figure 2a) in a time as short as 1 h when acting at a concentration of 16 μg/mL and in 2 h at 8 μg/mL. However, a few bacterial individuals were able to survive at lower concentrations (4 μg/mL). In contrast strain SaS16 was completely eradicated at this latter concentration (Paper 2, Figure 2). At a concentration of 8 μg/mL or higher, CAMP113 produced the rapid death of strain SaS16 (paper 2, Figure 2c) during the first hour whereas at an equal concentration of CAMP207 (Figure 2) 2 h were required. At 4 μg/mL, both peptides required 4 h to fully kill the cultures of strain SaS16. In experiments made with strain SaS18, a complete eradication was achieved within 4 h when using 8 μg/mL CAMP113 but within 6 h when using the same concentration of CAMP207 (Figure 2f). However, even at

4 μg/mL, CAMP207 completely failed to eradicate SaS18. Similar outcomes were obtained with the remainder of the tested strains.

Paper 2, Figure 2 also shows representative growth curves. With inocula up to 2 x 10^6 CFU/mL, the growth was fully prevented by both CAMPs irrespective of the concentration tested (4, 8 or 16 μg/mL). In contrast higher inocula (up to 2 x10^8 CFU/mL) gave initial growth delays when acting at 4 μg/mL, and a longer delay at 8 μg/mL. Finally, both CAMPs were able to fully inhibit the growth at concentrations of 16 μg/mL when using the bigger inoculum, demonstrating once again the concentration dependence.

S. aureus have evolved mechanisms to evade innate host defenses including proteolysis of natural peptides, secretion of lytic enzymes, modulation of the electrical charge of the cell surface to enhance repulsion, and biofilm formation. As we pointed out before, biofilms have been the focus of considerable research attention and their destruction the main goal of current antibacterial therapy. The MBECs of both CAMPs indicated the ability of these compounds to eradicate fully the S. aureus biofilms at relatively low concentrations, in contrast to colistin, which had no effect. Confocal imaging (Paper 2, Figure 3) revealed the effects

of the CAMPs (at 128 µg/mL) on *S. aureus* biofilm during short exposure periods (4 h). As expected no significant death occurred in the controls nor in colistin-treated biofilms, whereas CAMP113 and CAMP207 were able to kill roughly one-half the bacteria within the biofilm (47.27% and 50.46%, respectively).

Given the current interest in CAMPs in the search for new approaches in anti-biofilm treatment, our study demonstrates the capability and potency of these peptides in targeting biofilms of several clinical isolates of *S. aureus*. This open new perspectives in the use of AMPs as antibiofilm agents. In fact, under conditions in which a colistin dose of 2048 µg/mL was required to eradicate biofilm, both CAMP113 and CAMP207 were able to reach the same result (eradication of the biofilms of all strains tested) at much lower concentrations (Paper 2, Figure 3).

The use of new molecules in clinics requires a deep knowledge of their toxicological effects. CAMPs herein studied were tested for their cytotoxicity in the human hepatocellular cell line HepG2 (ATCC HB8065). Despite such an experiment is not enough to jump to the clinical use, preliminary experiments in this field are an indicator of the presumably expected interest. That is to say, if preliminary toxicological experiments demonstrate a high

toxic power the product may be discarded. As shown in Figure 4, colistin had relative low cytotoxicity in vitro as its mechanism of toxicity involves the accumulation of molecules in organs such as the kidney and others. At 5 μg/mL, a slightly higher concentration than the MICs of the CAMPs, none of the tested compounds had detectable toxicity. A concentration of 50 μg/mL yielded 89.49% HepG2 cell viability for colistin and 67.03% and 62.62% for CAMP113 and CAMP207, respectively. At 100 μg/mL, colistin resulted in 68.75% HepG2 viability, CAMP113 53.2% and CAMP207 57.3%. At an antimicrobial concentration of 250 μg/mL, HepG2 viability was 51.05%, 16.01% and 8.29%, respectively. At 500 μg/mL, whereas 38.39% of the cells survived in the presence of colistin both CAMP113 and CAMP207 were highly toxic (7.74% and 7.4%, respectively) (Paper 2, Figure 4). None the less, the selectivity index (the relationship between the IC50 and the MIC)[183] in *S. aureus* strains was inordinately high for the peptides (21.25 for CAMP113 and 23.75 for CAMP207 compared with 0.36 for colistin), i.e. CAMP113 and CAMP207 are much more selective than colistin because the concentrations needed to kill the bacteria are much lower. Moreover, colistin is nephrotoxic, as it increases tubular epithelial cell membrane permeability to cations, anions and water, leading to cell swelling and cell lysis.[184] The presence in the newly synthesized CAMPs of a disulphide bond facilitates their

