Development of new antimicrobial peptides and peptidomimetics and mechanism of resistance to peptide antibiotics

Xavier Vila Farrés

Programa Química Orgànica

Tesi doctoral

Development of new antimicrobial peptides and peptidomimetics and mechanism of resistance to peptide antibiotics

Xavier Vila Farrés

Revisada i dirigida per:

Dr. Ernest Giralt
(Universitat de Barcelona)

Dr. Jordi Vila
(Universitat de Barcelona)

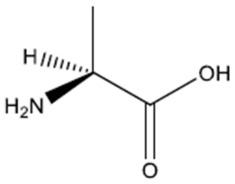
Barcelona, 2014

Contents

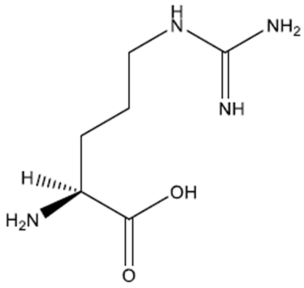
Contents

Annexes and abbreviations

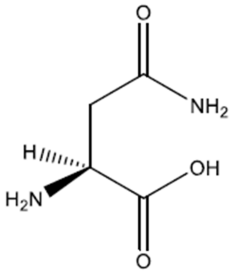
Annex I: Proteinogenic L-amino acids used



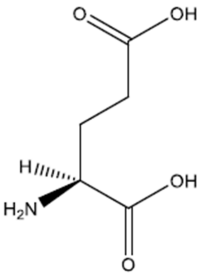
L-Alanine
Ala
A



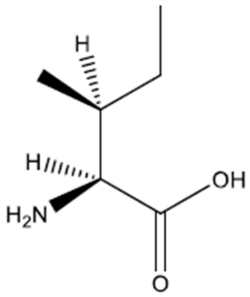
L-Arginine
Arg
R



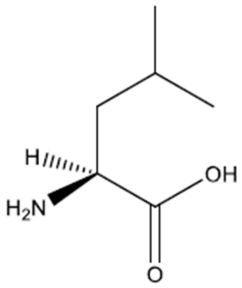
L-Asparagine
Asn
N



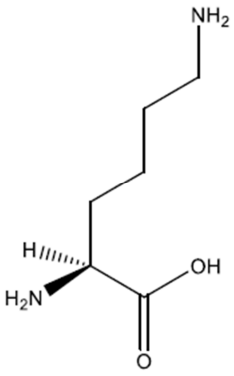
L-Glutamic acid
Glu
E



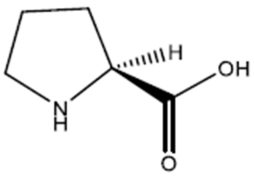
L-Isoleucine
Ile
I



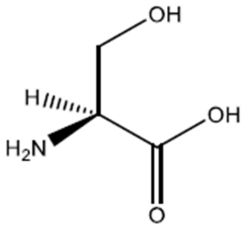
L-Leucine
Leu
L



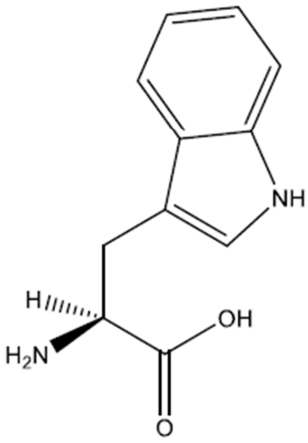
L-Lysine
Lys
K



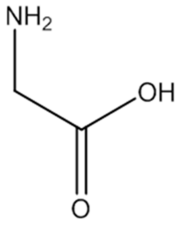
L-Proline
Pro
P



L-Serine
Ser
S



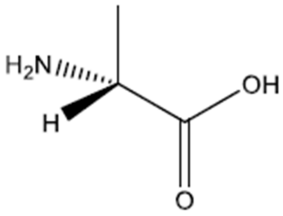
L-Tryptophan
Trp
W



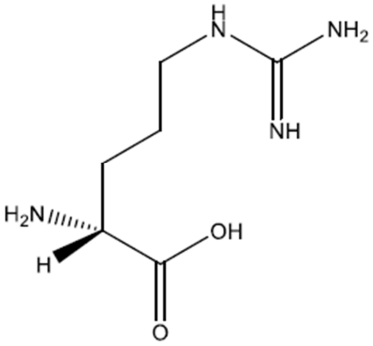
Glycine
Gly
G

* Amino acids abbreviations follow the rules of the Comission on Biochemical Nomenclature of the IUPAC-IUB as specified in *Eur. J. Biochem.* 1984; 138: 9-37 and *Eur. J. Biochem.* 1993; 213: 2.

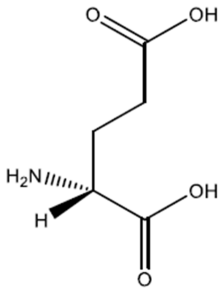
Annex II: D-amino acids used



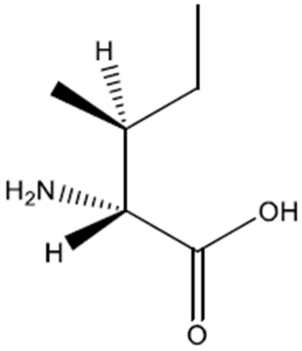
D-Alanine
d



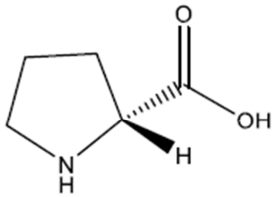
D-Arginine
r



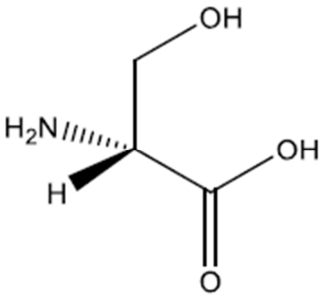
D-Glutamic acid
e



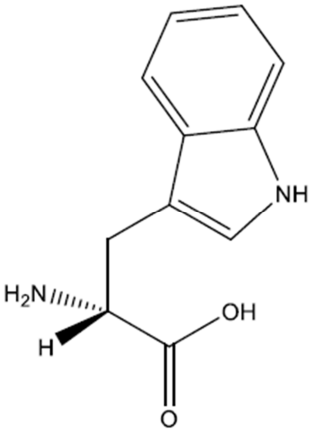
D-Isoleucine
i



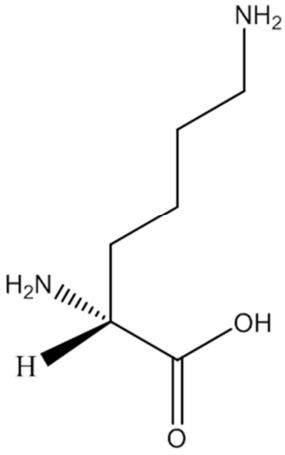
D-Proline
p



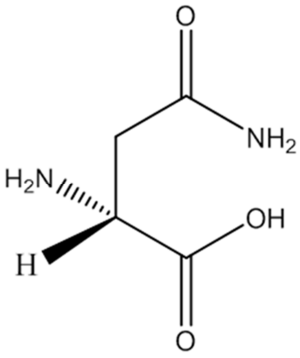
D-Serine
s



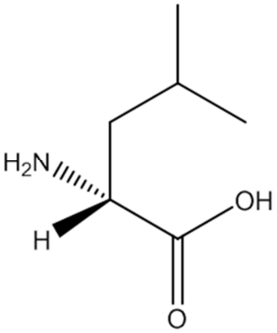
D-Tryptophan
w



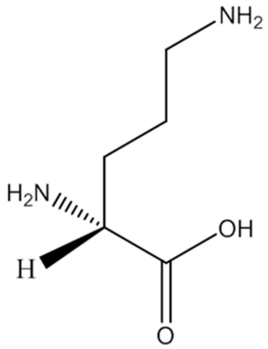
D-Lysine
k



D-Asparagine
n

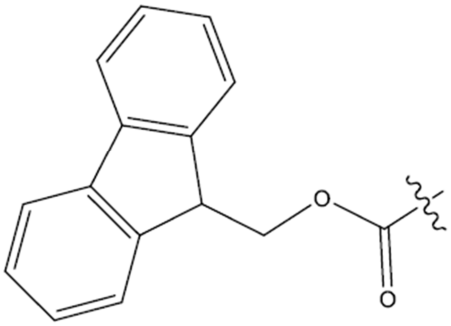


D-Leucine
l

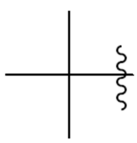


D-Ornithine

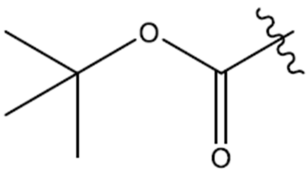
Annex III: Protecting groups used



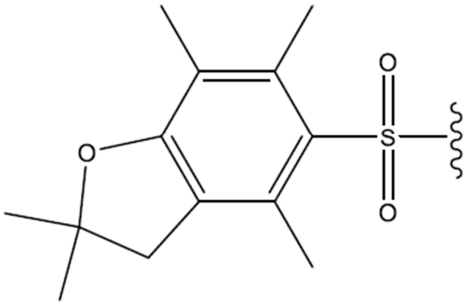
Fmoc



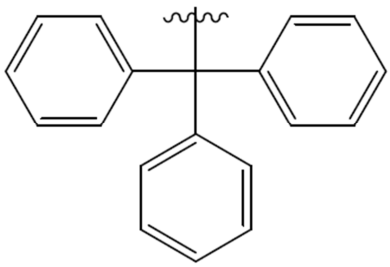
tBu



Boc

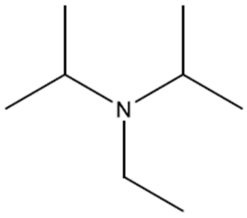


Pbf

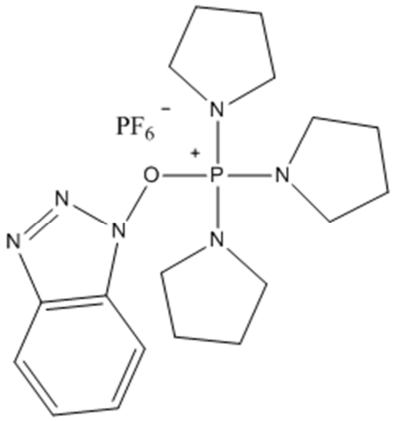


Trt

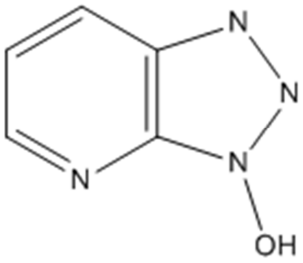
Annex IV: Coupling reagents used



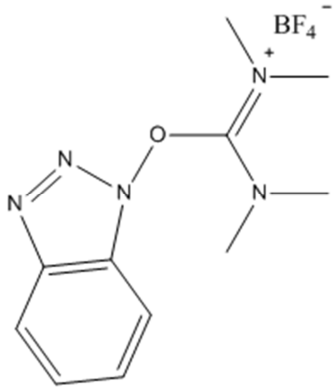
DIEA



PyBop

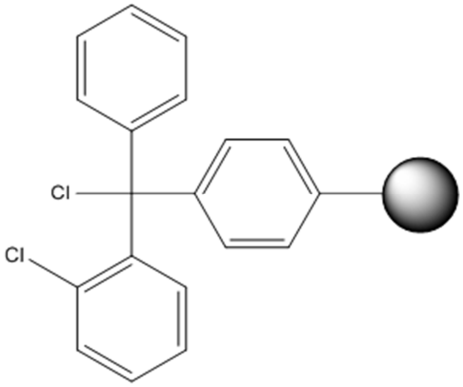


HoAt

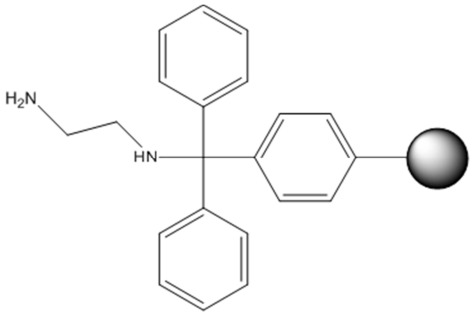


TBTU

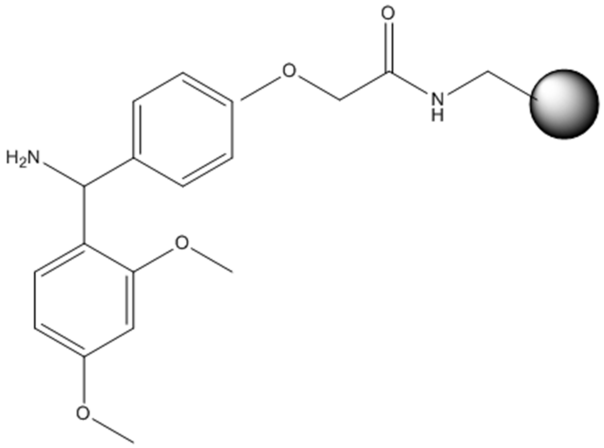
Annex V: Resins used



2-Chlorotrityl chloride



1,2-Diamino-ethane trityl



H-Rink amide ChemMatrix

Abbreviations

2-AIT	2-aminoimidazole–triazole
ACH	α-cyano-4-hydroxycinnamic acid
Ac_2O	Acetic anhydride
ACN	Acetonitrile
AHL	Acyl-homoserine lactone
AIF	Apoptosis-inducing factor
AMEs	Aminoglycoside-modifying enzymes
AMPs	Antimicrobial peptides
APS	Ammonium persulfate
BHI	Brain heart infusion
BMLD	Bacterial minimal lethal dose
BSA	Bovine serum albumin
CA	Cecropin A
CF	Carboxyfluoriscein
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate
CHDL	Carbapenem-hydrolyzing class D-β lactamases
CL	Cardiolipin
CMS	Colistin methane sulfonate
CSA	Cationic steroid antibiotic
Dab	Diaminobutyric acid
Dap	2,3-diaminopropionic acid
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIEA	*N*,*N*-Diisopropylethylamine
DIGE	Difference gel electrophoresis
DMEM	Dulbecco's Modified Eagle's medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
DTT	Dithiothreitol
ED_{50}	Effective dose 50
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Et_3N	Triethylamine
FA	Formic acid
FASP	Filter aided sample preparation
FDA	Foos and drugs administration
FDR	False discovery rate
Fmoc	Fluorenylmethyloxycarbonyl chloride
HD	High dose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HoAt	1-Hydroxy-7-azabenzotriazole
HP	N-terminal region of the *Helicobacter pylori* ribosomal protein L1
HPLC	High-performance liquid chromatography
HS	Human serum
ICU	Intensive care unit
i.p	Intraperitoneal
iTRAQ	Isobaric tags for relative and absolute quantitation

KCN	Potassium cyanide
LB	Luria Broth
LD	Lethal dose
L-PG	lysylphosphotidylglycerol
LPS	lipopolisaccharide
LUVs	Large unilamellar vesicles
MA	Magainin II
MALDI-TOF	Matrix-assisted laser desorption/ionization
MBLs	class B-metallo-β-lactamases
MD	Molecular simulations
MDR	Multi-drug resistance
ME	Meltittin
MeOH	Methanol
MH	Muller Hinton broth
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant Staphylococcus aureus
MS	Mass spectrometry
Mw	Molecular weight
NMR	Nuclear magnetic resonance
OMP	Outer membrane proteins
OMV	Outer membrane vesicles
PBPs	Penicillin binding proteins
PCR	polymerase chain reaction
PD	Pharmacodynamic
PDB	Protein data bank
PDR	Pan-drug resistance
PE	phosphatidylethanolamine
PEG	Polyethylene glycol
PG	phosphatidylglycero
PK	pharmacokinetic
PyBOP	benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate
QRDR	Quinolone resistance determining region
QS	Quorum sensing
RD	Recommended dose
RNA	Ribonucleic acid
RT	Room temperature
SNPs	single nucleotide polymorphisms
SPPS	Solid phase peptide synthesis
TBTU	O-(Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate
TEAB	triethyl ammonium bicarbonate
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
UTI	Urinary tract infection
VRE	Vancomycin resistant *Enterococci*
WHO	World health organization
XDR	Extended-drug resistance

Introduction

1. Current situation of antimicrobial resistance

Nowadays, we are facing a very important problem, the rapid and steady increase of resistance of most of the bacteria against the antibiotics available to treat the infections. In the last report of the WHO (World Health Organization) (1), they alert about this dramatic situation. The number of antibiotics approved by the FDA (Food and drug administration) has been decreasing since 20 or 30 years ago, with a very few effective antibiotics in the past years. If we take a look into the data published by the WHO in 2011, we have to be aware how big is the problem of the antimicrobial resistance, causing 3.2 millions of deaths per year just being lead by ischaemic heart disease and stroke with 7 and 6.2 millions respectively, and with higher numbers compared to AIDS or diabetes mellitus (Figure 1). In the EU the number of infections caused by multi-drug resistant (MDR) are approximately 400.000 per year, and of this number 25.000 per year are attribuible deaths, a part from the high number of people that acquire infections, there is an important burden which is the economical, these infections cost one billions per year in extra in-hospital days, and the losses of productivity are 600 millions per year. Taking into account the scenario of the present situation, they called ESKAPE (***E****nterococcus faecium*, ***S****taphylococcus. aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, ***E****ntereobacter* species) to the most resistance bacteria.

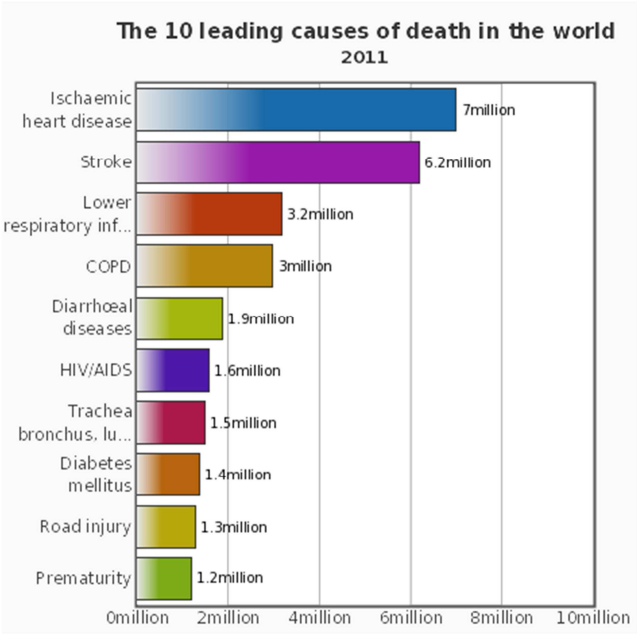


Figure 1: The 10 leading causes of death in the world in 2011. (WHO)

We can observe that ESKAPE group is composed by both Gram-positive and Gram-negative bacteria. ESKAPE bacteria are really attractive bacterial targets due to its high resistance profile of the components.

Another important issue is how to name the scale of resistance of the bacteria, because since a few years there was a lot of controversy about this topic, therefore it is very important to have the concepts of resistance very clear. We can define MDR when non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories, the next resistance grade is extensive drug-resistance (XDR) being non-susceptible to ≥ 1 agent in all but ≤ 2 categories, and pandrug-resistance (PDR) is defined when the bacteria is resistance to all the antibiotics in all the antimicrobial categories. (2)

Since a few years ago there was no controversy about the definition of these concepts because no pandrug-resistance were isolated, but in the last 10 years the number of pandrug-resistance strains isolated have been increasing rapidly, specially in the case of *A. baumannii* (3).
In the resistance profile of this specie it is possible to observe the evolution by comparing studies performed at different years, the percentage of resistant of imipenem was 3% in 1993 and increase up to 70% in 2007, the same effect was observed with quinolones, with an increase from the 30 to 97% in the same period of time (4). The same evolution is observed in Spain focusing in carbapenems, in 2001 the percentage of resistant was around 45% and ten years later was more than 80% (5). Observing this data we can imagine a really dramatic future in the war against these bacteria.

2. Current situation of antibiotics

As commented previously, there is an urgent need of new antibiotics, as we can observe in the figure 2, the antibiotics approved by FDA during the last 20-30 years are unuseful in some situations. In the figure 2 it is possible to observe that the last antibiotics are linezolid and daptomycin, these compounds are only active against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE). Indeed, even if they are the last antibiotics of

being released in the market some resistance to them have been reported, in the case of linezolid the most important mechanism of action are the alterations in the 23S rRNA target (6), and in the case of daptomycin is the modification of the lipids that composed the membrane of the Gram-positive bacteria, observed in *Enterecocci* (7), MRSA (8), and recently also some resistance to daptomycin in *Streptococcus mitis*, however the mechanism have not been elucidated yet (9). We want to highlight that the last antibiotics approved are only active against Gram-positive bacteria, so that means that the necessity of compounds active against gram-negative is more imperious.

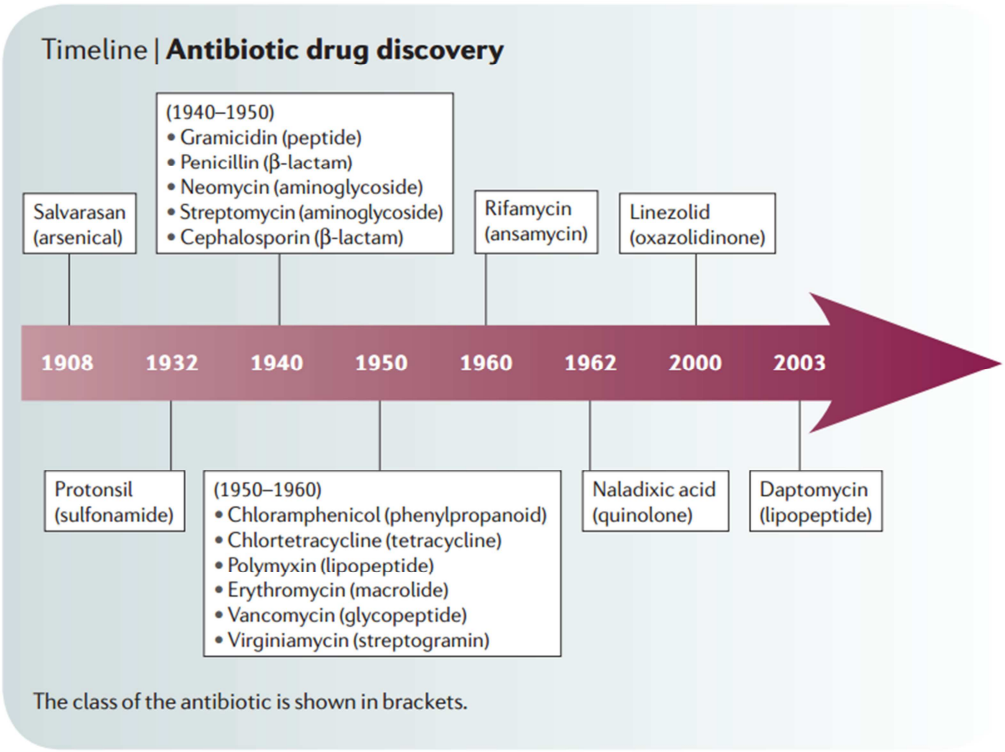


Figure 2: Timeline of development of new antibiotics. (**Wright GD**. The antibiotic resistome: the nexus of chemical and genetic diversity. Nat Rev Microbiol. **2007**. 5;175-86.)

3. *Acinetobacter baumannii*: The Archetype of resistance

The *Acinetobacter* species are mainly nosocomial opportunistic pathogens, that means that normally affects people with a low immune system admitted in the hospital. Between 2 and 10% of the gram-negative infections in the ICU (Intensive care unit) are caused by *Acinetobacter* species (10). They are normally one of the most frequent causative agents in nosocomial pneumonia but they are also implicated in bacteremia,

UTI (Urinary track infections) and meningitis. Another important feature of this specie is the ability to colonize any type of surface (11).