biodegradation and thus avoids their accumulation in the kidney. This is in agreement with previous studies, in which the LD50 for peptides of this family in CD-1 mice was 283 mg/kg, compared with the 59.5 mg/kg determined for polymyxin.[179]

The emergence of MDR strains of several species has posed a tremendous challenge in clinical practice and renewed interest in polymyxins, whose activity against Gram-negatives is well established. Colistin is considered the drug of last resort in the treatment of Gram-negative MDR bacteria such as *P. aeruginosa, A. baumannii* and *Klebsiella pneumoniae*.[185] None the less polymyxins are intrinsically inactive against Gram-positive bacteria because these bacteria lack outer membrane and consequently lipid A, which is the the primary binding site for all polymyxins. Novel peptides herein studied, despite they were synthesized in an attempt to evade the increasing polymyxin resistance of Gram-negative bacteria resulted to have low MIC values in Gram-positive organisms showing good activity against *S. aureus*.

In an attempt to explain such an activity, we did a search of molecules in Gram-positive organisms which may mimic the chemical structure of Lipid A. In figure 6.1 there is a reproduction of figure of the article (Velkov *et al.*)[186] in which a model of

colistin-lipid A binding is presented.

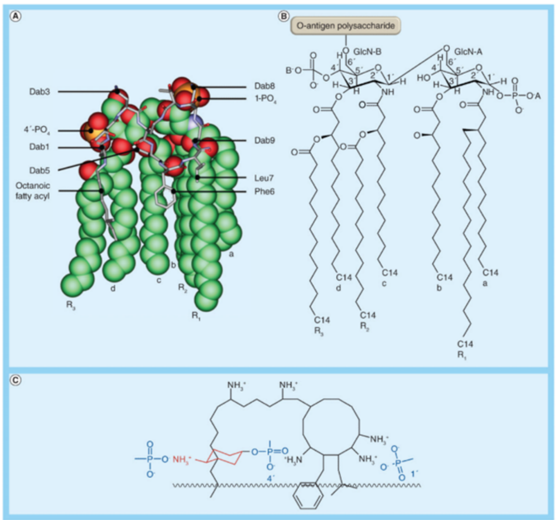

Figure 6.1: Molecular models of the complex between polymyxin B and the lipid A structure from *Klebsiella pneumoniae*. From Velkov *et al.*[186]

In figure 6.2 chemical structure teichoic acids is provided. Based on the apparent similarities between both formulas we have hypothesized that teichoic acids may act as primary receptors for pour colistin-like CAMPs. To determine whether teichoic acids were such targets for CAMP113 and CAMP207, we performed isothermal titration calorimetry experiments. The results showed a three-step reaction between the peptides and teichoic acid, two of which were

exergonic and the third endergonic (Paper 2 Figure S1). To examine the spectrum of action of the CAMPs studied, we explored their action against Gram-positive *Streptococcus pneumoniae*.

Figure 6.2: Teichoic acid molecule

Our results showed that in these species the MIC of colistin was consistently 32 μg/mL, whereas the MICs of both our peptides were between 2 and 4 μg/mL depending on the isolate. Polymyxins, regarded as inactive against Gram-positive organisms, after some small changes, have activity against *S. aureus* and *S. pneumoniae*; subject to more detailed determination of in vitro and in vivo toxicity as well as the development issues, peptides of this type may open up new frontiers in the treatment of “untreatable” infections.