3.1. Characteristics and taxonomy

The *Acinetobacter* genus is formed by several species, they are Gram-negative, cocobacillus, nonfermenting, unable to reduce nitrates, catalase positive, oxidase negative (12) and without mobility, that is were the name Acinetobacter is derived, in Greek the term (*a-kineto*) means a lack of motility, so its name means that this bacteria does not have motility on surfaces (13).

In 1911 it was isolated the first *Acinetobacter* strain, it was from the soil and at that time it was named *Micrococcus calcoaceticus*, after 43 years, in 1954 Brisou and Prévot created the genus *Acinetobacter*, (14). In 1986 Bouvet and Grimont performed a really exhaustive taxonomic classification of this genus with 12 different species (15). Nowadays and due to the new techniques used for bacterial taxonomy new species are proposed, and now in 2014, 34 different species have been defined (16).

3.2. Morphological and phenotypical aspects.

Acinetobacter species (Figure 3) form smooth colonies in solid media, their colour can go from yellow to white-grey and from 1 to 2 mm of diameter (11,17). The oxidase test is the one used to differenciate the *Acinetobacter* genus from other related none fermentative Gram-negative bacteria (17). The ability to use different carbon sources in their metabolism give them an intrinsic resistance to the enviroment (18).

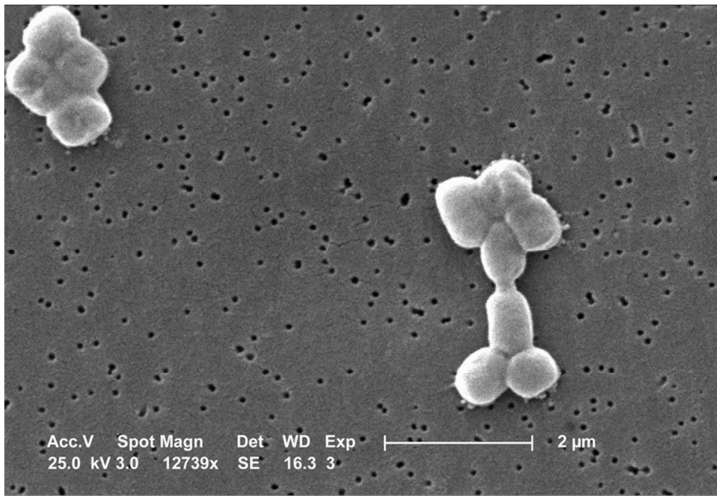


Figure 3. *Acinetobacter baumannii* morphology grouped. (Wikipedia *Acinetobacter baumannii*)

For the direct isolation of clinical strains, selective solid media, such as MacConkey can be used, but is preferable to use other solid media that do not allow the growth of other microorganisms (11, 17). This type of selective media that suppresses the growth of other bacteria are Herellea agar and Leeds *Acinetobacter* media, both of them are selective media and they are uselfull for most of the genospecies of *Acinetobacter*, both clinical and environmental (19).

3.3. Natural and clinical reservoirs

The two main features that make *Acinetobacter* such a difficult bacteria to fight against, are the ability to develop multiresistant patterns and the ability to survive in different conditions.

Acinetobacter species can be found in soil, water, animals and humans, growing normally in all the samples collected from soil or water (20). The places where or in which these bacteria have been isolated can go from fresh water ecosystems (21), to air samples from hospitals (22) or in kindergarden or high schools (23). This high resistance to environmental conditions comes from the ability to use different carbon sources, as previously commented. It has been observed that the ability of survival in different surfaces is different if we compare clinical isolates and standard strains from ATCC collection (24-28).

If we compare all the species of *Acinetobacter*, *A. baumannii* is the one which present a higher ability to survive to desecation, therefore with this information and also knowing that *A. baumannii* is the specie that presents more resistant profile patterns, we have the answer of why is *A. baumannii* the most common specie that cause nosocomial outbreak in hospitals (26, 27, 29). If we compare the ability to survive in dry surfaces we can observe that *A. baumannii* can live during more than 25 days, while *A. lwofii* can only survive during 7 (25).

If we compare their survival with other bacteria the difference is even higher, *P. aeruginosa* can survive less than 24 hours and *Escherichia coli* at around 24 hours (30).

3.4. Pathogenesis and virulence factors of Acinetobacter baumannii

This part of the introduction is a summary of the review. (Part 3.4)

Roca I, Espinal P, Vila-Farrés X, Vila J. The *Acinetobacter baumannii* Oxymoron: Commensal Hospital Dweller Turned Pan-Drug-Resistant Menace. Front Microbiol. **2012**. 3;148

Virulence factors are required for the pathogenesis of infections caused by *A. baumannii*, these virulence factors allow the bacteria to colonize and infect the patient. There are not many studies about the pathogenicity of this microorganism (31). These are the most important virulence factors in *Acinetobacter*.

3.4.1. Biofilm

The first step in bacterial colonization is the adherence to the cell host. Once the first bacteria is adhered to the cell host a biofilm is build. Biofilm can be defined as a group of bacteria stacked to each other with a polymeric matrix, this matrix do not allow the antibiotic to be efficient against bacteria and also help the bacteria to survive. This polymeric matrix can be formed by carbohydrates, nucleic acids, proteins and other macromolecules (32). The ability of a strain to create biofilm can be one of the factors that can influence in its virulence. (33-35). The ability of each strain to perform biofilm can be influenced by different factors such as quorum sensing, bacterial surface components, amount of nutrients, macromolecular secretions (36-37). The two-component regulatory systems and transcriptional regulators which have relation with

the genes associated with biofilm are also very important (38). The most important gene is *CsuE*, which is a component of the CsuA/BABCDE chaperone-usher complex, this gene is involved in the production of the pili, and when the inactivation of the gene is produced no biofilm formation is observed (39).
A. baumannii can form biofilm also in the environmental surfaces not only in human cells, even in dry surfaces and with low amount of nutrients (40).

3.4.2. Motility and adherence

As previously commented the name *Acinetobacter* comes from the non-motility, this fact is due to the lack of flagella (41). This absence of flagella means that this bacteria does not move using swarming (42), however *A. baumannii* spreads really fast over surfaces, this fact may be as the twitching already described in *A. calcoaceticus* (43). There are large number of genes involved in everything related to the type IV pili, which is the one associated with twitching motility (44). It has been observed that *pilT* gene it is also involved in the twitching (42). Twitching motility is also controlled by two component sensor-regulators and complex chemosensory system (45).

3.4.3. Surface polysaccharides

LPS (lipopolysaccharide) plays an important role in the virulence of *A. baumannii*, this fact it is showed in the study performed by García *et al*. were they claimed that LPS and lipid A showed lethal toxicity in mice, pyrogenicity in rabbits and complement inactivation *in vivo* (46). The same results are obtained confirming the important role of the LPS in terms of virulence (47). Another study that confirms the importance of LPS in the virulence of *A. baumannii* was based on the study of a colistin-resistant strain. All mechanisms of resistance to colistin are related to LPS, modification by mutations in the gene *pmrAB* or *lpxACD* and this fact also make the strain less virulent (48)

3.4.4. Outer membrane proteins

When we talk about outer membrane proteins (OMPs) in terms of virulence and pathogenesis we have to highlight OmpA. OmpA is the most abundant protein in the

bacterial surface, it plays a major role in the adherence and invasion of epithelial cells and induce apoptosis (37).

One of the mechanisms of *A. baumannii* is to induce cell death (HEp-2 cells) by cell surface death receptors and mitochondrial disintegration. It has been observed OmpA in the mitochondria, where it generates a release of pro-apoptotic molecules such as cytochrome *c*, and apoptosis-inducing factor (AIF), these apoptosis of the cells make the bacteria able to internalize trough the disrupted mucosal lining (49).

Regarding the different outer membrane proteins found in *A. baumannii*, Smani *et al*., discovered that Omp33, a protein involved in carbapenem resistance, was also involved in the virulence and fitness of this microorganism (50).

3.4.5. Outer membrane vesicles

Another virulence mechanism which is also common to all Gram-negative bacteria are the outer membrane vesicles (OMVs). OMVs are spherical nanovesicles with a diameter between 20 and 200 nm, and are composed by DNA, RNA, lipids, lipopolysaccharides and even OMPs (51, 52). The role of these OMVs is different, they can transport virulence factors, they can participate in biofilm formation, and they can also be involved in quorum sensing and gene transfer (53,54). OmpA is the most abundant protein of the OMVs, these allow OmpA to adhere to the host cells (55). OMVs have also the ability to carry genetic material, like the $bla_{\text{OXA-24}}$ gene which encodes a oxacillinase which produce resistance to carbapenems (54). With this information it is shown the importance of the OMVs, not because they are important in the interaction host-pathogen, but also because they are also able to spread resistant genes.

3.4.6. Siderophores

Siderophores are compounds that have the ability to chelate iron, as it is known, iron is essential for the growth of bacteria, therefore bacteria secrete these compounds in order to obtain iron from outside the bacteria. Just two different siderophores have been identified in *Acinetobacter species*, which are Acinetobactin (56) and Fimsbactin A-F (57). An *A. baumannii* isolate during an outbreak was deeply analyzed due to its high-affinity iron-uptake, it was demonstrated that the genes *dhb* were the ones involved in

iron-uptake. It was also detected some proteins highly-related to iron transport like OM73, P45 and P114 (58).

3.4.7. Quorum sensing

Bacteria excreted chemicals compounds in order to perform communications with the other bacteria and environmental adaptation, this ability is called quorum sensing (QS). (59) These compounds secreted by the bacteria are normally acyl-homoserine lactone (AHL)-like signal molecules produced by the LuxI family of AHL synthases. The production and secretion of these compounds is controlled by the cell depending on the cell density and the growth phase, and it has been linked with the production of certain virulence factors such as motility or biofilm (59,60). All these processes mediated by AHL system in Gram-negative bacteria involve two important family proteins, LuxI which interact with LuxR. This complex binds to a specific promoter sequence known as lux-box, which regulates the expression of QS target genes. Among the LuxI proteins, AbaI protein, found in *A. baumannii*, is the one involved in the production of N-(3-hydroxydodecanoyl)-$_{L}$-HSL and (3-hydroxy-C(12)-HSL) (61), It is also important to point out the high promiscuity if these receptors, as it is observed the similar effect when the same compound (R) and (S)-stereoisomer is tested (62).
It was also observed that to isogenic strains with mutations in the *abaI* gene led to a reduction of biofilm production (36,62).

3.5. Antimicrobial resistance of *Acinetobacter baumannii*

This part of the introduction is a summary of the review. (Part 3.5)
Roca I, Espinal P, Vila-Farrés X, Vila J. Front Microbiol. **2012**. 3;148

Acinetobacter have innate ability to survive in difficult conditions and in difficult places, but another important feature that makes *Acinetobacter* such a successful pathogen is the ability to acquire and keep different mechanisms of resistant that makes it able to survive against all the antibiotics used to treat the infections caused by this pathogen.

Acinetobacter has become resistant to all the antibiotics used to treat infections, here we report the mechanisms of resistance used by this pathogen to survive to the action of the antibiotics.

3.5.1. β-lactams

The mechanisms of resistance against this group of antibiotics can be divided into two main groups:

i) Enzymatic mechanism (β-lactamases)

ii) Non enzymatic mechanism.

i) Enzymatic mechanisms.

β-lactamases are enzymes produced by some bacteria that provide resistance to β-lactams. These antibiotics family all have a common element in their molecular structure which is four atom ring with an amide group, known as β-lactam. The effect of the enzyme is opening this β-lactam ring by hydrolysis, deactivating the molecule antibacterial activity.

Chromosomal cephalosporines (AmpC /ADC)

AmpC enzymes belong to class C β-lactamases, and they are responsible of the resistance to penicillins and extended-spectrum cephalosporins, except cefepime, as well as to β-lactam-β-lactamase inhibitor combinations (63). The basal level of expression of these enzymes in *Acinetobacter* do not affects the susceptibility to the most of β-lactams, however the presence of an insertion sequence (IS*AbaI*) upstream of the bla_{ADC} gene promotes the increase of its expression and increase the minimal inhibitory concentration to expanded-spectrum cephalosporins (64). This is due to the fact that the IS element carries a promoter that can be used for the RNA polymerase to express the bla_{ADC} gene.

Oxacillinases

Oxacillinases, also called Ambler Class D enzymes, as their name shows are able to hydrolyzed oxacillin and cloxacillin, apart from some oxyimino-β lactams, but they are weakly inhibited by clavulanic acid, however there is a subgroup called carbapenem-hydrolyzing class D-β lactamases (CHDL) which are able to inhibit the action of imipenem and meropenem but not the action of extended-spectrum cephalosporins and aztreonam (65).

We can divide the OXA found in *A. baumannii* into five groups: i) OXA-51/49 which is the only one naturally occurring, found in the chromosome of all strains of *A: baumannii*. ii) The other four groups are acquired CHDLs (OXA-23, OXA-24/40, OXA 58, and OXA-143).

Metallo-β lactamases

Nowadays carbapenems are the most used antibiotics to treat infectious caused by *Acinetobacter* species, however during the last decades resistance to carbapenems is increasing worldwide (66,67), there is also a big problem when dealing with carbapenem-resistance *Acinetobacter*, most of the strains are resistant to almost all the antibiotics classes, except intermediade resistance to rifampicin and being susceptible to tigecycline and colistin.

The main feature of class B-metallo-β-lactamases or MBLs is their broad spectrum, they are able to hydrolyze all the β-lactams except aztreonam (68). There are several MBLs described such as IMP metallo- β-lactamases (69), VIM enzyme (70), SIM-1 (71) or the most recent one which is NDM (72), This MBL enzyme has the ability to spread really fast (73).

ii) Non enzymatic mechanism.

When we talk about non enzymatic mechanism of resistance to β-lactams we refer to membrane alterations, normally by alterations in OMP or overexpression of efflux pumps. In addition modifications in the penicillin binding proteins (PBPs) have also been shown as a potential mechanism of resistance. An example is the protein CarO that it was associated to carbapenem resistance, with out the presence of any known

carbapenemases (74), the same effect was observed in 33- to 36- kDa OMP in a *A. baumannii* strain isolated in Spain (75). Other proteins like the heat-modifiable HMP-AB is also involved in resistant to carbapenemases in *A. baumannii*, and it is very similar to OmpA from Enterobacteriacae. (76) Another mechanism of action that could be also compatible with the production of β-lactamases, is the change of affinity or expression of the PBPs (77).

3.5.2. Aminoglycosides

There are two different ways to achieve resistance to aminoglycosides, the first way is by the expression of aminoglycoside-modifying enzymes (AMEs), the most common are, acetyltransferases, nucleotidyltransferases, and phosphotransferases (78), the acivity of this AMEs result in the modification of the amino or hydroxyl group present in the aminoglycosides which make them to decrease their affinity for the target site (79). The second way is by efflux proteins, these efflux pumps, and specially RND family plays an important role in MDR (80). We want to focus in the AdeABC which confers resistance to aminoglycosides apart from other antibiotics families (81). There is a third mechanism which has not been reported in *A. baumannii* which is the modification of some ribosomal proteins.

3.5.3. Quinolones

The most important mechanism of resistance is, as in all the species, the mutations in the quinolone-resistance determining region (QRDR) in the A subunit of the DNA gyrase and DNA topoisomerase IV. Both mutations in the *gyrA* (encoding the A subunit of the DNA gyrase) and *parC* (encoding the A subunit of topoisomerase IV) have been described. The substitution of Ser83 to Leu83 in GyrA and the substitution of Ser80 to Leu80 in ParC are the most frequently found (82). It is important to mention that mutations in the *parC* gene are always accompanied by mutations in the *gyrA* gene, that suggests that *gyrA* is the preferred target when compares to *parC* (83). Efflux pumps are also important as an alternative mechanism of resistance to quinolones, being the most common these belonging to the RND family such as AdeIJK (84) and AdeFGH (85).

3.5.4. Polymyxins

In spite of their toxicity, polymyxins are the last option in the treatment of infections caused by *A. baumannii*. Up today most of the strains of *A. baumannii* are still susceptible to colistin, however in the last years colistin-resistant *A. baumannii* clinical isolates have been reported (86). Regarding the mechanism of action of colistin not many things are known, but all the hypothesis indicates that it acts in the outer membrane, however recently some inhibition of the NADH-quinone oxidoreductase have been descrived as a secondary mechanism of action (87). Two different mechanisms of resistance to colistin have been elucidated, they are related to each other because somehow they have some relation with membrane components. Adams *et al*. observed mutations in *pmrABC, pmrAB* is a two component regulatory system that regulates the expression of *pmrC* which encodes a phosphoethanolamine transferase enzyme involved in lipid A modification (88-90).
The second mechanism of resistance is the complete loss of the lipopolysaccharide by mutations in *lpxACD* genes, which are involved in the lipid A biosynthesis (91).

4. Colistin and daptomycin

Currently, two peptidic antibiotics are widely used, colistin and daptomycin. The first is an old antibiotic that was not used for a long time since it showed an important toxicity and other antibiotics were available, however it is now used as a last resort to treat infections caused by multidrug resistant bacteria such as *A. baumannii, P. aeruginosa* or *K. pneumoniae.* The second one, is together with linezolid the two antibiotics launched during the last 30 years, both having activity only against Gram-positive bacteria. Both compounds have a similar structure being both lipopeptides, with a cyclic peptidic part and a fatty acyl tail.

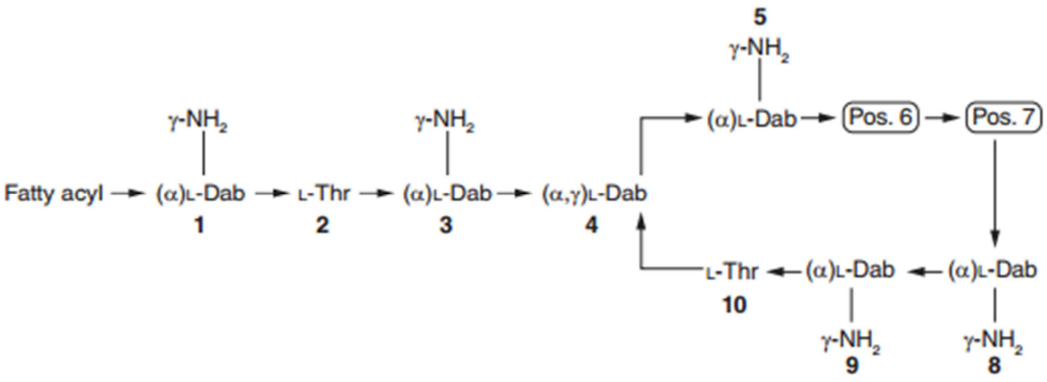
4.1. Colistin

Colistin, also called polymyxin E, is a lipopeptide isolated from *Bacillus polymyxa*. It has a very narrow spectrum of activity, only being active against some Gram-negative bacteria, so it is used as “salvage” to treat multidrug-resistant infections. It was used before the 1970s but then was replaced by aminoglycosides due to its nephrotoxicity

and neurotoxicity (92, 93). In 1959 it was the beginning of its use in the clinical setting The procedures carried out by the Food and Drugs Administration (FDA) at that time were not so strong as today, and colistin became an antibiotic easily (94). During the last decades we have witnessed a dramatic increase both in the proportion and absolute number of pathogens such a *A. baumannii* presenting extensive-drug-resistance to antibacterial agents, being susceptible only to tigecycline and colistin. Therefore, colistin is used widely and hence the emergence of colistin-resistant *A. baumannii* has been observed. Two aspects are important to be studied, both are related to each other, these aspects are potential nephrotoxicity and neurotoxicity and optimizing dose (PK/PD). In terms of nephrotoxicity and neurotoxicity, we want to highlight that neurotoxicity is not as common as nephrotoxicity. It has been proved that colistin has the same nephrotoxicity levels as tobramycin, an aminoglycoside (95, 96). In another more extensive study, the results were that patients who received colistin more than 14 days had a 3.7 fold increased risk of nephrotoxicity, thereby what is important is the accumulative dose more than the daily dose, in this sense it is important to monitor the kidney function in long term treatments (97). It is now accepted that renal injury caused by colistin is not as strong as it was though in the beginning and is also totally reversible over weeks to months after ceasing therapy (97). The other important aspect is the adjustment of the dose treatment. In the last decade, the pharmacokinetic and pharmacodynamic (PK/PD) has been examined in different ways like mouse model or just *in vitro*, these ways try to find out the right dose that should be given (98-101). It should be noted that colistin is administered as a prodrug colistin methane sulfonate (CMS), and the conversion to the active specie, colistin, is really slow, therefore during the first dose, the concentration of colistin is below the break point (2 mg/L) for that reason monodose therapy is not useful to treat *A.baumannii* (102). The dosage of intravenous CMS recommended by the manufacturers in the USA is 2.5–5 mg/kg (31,250–62,500 IU/kg) per day, divided into two to four equal doses, however the dose in the UK is 4–6 mg/kg (50,000–75,000 IU/kg). CMS is normally administered during 10-14 day, however it can change depending on the renal function of the patient

The chemical structure of the colistin, and all the polymyxins, is formed by a cyclic peptide and a fatty acyl group, as commented previously. It is a decapeptide and the loop is produced between the side chain of the diaminobutyric acid (Dab) at position 4 and the carboxyl group of the C-terminal threonine residue at position 10. Some

different polymyxins have been isolated, the 3 differences are, positions 6 and 7, and the different fatty acyl group present in the N-terminal of the peptide, all of them are showed in Table 1.



Polymyxin	Fatty acyl group	Pos. 6	Pos. 7
B_1	(*S*)-6-methyloctanoyl	D-Phe	Leu
B_1-Ile	(*S*)-6-methyloctanoyl	D-Phe	Ile
B_2	6-methylheptanoyl	D-Phe	Leu
B_3	Octanoyl	D-Phe	Leu
B_4	Heptanoyl	D-Phe	Leu
B_5	Nonanoyl	D-Phe	Leu
B_6	3-hydroxy-6-methyloctanoyl	D-Phe	Leu
E_1	(*S*)-6-methyloctanoyl	D-Leu	Leu
E_2	6-methylheptanoyl	D-Leu	Leu
E_3	Octanoyl	D-Leu	Leu
E_4	Heptanoyl	D-Leu	Leu
E_7	7-methyloctanoyl	D-Leu	Leu
E_1-Ile	(*S*)-6-methyloctanoyl	D-Leu	Ile
E_1-Val	(*S*)-6-methyloctanoyl	D-Leu	Val
E_1-Nva	(*S*)-6-methyloctanoyl	D-Leu	Nva
E_2-Ile	6-methylheptanoyl	D-Leu	Ile
E_2-Val	6-methylheptanoyl	D-Leu	Val
E_8-Ile	7-methylnonanoyl	D-Leu	Ile

Pos.: Amino acid position.

Table 1. Different structure of all the polymyxins.

Velkov T, Roberts KD, Nation RL, Thompson PE, Li J. **Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. Future Microbiol**. 2013. 8;711-24.

We want to focus only in polymyxin E. It has a D-Leu in the position 6, being the only D-amino acid in all the peptide, and Leu also in the position 7. We have to take into account that polymyxin E, contain several species, but the majority (more than the 80%) belongs to a mixture of polymyxin E_1 and polymyxin E_2, they just differ in the fatty acyl group, having (S)-6-methyloctanoyl for polymyxin E_1 and 6-methylheptanoyl for polymyxin E_2.

The mechanism of action of colistin seems to be in the outer membrane, due to its big size, probably unable to penetrate inside the bacteria, however some authors have suggested that it can accumulate inside the bacteria and interact with a intracellular target, as commented previously. First of all we need to have very clear which are the components of the Gram-negative bacteria, and why its not effective against Gram-positive. The main difference between the cell wall of Gram-negative and Gram-positive bacteria is that Gram-positive bacteria is composed by a lipid bilayer, and protected by a thick peptidoglycan layer, while the Gram-negative bacteria have a more complex arquitecture having inner and outer membrane separated by periplasmic space. In the outer membrane layer is where lipopolysaccharides are located, and especially lipid A which is one of the components. The structure of lipopolysaccharides is divided in three parts: the variable O-antigen chain (repited saccharide units), a core oligosaccharide domain and the lipid A, Figure 5 (103).