7 . CONCLUSIONS

1. Combinations of colistin and imipenem are indifferent in imipenem-susceptible strains and strongly synergistic in imipenem-resistant strains.

2. Synergism between CAMPs and imipenem lies on the ability of CAMPs to open ways through both sides of outer membrane which are going to be used for carbapenems to penetrate.

3. AMP38 alone has a bacteriostatic effect, at low concentrations while the combination of AMP38 plus imipenem result in a bactericidal effect.

4. A true synergism of imipenem and AMP38 exists able to remove biofilm of *P. aeruginosa*.

5. It is feasible to kill imipenem-resistant *Pseudomonas aeruginosa* with imipenem (apparently a paradox) when combined with AMP38.

6. AMP38 disrupts outer membrane of Gram-negative bacteria.

7. CAMP113 and CAMP207 result to be active on *Staphylococcus aureus*. Both peptides kill the bacterium in a concentration-dependent manner, bacterial death also strongly depended upon the initial inoculum.

8. Both CAMPs (113 and 207) have the ability to fully eradicate *Staphylococcus aureus* biofilms.

9. The selectivity index in *Staphylococcus aureus* strains is inordinately high for the peptides CAMP113 and CAMP207 compared with colistin.

10. Isothermal titration calorimetry experiments strongly suggest that teichoic acids are the primary receptor for colistin-like peptides.

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APPENDIX

During the course of this doctorate we succeed in characterising antimicrobial peptides synthesized by in Organic Chemistry Faculty of University of Barcelona. There, I learnt the basic principles of biomolecule synthesis, carrying out solid phase synthesis technique. All peptides studied in this PhD thesis were synthesized using manually standard Fmoc/tBu procedures. Moreover, chromatography techniques were used in order to characterize peptides.

1.1 Background

Emil Fisher was the first in synthesize a protein (glycyl-glycine) in 1901, checking that the amino acids were linked together by the link peptide. Technique evolution was very slow because of it should protect amino acid group to avoid self-linking. Finding a compound able to block amino group and able to be removed easily after link peptide formation do not achieve until 1932 by Max Bergamnn y Leonidas Zervas. Moreover, another handicap was to isolate the peptide and obtain a relatively high yield.

1.2 Synthesis

1.2.1 Protective groups

Protecting functional groups is one of the main steps in peptides synthesis in order to avoid that carboxyl group reacts with amino group of the same amino acid. In addition, amino acid side chains must be protected because they are able to react with carboxyl group and/or amino group as well. One of the most used group to protect α-amino group is fluorenyl-9-methoxycarbonyl (Fmoc) which is very stable in the presence of acids and in addition it can be removed in light basic conditions such as 20% piperidine in dimethylformamide (DMF).

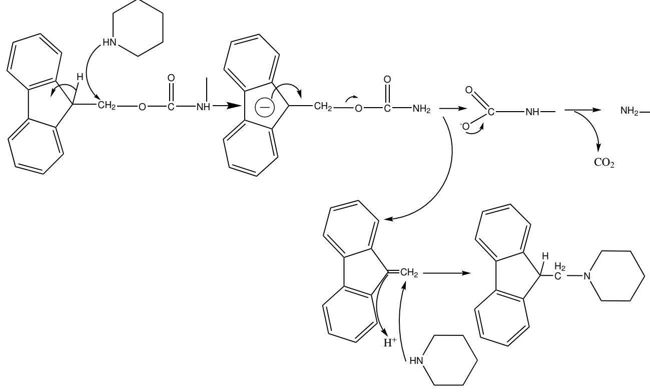

Figure 1.1: Fmoc deprotection

Carboxyl group it is not mandatory to protect due to it is less nucleophilic. Nevertheless, it is convenient to protect it in order to increase peptide solubility in organic solvents. Moreover, it is important to block carboxyl group which is nor forming part of the peptide link whereas the one that participates in the link must be unprotected ready to be activated. For this purpose, we used t-butyl ester technique.

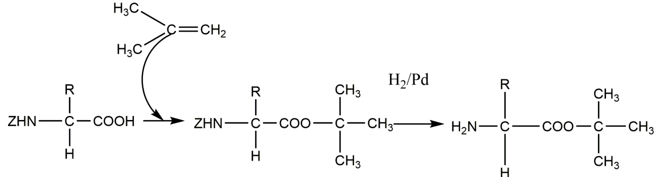

Figure 1.2: tbutyl ester deprotection

1.2.2 Peptide bond

Peptide bond is an endergonic reaction, addition of carboxyl acid with an amine will result in the correspondent organic salt. However, peptide bon consist in an amide, thus it is mandatory to activate carboxyl group. This reaction is called aminolysis and it is necessary an electrophilic activating agent (X).

$$R_1-COOH \longrightarrow R_1-\overset{O}{\overset{\|}{C}}-X \longrightarrow R_1-CONH-R_2 + HX$$

$$R_2-NH_2$$

Figure 1.3: amide bond formation

1.2.3 **Solid phase synthesis**

The principal idea of the solid phase synthesis is based on anchoring the growing peptide by C-terminal to an insoluble solid support. Wherewith, peptide elongation occurs in several cycles of unprotect and attach of amino acids. Here, there is a scheme of the procedure.

One of the most important steps in solid phase synthesis is the anchor a molecule called “linker” to the resin. Usually, linkers are bifunctional molecules which attach permanently to the resin by a amide bond, and the other side they attach temporarily to the growing peptide by a ester bond. This linker should be very stable in order to withstand deprotection reactions but at the same time should be easily to detach to the resin when reactions finish. Some likers are: 4-hydroxymethylbenzoic acid, methoxy-4-hydroxymethyl benzoic acid or 1,3dimethyl-4-hydrozymethylbenzoic acid.

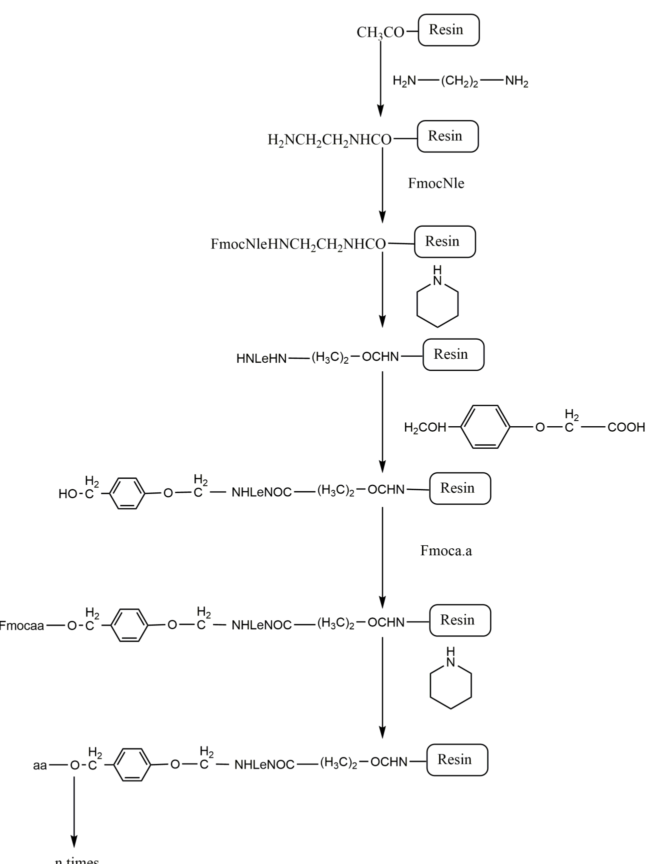

Figure 1.4: solid phase synthesis scheme

Once peptide is synthesized and detached from resin, it is cycled by two cysteine residues to form a disulphide bond. Finally, peptide is purified by HPLC and characterized by MALDI-TOF.

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