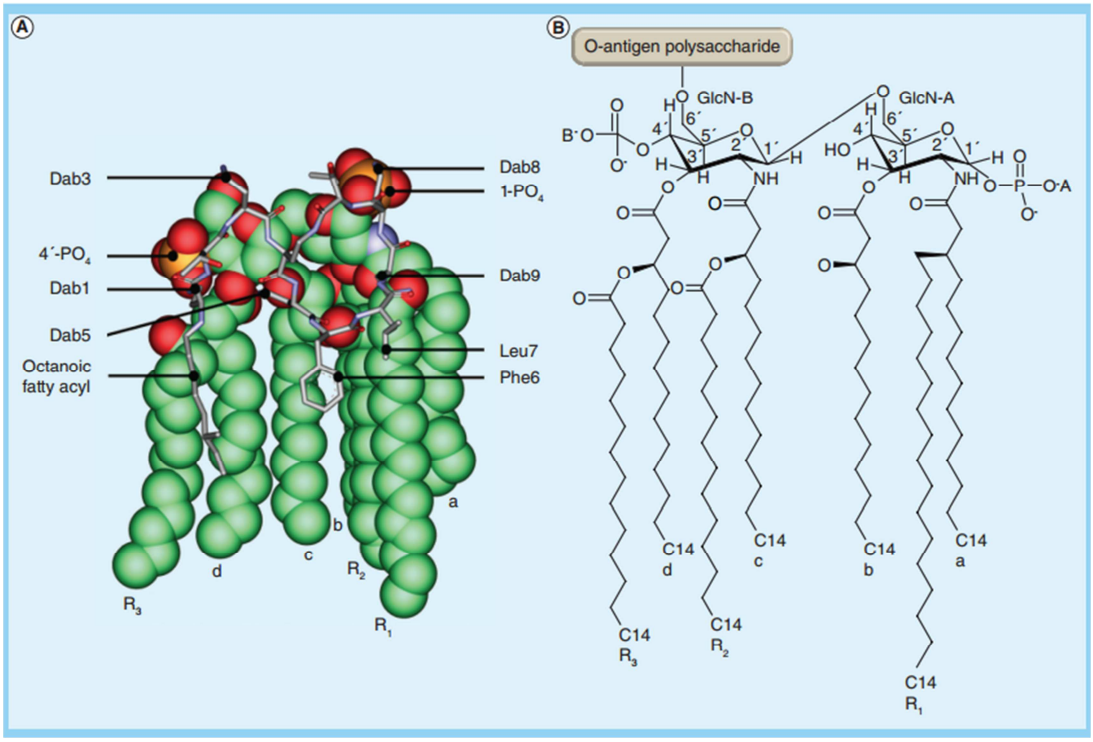


Figure 5. A) Interaction between polymyxin and lipid A. B) Components of the Lipopolysaccharide (LPS).

Velkov T, Roberts KD, Nation RL, Thompson PE, Li J. **Pharmacology of polymyxins: new insights into an 'old' class of antibiotics. Future Microbiol**. 2013. 8;711-24.

Lipid A is formed by a β-1´-6-linked D-glucosamine disaccharide which is phosphorylated at positions 1´and 4´ (104). It is important to mention the function of the cations Mg^{2+} and Ca^{2+}, which are able to link adjacent LPS moieties (105), however as it has been commented previously there is a suspicion that it could also have an intracellular target (87).

If we take a look into the peptidic structure of polymyxin B and colistin, just two residues are different, due to its different activity, that means that these variable residues are important in the mechanism of action, also the amphipathicity of the molecule has a crucial role. Several theories of the mechanism of action have been proposed, but nowadays the accepted model is the one called 'self-promoted uptake' (Figure 5), the action of the amines of the Dab residue interact electrostatically with the anionic lipid A phosphates, this interaction leads to the displacement of the cations commented previously, that allows the two key residues (amino acids 6 and 7) and the fatty acid acyl tail to internalize into the outer membrane, this is the part where most the articles published about this topic agrees, the rest of the mechanism remain unclear. (106).

4.2. Daptomycin.

Daptomycin is one of the lastest antibiotics released in the market, it was discovered by Lilly in the late 80s from *Streptomyces roseosporus* a saprotroph isolated in soil. Lilly researchers observed a really good “in vitro” activity against Gram-positive bacteria but they gave up the development of the antibiotic due to the fact that surfactants found in the lung inhibit the effect of this antibiotic, therefore can not be used to treat respiratory tract infections such as pneumoniae. Later on the rights were sold to Cubist Pharmaceuticals. In 2003 this antibiotic was approved by the FDA (107). Daptomycin has a similar structure as polymyxins, a cyclic peptide with a fatty acyl chain, daptomycin has 13 residues, 10 of them are forming the cycle and the rest are forming the lipophilic tail. From the 13 amino acid residues, five of them are nonproteinogenic, such as L-ornithine (L-Orn), L-*threo*- 3-methylglutamic acid, L- Kyrunenine (L-Kyn) and two D-amino acids. In the begining one of the D-residues was assignated as L-, but recently they corrected into D-configuration (108). The cyclization is produced between the carboxyl group of the L-Kyn (residue 13) and the hydroxyl group of the Thr (residue 4), and the fatty acid is formed by a decanoyl. Figure 6

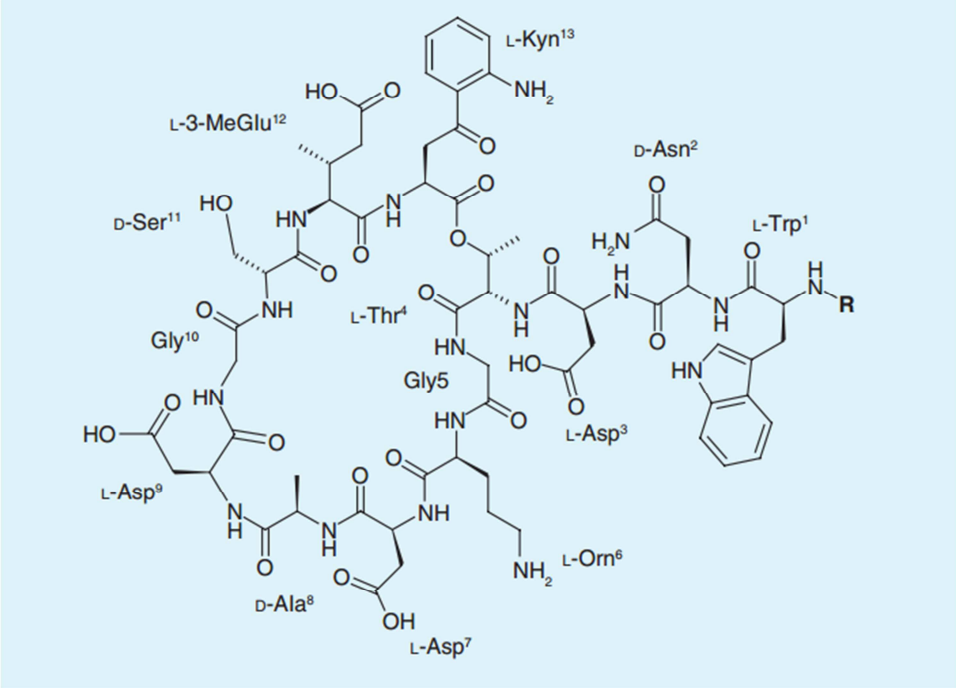


Figure 6. Chemical structure of daptomycin.

Bionda N, Pitteloud JP, Cudic P. Cyclic lipodepsipeptides: a new class of antibacterial agents in the battle against resistant bacteria. Future Med Chem. **2013**. 5;1311-30.

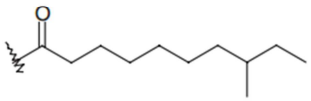
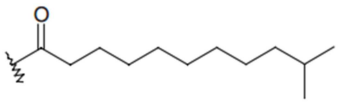
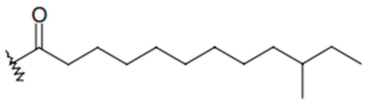
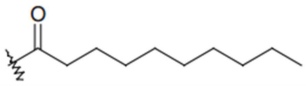
Compound	R	MIC (μg/ml) *Staphylococcus aureus*
1 A21978C$_1$		1
2 A21978C$_2$		0.5
3 A21978C$_3$		0.13
4 Daptomycin		0.125–0.5

Table 2. Chemical structure of daptomycin and analogs, and MIC against *S. aureus*.

Bionda N, Pitteloud JP, Cudic P. Cyclic lipodepsipeptides: a new class of antibacterial agents in the battle against resistant bacteria. Future Med Chem. **2013**. 5;1311-30.

Several compounds were found when they discover daptomycin (109), all of them had the same scaffold just changing the fatty acid side chain, in table 2, the activity of the other daptomycin analogues can be observed (108). When comparing all the analogues and daptomycin they observed that the penetration into the lipid bilayer was inversely proportional to the length of the fatty acid chain, however the length of the fatty acid chain was also really important in the toxicity of the compounds, longer side chain fatty acid exhibited higher toxicity (110)

The mechanism of action of daptomycin is in the same stage as polymyxins, it is known that the mechanism of action is very similar, first of all it was demonstrated that the activity of daptomycin and their analogues are intrinsically related to the amount of Ca^{2+}, present in the serum, no activity is present when divalent cations such as Mg^{2+}, Zn^{2+} or Ba^{2+} are present in the medium (111), this calcium may induce conformational changes into daptomycin (112,113), or neutralize the repulsion between the daptomycin (anionic) and anionic phospholipid heads of the membrane (114). The initial step is the interaction of the polar head groups of the phospholipid and the fatty acid chain of the lipopeptide, and after the accumulation of the compound, oligomers can be formed, these oligomers are the responsible of the depolarization of the membrane, but there is not a clear evidence of this hypothesis.

In terms of pharmacokinetics, daptomycin is a bactericidal antibiotic with a really long half-life (8h), and showed a postantibiotic effect of up to 6.8 hours (115). The fact that it has this high values may be due to its high affinity with the serum proteins (90%) (116). Doses approved by the FDA are 4 mg/Kg for soft tissue infections and 6 mg/Kg for *S. aureus* bacteremia, however the data obtained in animal model and *in vitro* suggest that the doses could be higher, and that will reduce the development of resistance and will increase the activity (117).

As we have commented previously the spectrum of activity of daptomycin is in front Gram-positive bacteria, before being approved by the FDA, it was tested in over 70 medical centers located in Europe and America, and the results were that 99.4% of the isolates were inhibited by ≤2 mg/L daptomycin (118). The spectra of activity of daptomycin include both methicillin-susceptible and –resistant *S. aureus*, vancomycin –susceptible and –resistant *Enterococcus*, coagulase-negative *Staphylococcus*, and streptococci.

Although daptomycin is a really new antibiotic, some resistance have already been reported in the Gram-positive bacteria against which daptomycin is more commonly used, such as *S. aureus*, *Enterococcus* and *Streptococcus*. Since the mechanism of action of daptomycin is associated with the depolarization of the cell membrane and the cell wall, the mechanisms of resistance of the bacteria commented below are related to modifications of the cell wall and cell membrane.

In *S. aureus*, the first gene identified when a daptomycin resistant strain was isolated was the *mprF gene* with some single nucleotide polymorphisms (SNPs) observed, (119,120). When the MIC of daptomycin start to increase the amount of SNPs in different genes are increasing, these genes are the *yycFG* gene, were the *yyc* operon is involved in the generalized response to stressors such as antimicrobials, and consequently the *rpoB* or *rpoC* genes, beta and beta' subunits of RNA polymerase. The SNPs observed in these genes, specially in the *mprF* gene, lead to an increase of the relative positive surface charge. The *mprF* gene encodes a protein which has a double function being the responsible for the lysinylation of the lysylphosphotidylglycerol (L-PG) to generate the positively charged cell membrane phospholipid, L-PG, and the second function which is involved in the inner-to-outer cell membrane translocation of L-PG (121), apart from the SNPs in the *mprF* gene, the same genes lead to a change of the rigidity/fluidity of the cell membrane, that do not allow the interaction of daptomycin with the bacteria (119), this rigidity can be associated to the overproduction

of staphyloxantin as well (122). It has been observed that the thickness of the cell wall is also an important fact of the daptomycin -resistant, the increase of the thickness may act as physical barrier in the interaction of daptomycin. (123)

Resistance in Enterococcus have also been observed , the group of Arias CA *et al.* (7) isolate a strain of *E. faecalis* after the therapy with daptomycin, they observed by comparing genomically both daptomycin-susceptible and daptomycin-resistant the mutations in three genes that were the ones involed in this resistance. The genes associated with daptomycin resistance in *E. faecalis* were the *LiaF* gene, this gene belongs to a three component regulatory system (LiaFSR) which is involved in the cell envelope stress-sensing response to antibiotics. The second and third gene (*GdpD* and *Cls*) encodes proteins involved in the metabolism of membrane phospholipids (7). The mechanism of resistance to daptomycin in both *S. aureus* and *E. faecalis* involves components of the membrane such as lipids or proteins.

The Dr. Miro's group from Hospital Clinic of Barcelona (9), observed also resistance to daptomycin in *S. mitis* when it was exposed to daptomycin in an animal model. To elucidate the mechanism of resistance of *S .mitis* to daptomycin is a chapter of the thesis.

5. New antibacterial drugs.

The discovery of new antibacterial agents is one of the strategies to be used to control the emergence and spread of multi-drug resistant bacteria. Here we describe different options in order to search new antimicrobials.

5.1. Antimicrobial peptides (AMPs)

This part of the introduction is a summary of the review. (Part 5.1.1 and 5.1.2)

Vila-Farrés X, Giralt E, Vila J. Update of peptides with antibacterial activity. Curr Med Chem. **2012**;19;6188-98

Hundreds of peptides have been tested in order to know if they have the ability of being able to beat bacteria. It would be really difficult to explain all the peptides active against all types of bacteria, therefore we are going to focus only in the peptides tested against *A. baumannii*, which is the main bacteria of this thesis.

There are different ways to obtain an active peptide, some of them use natural extracts and perform a high troughput screening against the desired bacterial species, some others have just a peptide or a few peptides, totally synthetics, and try to find out their activity. The use of peptides as antimicrobial agents has several advantages such as the broad spectrum activity (active against even virus and fungi) compared to normal antibiotics, or the less specific target, that reduce the ability of the bacteria to acquire resistant to the peptide. Apart from the advantages, it has also some drawbacks like poor stability in human serum, due to the activity of proteases and peptidases and the rapid clearance. This fact could also been solved by using D-amino acids (124), by the methylation of some amino acids (125), by the cyclization of the peptide (126), by the introduction of some fluorinated residues, (127) or by introducing a PEG chain in order to protect the peptide (128). Another disadvantage is the most expensive production of the antimicrobial peptides compared to normal antibiotics (129).

When trying to find out an active peptide against a certain bacteria, the strategy adopted could be different, trying to find a natural peptide, and afterwards try to optimize the important features that generates the activity of the peptide against bacteria. The other strategy is to synthesize a *de novo* peptide, with a new sequence that somehow could be active against bacteria, therefore we are going to divide the antimicrobial peptides active against *A. baumannii* into two different groups, the natural and the synthetics.

5.1.1. Natural and optimized antimicrobial peptides

First of all we will start highlighting some of the typical antimicrobial peptides, which most of them are commercially available, such as indolicidin, buforin II, cecropin P1, magainin II, ranalexin, cecropin A and melittin. We have to point out the importance of the kind of strain used to test the peptide. It has not the same importance to test a peptide against an ATCC strain or against a pandrug-resistant strain. All these peptides commented previously were tested against multidrug-resistant *A. baumannii* strains, the best results were for buforin II and magainin II with a MIC_{50} and MIC_{90} of 2 and 16 mg/L respectively, the same MIC_{90} was observed in the case of melittin and cecropin P1, but the MIC_{50} is one dilution higher (4 mg/L). The other three peptides, indolicidin, ranalexin and cecropin A have slightly higher values for MIC_{50} with 8 mg/L, and the MIC_{90} is 32 mg/L for cecropin A and ranalexin and 64 mg/L for indolicidin (130,131).

Two of the peptides commented previously, cecropin A and melittin, were mixed in order to achieve higher activity against colistin-resistant *A. baumannii* strains. First of all mention that the same peptide cecropin A(1-7)melittin (2-9) (CA(1-7)M(2-9)) was tested in two different studies against multidrug-resistance *A. baumannii* and colistin-resistance *A. baumannii*, and is curious that the best results were obtained (MIC_{50} 2 mg/L, MIC_{90} 4 mg/L)(130) when tested against colistin-resistant bacteria, compared to the multidrug-resistant strains (MIC_{50} 4 mg/L, MIC_{90} 12 mg/L) (131). Several versions of this hybrid peptide were tested against colistin-resistant *A. baumannii* strains, the results were indentical for all the analogs (MIC_{50} 4 mg/L, MIC_{90} 8 mg/L), the analogues were CA(1-8)M(1-18), CA(1-7)M(5-9) and Oct-CA(1-7)M(2-9) (132).

The original hybrid CA(1-7)M(2-9) was also improved in terms of selectivity and stability by methylating the lysines present in the peptide, however the *in vitro* activity decreased (125). The method of mixing two or more chimeric parts of natural antimicrobial peptides is done also in the article published by Gopal *et al.* (133), they synthesize hybrid peptides mixing cecropin A (CA) and melittin (ME), as the same strategy as commented previously but adding also the N-terminal region of the *Helicobacter pylori* ribosomal protein L1 (HP) (134), and magainin II (MA). The four hybrid peptides HP(2-9)-ME(1-12), HP(2-9)-MA(1-12), CA(1-8)-ME(1-12) and CA(1-8)-MA(1-12), have a MIC slightly higher compared to polymyxin but better than some antibiotics, which the strains tested are resistant to (133). The source where antimicrobial peptides can be found is very diverse, they can be isolated from frog skin secretions. A peptide obtained from there with moderate activity against *A. baumannii* has been found. They tried to improve it by changing some residues but they did not success, just similar values but with less hemolytic activity (135). Other antimicrobial peptides such as hymenochirin-1B can be found in frog skin secretions as well. It has a good activity against multidrug-resistant *A. baumannii* (136). Frog skin seems to be really good source for the isolation of active peptides against *A. baumannii*, as well as other peptides which have not been tested against this microorganism (137,138). Another natural source of antimicrobial peptides is the venom of the Australian scorpion in which several antimicrobial peptides with an interesting activity against *A. baumannii* has been found (139).

There are other natural sources which could lead to an interesting hit when a good optimization is done, for instance, apidaecin, a peptide isolated from honeybee. The activity of the original compound was optimized and it led to an antimicrobial peptide

apidaecin 88 (Api88) with good activity *in vitro* against several multidrug-resistant bacteria. It was also tested *in vivo* against *E. coli*, observing good activity when dealing with systemic infections (140). Even though if it was active *in vivo*, the half-life time was really low, therefore the sequence of Api88 was optimized in order to obtain a more stable peptide, leading to apidaecin 137 (Api137), which should a better compound *in vivo* compared to Api88 (141).

5.1.2. Synthetic Antimicrobial peptides

Some other peptides active against *A. baumannii* did not have any relationship with natural sources, they are fully synthetic, this is the case of the two peptides which only contains leucines and lysines (LKLLKKLLKKLLKLL), one with all L-amino acids and the other one with 33% of D- amino acids (^{3}Lys, ^{7}Lys, ^{8}Leu, ^{10}Lys, ^{13}Leu). The peptide containing D-residues has a better activity (5.6 mg/L) compared to the peptide with just L-amino acids (11.2 mg/L), it was also more stable to hemolysis, at 180 mg/L goes from 0 to 100% (142). In the work performed by Huang Y *et al*. (143) also two peptides with Leucines and Lysines were synthesized, but in this case they also have one or two cystein that are funcionalized. C(LLKK)$_2$C had a MIC_{50} and MIC_{90} of 50 and 75 mg/L respectively, meanwhile for the other peptide, (LLKK)$_2$C, the values were MIC_{50} 30 mg/L and MIC_{90} 40 mg/L. If the values of these peptides are compared with the other values commented previously it is possible to observe that this values are significantly higher, however they have a really low toxicity *in vivo* with a lethal dose of 173 and 192.4 mg/Kg for C(LLKK)$_2$C and (LLKK)$_2$C, respectively. This low toxicity allows to obtain the ED_{50}, which the effective dose that keep alive the 50% of the mice, this ED_{50} is 5,05 and 6,75 for C(LLKK)$_2$C and (LLKK)$_2$C, respectively, which is not so common when working with AMPs (143).

Another peptide which was a synthetic hybrid was the one synthesized by the group of Pini (144), their idea was to use dendrimers, in this case tetrabranched with a lysine core, and in each branch to have the active peptide which was the KKIRVRLSA, both L- and D- amino acids were synthesized but D-version was more active and more stable. The MIC of M33 (D-amino acids) for *A. baumannii* was 1.5-3 μM, more or less at the same level as polymyxin B. The mechanism of action of this dendrimer was to neutralize the LPS, that as has previously been commented is one of the virulence

factors of *A. baumannii*. It had a good activity *in vivo* against *E. coli* and *P. aeruginosa* (144).

5.2. New polymyxins.

As we have commented previously, polymyxins are the last option in the treatment of carbapanem-resistance *A. baumannii* infections. Another method to find out new antibiotics is by modifying the original polymyxin. Several groups have been working in the development of new polymyxins. The strategy followed to synthesize these new polymyxins could be different, due to the uncomplete understanding of the mechanism of action. The group from the Monash University (Australia) suggested that the problem of resistance to polymyxins could be overcome by introducing new hydrophobic groups in the positions 6 and 7, and in the fatty acyl tail. The groups introduced were octylglycine, bipheylalanine, O-Benzyltyrosine, S-Benzylcysteine, S-Hexylcysteine. It is important to mention that they kept the same stereochemistry of the original compound, which is D-amino acid for the residue in the position 6 and L- amino acid for the position 7, L-Ala and L-Phe were also introduced. If the results with polymyxin B and colistin are compared, it is possible to observe the same behaviours when the strain has lower (0.5-1 mg/L) and higher MICs (16 and 128 mg/L) of polymyxins, the MICs of the analogues were between 1 and 32 mg/L for both type of strains (145). The pharmaceutical company Pfizer developed also new polymyxin analogues trying to improve the potency against *A. baumannii*, as well as reduce its toxicity, these analogues were obtained by changing in all of them the Dab in position 3 for a Dap (2,3-diaminopropionic acid), this was the scaffold they use in order to test different motifs in the position of the fatty acyl tail, most of them containing two aromatic rings, the most active compound was the one with a relatively polar 6-oxo-1-phenyl-1,6-dsihydropyridine-3-carbonyl, this compound showed the same results as polymyxin B in polymyxin-susceptible strains, and better results in polymyxin-resistant *A. baumannii* strains, it showed also less renal cytotoxicity, however the efficacy of this new compound compared to polymyxin B in a murine neutropenic tight model of *P. aeruginosa* was worst (146).

Another strategy, but in this case with worse results is the one followed by Vaara *et al*. (147), their analogs have just three positive charges only compared with the five that original polymyxins and the other analogs have, they remove the Dab next to the fatty

acyl chain, and they substitute the other Dab of the side chain by a D-serine, the fatty acyl chain remains as octanoyl. These changes removed all the positive charges in the side chain, however the results were not so promising, for polymyxin-susceptible strains, the MIC_{50} and MIC_{90} were 4 and 8 mg/L, respectively. These results were not so bad if they would have the same for the polymyxin-resistance, but this was not the case, the results for polymyxin-resistance were >64 mg/L for both MIC_{50} and MIC_{90} compared to 32 and 64 mg/L, respectively for polymyxin B with the same strains (147).

5.3. Novel therapies against *A. baumannii*

There also are emerging therapies to treat infections caused by *A. baumannii*, that take another point of view completely different from normal antibiotics.

- Iron quelation.

The siderophores are really important compounds for the survival of bacteria, therefore to inhibit their activity is an alternative of antibiotics or can even be a complement for the treatment with antibiotics. Several compounds such as deferoxamine, deferiprone, VK28, Ap6619 and 2,2 dipyridyl have been tested as iron quelators in order to inhibit *A. baumannii* growth (148,149). All of them present moderate values, observing some inhibition but not achieving the level of most of the compounds commented below. Another method used is to change the cation Ga^{3+} for the Fe^{3+} that bacteria need to survive, Ga^{3+} have the same valence, and similar atomic radius as Fe^{3+}, this fact allows Ga^{3+} to compete against Fe^{2+}, with the advantatge that it can not do the same processes as Fe^{3+}. It is administered as the salt gallium nitrate, and it was has a good activity *in vivo* (*galleria mellonela* infection model) against multidrug-resistant *A. baumannii* (150). In the article published by Wencewicz and Miller (151), they found another application for the siderophores, they use it as drug a “Trojan horse”, in the way to use biscatecholate−monohydroxamate mixed ligand sideromycins as a drug delivery that allow the compound to cross all the biological barriers and go inside the bacteria. They use biscatecholate−monohydroxamate mixed ligand sideromycins plass a succinyl linker and different antibiotics already known, in this case they use quinolones and β-lactams, the MICs using quinolones are very high, however when β-lactams are attached to this “Trojan horse” the results obtained against *A.baumannii* were extremely good with a MIC of 0.0078 μM (151).

- Biofilm

Another virulence factor is biofilm, therefore it is also a interesting target to beat *A. baumannii*. Some oroidin analogs, natural marine compound, have been synthesized, they have the common feature of having a 2-aminoimidazole ring, but this motif remain untouchable, just changing a substituent in the N-pyrrole group. The best compound of all the analogs is the one with 3-bromophenyl as N-substituent in N-pyrrole (152), the same group optimized the biofilm inhibitor activity of the original compounds, in this case the 2-aminoimidazole ring was disubstituied in position 4 and 5, they keep the same moiety in position 5 as found in the article published in 2008 (153) named (2-AIT) 2-aminoimidazole–triazole conjugate. In all the analogs synthesized the most active were the ones that had a 3-propylphenyl, 3-flurorphenyl and a biphenyl group, obtaining IC_{50} values of 1.42, 1.39 and 1.43 μM, respectively (154).

- Phage therapy

It is known that phages are virus able to infect and sometimes lysate the bacterial cells. The utility of phages as antimicrobial agents was known a long time ago, however due to the effectivity of the actual antibiotics no further development was done. Since a few years ago phages active against *A. baumannii* started to appear but they had a big problem which was the ability to infect, at the beginning the effectiveness of these phages, was really low less than 30% in multidrug-resistant strains (155,156), however the improvements on this field make achieve a 90% of effectiveness of the strains tested (157). Moreover some *in vivo* studies have been performed achieving good results (158).

- Vaccines

Nowadays, vaccines are a really hot topic and the study of them is increasing a lot. The creation of vaccines to deal with *A. baumannii* infections has an interesting point which is the nosocomial aspect of A. baumannii. Most of the vaccines currently in the market are devoted to microorganisms causing community-acquired infections, therefore it will be another approach of the vaccine development. Multidrug, extended-drug and pan-drug-resistant *A. baumannii* cause mainly infections in immunocompromised patients in ICU, therefore its really interesting idea to perform a prophylactic vaccine against *Acinetobacter*. The first idea is to choose a highly conserved molecule in *Acinetobacter*

species in order to have a broader spectrum of action. The first prophylactic vaccine was targeting multiple antigens, and it had a good activity by proctecting mice to develop infections by pandrug-resistant strains (159). Other vaccines were done using outer membrane veiscles as antigens (160, 161). There is one may drawback of these vaccines and is the fact that most of them carry a high amount of LPS which is not good for humans. The vaccine created using the virulence factor ompA as the antigen was also active, protecting mice from disseminated sepsis (162).

- Photodynamic therapy

Photodynamic therapy was developed long time ago, but it was used for other purposes like cancer or ophthalmology, however, due to the lack of antimicrobial agents, the research using these therapies has currently increased. The concept of this therapy is to generate a reactive oxygen, this oxygen will interact with some components of the bacteria such as DNA or membrane proteins that will lead to bacterial death. In order to generate this reactive oxygen, it is necessary to have, oxygen, light (visible or near infrared light) and a photosensitizer. The photosensitizer could be porphyrins, chlorins, pthalocyanines, xanthenes or phenotiazines, these compounds are commonly used due to its high singlet oxygen quantum yields. Some of these photosensitizers such as tetrapyrroles, deuteroporphyrin, Cd-texaphyrin, tetramethylpyridyl porphine, and propyl gallate are active against multidrug-resistant bacteria (163,164). Another option is to link a compound active against bacteria such as AMPs to the photosensitizer in order to help the oxygen, one example of this option is to attach the AMP $(KLAKLAK)_2$ to the photosensitizer Eosin Y, it is demonstrated that they have a really good activity against both Gram-positive and Gram-negative bacteria and with low activity against human cells (165).

Objectives

The general aim of this thesis is to contribute to the development and evaluation of the antibacterial activity of peptides and peptidomimetics against MDR-bacteria aswell as to the knowledge of the molecular bases of the resistance to colistinin *A. nosocomialis* and resistance to daptomycin in *S .mitis*.

One strategy to design new antibacterial agents is based on the mechanism of resistance in order to develop derivatives of the known antibacterial agents which circunvents these resistant mechanism. We have mainly focused on *A .baumannii* since multi-, extended- and pan-drug resistant strains have been descrived, therefore there is a need of new therapeutic alternatives

Specific objectives:

1. Elucidation of the mechanism of resistance of *S. mitis* to daptomycin using a proteomic approach.

2. Generation of *A. nosocomialis* colistin-resistant strains, and complete analysis of these mutants using genomic and proteomic approaches.

3. Screening of lineal peptides against colistin-susceptible and colistin-resistance *A. baumannii* strains, and optimization of the best candidates.

4. *In vivo* studies of the best candidates against pandrug-resistance *A. baumannii* strains.

5. Test several frog skin secretion peptides against colistin-susceptible and colistin-resistance *Acinetobacter* species.

6. Investigate the activity of several peptidomimetics (ceragenins) against colistin-susceptible and colistin-resistance *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*.

7. *In silico* design and synthesis of a library of cyclic hexapeptides able to bind ompA.

8. *In vitro* and *in vivo* (mice) studies of the antivirulence activity of the above cyclic hexapeptides.

Chapter 1

Mechanism of resistance of *Streptococcus mitis* against daptomycin

Context.

Daptomycin is one of the last antibiotics released, and it is used to treat several infections such as those caused by both methicillin-susceptible and -resistant *S. aureus*, vancomycin-susceptible and -resistant *Enterococcus*, coagulase-negative *Staphylococcus*, and streptococci. Up to today only resistance to *S. aureus* and *Enterococcus* have been reported. As it is known daptomycin's target is the cell membrane or cell wall, therefore the main mechanisms of resistance to daptomycin are associated with that. In previous studies performed by the group of Dr. Miró, it was found that when comparing the efficacy of vancomycin (30 mg/Kg in two doses) and daptomycin (6 mg/Kg in one dose) for endocarditis caused by *S. mitis*, the strains recovered from the vegetations after 2 days of treatment had a high-level of daptomycin resistance (MIC>256 mg/L). Taking into account these results, the main idea was to know if that tendency of acquiring resistance was the same for all the viridans streptococci group. All the strains from the viridans groups were incubated with 0.5 and 1 mg/L of daptomycin, and after 48h the strains were subcultured on blood agar and the MIC experiments were performed in order to observe the new MIC, the results are showed in the table 1.1

Microorganism(s)	No. of strains	No. (%) screening positive[a]	No. (%) that were[b]: DNS (MIC, ≥2 mg/liter)	HLDR (MIC, ≥256 mg/liter)
Mitis group	92	74 (80)	61 (66)	25 (27)
S. mitis	51	35 (69)	30 (59)	14 (27)
S. oralis	19	18 (95)	14 (74)	9 (47)
S. sanguis	15	15 (100)	11 (73)	2 (13)
S. gordonii	4	4 (100)	4 (100)	0 (0)
S. parasanguis	3	2 (67)	2 (67)	0 (0)
Bovis group	54	2 (4)	0	0
Anginosus group	10	5 (50)	5 (50)	0
Mutans group	8	0	0	0
Salivarius group	4	0	0	0

[a] Screening was considered positive if the microorganism grew in the presence of 0.5 mg or 1 mg/liter daptomycin.
[b] DNS, daptomycin nonsusceptible; HLDR, high-level daptomycin resistance.

Table 1.1. Rates of selection of resistance and high-level resistance after exposure to daptomycin. (9)

Daptomycin non-susceptibility strains were indentified from mitis group in 61 cases (66%) and 5 (50%) from the anginosus group, the global amount of daptomycin non-

susceptible isolates were 39% (66/168). The highest rates of resistance were observed in mitis group: 74/92 (80%) were resistant, 61/74 (82%) were daptomycin non susceptible, and 25/61 (41%) developed high level of daptomycin resistant.

Some *in vivo* studies with rabbits were also performed. Rabbits were infected with *S. mitis*, causing endocarditis. Two types of dosing were used recommended dose (RD, 6mg/kg iv once daily) and high dose (HD, 10 mg/kg iv once daily) for daptomycin and the previous dose commented for vancomycin. The results are showed in table 1.2

Treatment group	No. of sterile vegetations/ total no. of vegetations (%)	Log_{10} CFU/g vegetation [median (IQR)]	No. of recovered isolates with HLDR MIC/ total no. of animals treated (%)[l]
Control[a]	0/15 (0)	9.1 (9–9.6)	0
RD-daptomycin	1/11 (9)[b]	6.7 (5.9–7.8)[c]	7/11 (63)[d]
HD-daptomycin	1/12 (8)[e]	6.1 (5.2–7.2)[f,g]	8/12 (67)[h]
Vancomycin	0/12 (0)[i]	3.4 (2–4)[g,j]	NA

Table 1.2. Treatment of experimental endocarditis caused by *S. mitis* 351 strain. (9)

Results obtained using both types of dosing were the same. When daptomycin and vancomycin were compared, the effectiveness of the last one is higher, observing 3.4 log CFU/g vegetation, whereas for daptomycin was between 6 and 7 log CFU/g. Moreover, between 60 and 70 % of the vegetations recovered presented high level of daptomycin resistant.

With the scenario commented previously and with the idea that it was the first time that daptomycin resistant against *S. mitis* was reported, we though it would a good idea to study the mechanism of resistance.

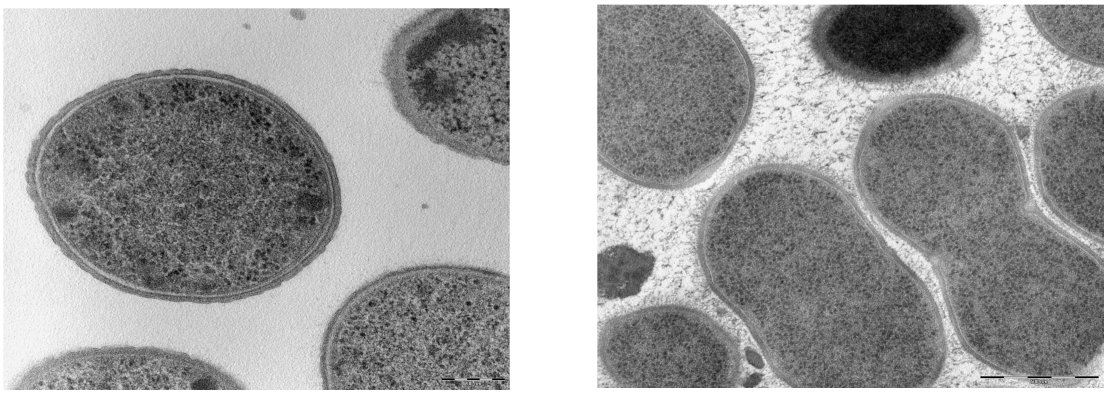
1.1. Cell wall thickness in daptomycin resistant *S. mitis.*

The mechanisms of resistance to daptomycin might be associated with the cell wall or the cell membrane, in fact previous mechanisms of resistance to daptomycin reported in *S. aureus* and *Enterococcus faecium* reinforce this hypothesis, therefore the first thing was to find out if there was an increase of the thickness of the cell wall that did not allow daptomycin to disrupt or to permeabilize the membrane of this Gram-positive bacteria.

The technique used to calculate the cell wall thickness was transmission electron

microscopy (TEM). Figure 1.1. Following the procedures developed in the manuscript published by Yang *et al*. (166), 100 cell wall thickness measurements were taken from a minimum of 50 cells at X190,000 magnification. The results obtained of these measurements are showed in Table 1.3 and the images of the cells in Figure 1.1

a) D6-7 b) D6-14



c) 351 d) D6-2

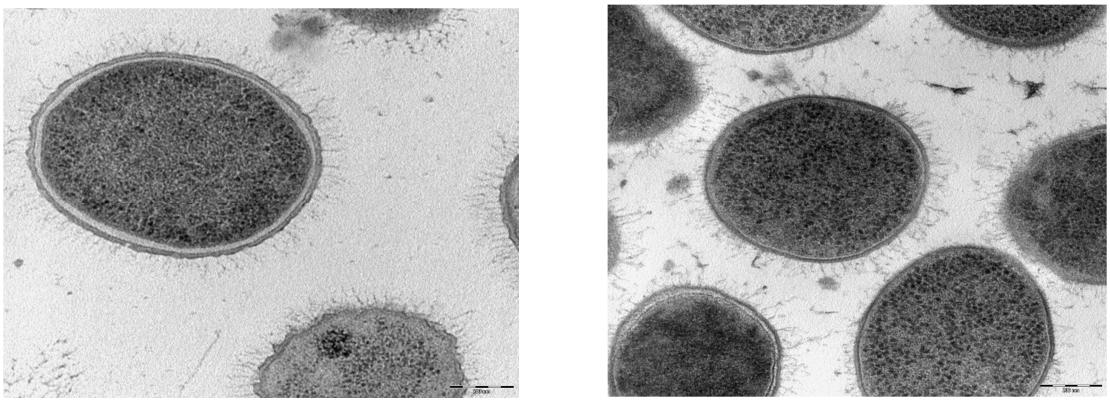


Figure 1.1. TEM images of *S. mitis*. a), b) and d) are daptomycin-resistant *S. mitis*; c) is a daptomycin-susceptible *S. mitis* strain..

Strains	**Membrane thickness (nm)**	**MIC to daptomycin (mg/L)**
351	20.03±1.47	0.38
D6-2	25.21±2.05	>256
D6-7	22.35±2.84	>256
D6-14	24.44±2.56	>256

Table 1.3. Membrane thickness and MIC to daptomycin to different *S. mitis* strains.

The differences in the thickness of the cell wall in all the strains measured (1 daptomycin-susceptible and 3 daptomycin-resistant) were not statistically significant using ANOVA and Dunnet tests. The cell wall thickness of the daptomycin-susceptible strain was 20.03±1.47 nm, and the rest of strains, daptomycin-resistant, were 22.35±2.84 nm, 24.44±2.56 nm and 25.21±2.05 nm (Table 1.3).

1.2. Comparative proteomics of daptomycin-susceptible and daptomycin-resistant *S. mitis* strains.

Two different approaches can be used in order to have an initial idea about which are the proteins or the genes involved in a mechanism of action, these two approaches are proteomics and genomics. Both approaches could be complementary but we decided to start by using the proteomic approach.

Whole-cell proteins of daptomycin-susceptible (strain 351), and daptomycin-resistant (strain D6-14) *S. mitis* strains were extracted following the procedure described in the materials and methods Section. Three different experiments with the abovementioned extracts were carried out. Isobaric tag for relative and absolute quantitation (iTRAQ) was performed, thus we had methodological triplicates. The iTRAQ experiment is based on the covalent labeling of the N-terminus and side chain amines of peptides from protein digestions with tags of varying mass. There are currently two reagents mainly used: 4-plex and 8-plex, which can be used to label all peptides from different samples/treatments. These samples are then pooled and usually fractionated by nano liquid chromatography and analyzed by tandem mass spectrometry (MS/MS). A database search is then performed using the fragmentation data to identify the labelled peptides and hence the corresponding proteins. The fragmentation of the attached tag generates a low molecular mass reporter ion that can be used to relatively quantify the peptides and the proteins from which they originated.

The proteins which have a significantly difference of expression are showed in the table 1.4 and table 1.5

NCBI GenInfo Identifier	Replicates for quantitation	Replicates % p<0.05	Protein ratio (Fold change) Dapto R/Dapto S
gi|77409507|	1	100,0	0,24
gi|419780699|	3	33,3	0,30
gi|15900151|	1	100,0	0,36
gi|15900549|	1	100,0	0,55
gi|293364197|	1	100,0	0,62
gi|417793472|	1	100,0	0,64
gi|306825386|	1	100,0	0,65
gi|293365248|	1	100,0	0,65
gi|315612275|	1	100,0	0,69
gi|322376983|	2	100,0	0,72
gi|293364663|	2	100,0	0,73
gi|406576657|	1	100,0	0,74
gi|293365311|	1	100,0	1,19
gi|293364514|	3	33,3	1,22
gi|289168021|	2	50,0	1,27
gi|270292667|	1	100,0	1,29
gi|293364272|	3	66,7	1,33
gi|194396827|	2	50,0	1,36
gi|315611998|	1	100,0	1,36
gi|331266333|	1	100,0	1,48
gi|419781125|	1	100,0	1,50
gi|293365494|	3	66,7	1,59
gi|306830250|	2	100,0	2,26

Table 1.4. Proteins identified by iTRAQ which have different expression rate.

NCBI GenInfo Identifier	Protein description
gi|77409507|	NADP-specific glutamate dehydrogenase [Streptococcus agalactiae COH1]
gi|419780699|	PF08006 family protein [Streptococcus oralis SK100]
gi|15900151|	30S ribosomal protein S3 [Streptococcus pneumoniae TIGR4]
gi|15900549|	PTS system transporter subunit IIB [Streptococcus pneumoniae TIGR4]
gi|293364197|	glutamate--ammonia ligase [Streptococcus oralis ATCC 35037]
gi|417793472|	ABC transporter, ATP-binding protein, partial [Streptococcus oralis SK255]
gi|306825386|	peptidyl-prolyl cis-trans isomerase [Streptococcus sp. oral taxon 071 str. 73H25AP]
gi|293365248|	peptidyl-prolyl cis-trans isomerase [Streptococcus oralis ATCC 35037]
gi|315612275|	ABC superfamily ATP binding cassette transporter, binding protein [Streptococcus sanguinis ATCC 49296]
gi|322376983|	hypothetical protein HMPREF0851_00776 [Streptococcus sp. M334]
gi|293364663|	PTS family maltose and glucose porter, IIABC component [Streptococcus oralis ATCC 35037]
gi|406576657|	peptidyl-prolyl cis-trans isomerase [Streptococcus sp. GMD6S]
gi|293365311|	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase [Streptococcus oralis ATCC 35037]
gi|293364514|	serine protease HtrA [Streptococcus oralis ATCC 35037]
gi|289168021|	phosphotransacetylase [Streptococcus mitis B6]
gi|270292667|	putative PTS system, IIC component [Streptococcus sp. M143]
gi|293364272|	**conserved hypothetical protein [Streptococcus oralis ATCC 35037]**
gi|194396827|	L-lactate oxidase [Streptococcus pneumoniae G54]
gi|315611998|	**group B streptococcal surface immunogenic protein [Streptococcus sanguinis ATCC 49296]**
gi|331266333|	glycogen biosynthesis protein,glucose-1-phosphate adenylyltransferase [Streptococcus oralis Uo5]
gi|419781125|	pyruvate oxidase [Streptococcus oralis SK100]
gi|293365494|	glucose-1-phosphate adenylyltransferase [Streptococcus oralis ATCC 35037]
gi|306830250|	**group B streptococcal surface immunogenic protein [Streptococcus mitis ATCC 6249]**

Table 1.5. Proteins description of the proteins identified by iTRAQ which have different expression rate.

Taking into account that the mechanism of action of daptomycin is associated with the membrane, the initial idea would be to search for proteins related to membrane compounds or metabolites involved in the membrane synthesis in the iTRAQ.

Two proteins fulfill the requirements, both proteins were overexpressed in the daptomycin-resistant *S. mitis* strain. The protein group B streptococcal surface immunogenic protein, is overexpressed 2.26 fold. Moreover, this protein have also been found belonging to *Streptococcus sanguinis*. The protein group B streptococcal surface immunogenic protein was quantified twice from the three replicates, and all of the quantifications were significantly different. The second interesting protein was, conserved hypothetical protein found in *Streptococcus oralis*, this protein was found in

all the three replicates performed and in two of the three replicates was significantly different, and over expressed 1.33 fold.

1.3. Development of mutants with a knockout in the interesting genes.

The information acquired in the iTRAQ is interesting but other experiments need to be performed in order to check that these two proteins are really the ones involved in the daptomycin resistant. One of the options to prove that these proteins are important is to truncate their genes and check out that the MIC of daptomycin in the generated mutant is different.

Two knockouts were tried, one with each gene mentioned above, the constructions of the plasmid with the truncated genes were built perfectly and it was confirmed by PCR, however the transformation of these plasmids into *S. mitis* did not worked. Further experiments to obtain a *S. mitis* transformant with the recombinant plasmid will be performed in the future.

In summary, from the work in this chapter we can conclude that daptomycin resistance did not imply an increase in the thickness of the cell wall. Using proteomic techniques, several proteins with a different rate of expression, comparing daptomycin-resistant and daptomycin-susceptible S. mitis, have been identified. We have the hypothesis, based on the mechanism of daptomycin resistance reported in other microorganisms, that two of them are involved in daptomycin resistance. However until the knockout of the genes enconding these proteins will not be completed its role in the resistance remains a working hypothesis.

Chapter 2

Mechanism of resistance of *Acinetobacter nosocomialis* against colistin

Context.

As commented in the introduction, among the different species of the *Acinetobacter* genus, *A. baumannii* is the most frequently reported as a cause of nosocomial infections. However the number of non-baumannii isolates have been increasing during the last years (167), being *A. nosocomialis* (previously named *Acinetobacter* genospecies 13) and *A. pittii* (previously named *Acinetobacter* genospecies 3) the most important species apart from *A. baumannii*. It is not easy to differentiate phenotypically these three species which constitute the *A. baumannii* group (*A. baumannii, A. nosocomialis and A. pittii)*, therefore and due to the increase of non-baumannii strains isolated, the techniques of identifying each species have been improved (168) and updated with new techniques such as MALDI-TOF (169). Indeed, the treatment used to fight against the new Acinetobacter species is controversial (170,171).

Although *A. nosocomialis* is a relatively new specie some resistance to several antibiotics have been published such as the acquisition of the $bla_{\text{OXA-23}}$ (172) and $bla_{\text{OXA-51}}$ genes (173) which confer resistance to carbapenems, or the famous bla_{NDM} gene, encoding a metallo-beta-lactamase, which makes the bacteria highly resistance to all β-lactam antibiotics but aztreonam (174). Most of the NDM-producing *Acinetobacter* sp. strains carried other mechanisms of resistance conferring XDR, thus being resistance to all antimicrobial agents except tigecycline and colistin (polymyxin).

Until now, no colistin resistance has been reported for *A. nosocomialis*, therefore it will be interesting to know if the mechanisms of resistance of this bacteria are similar to *A. baumannii* reported.

2.1. Selection of colistin-resistant *A. nosocomialis* mutants.

No colistin-resistant *A. nosocomialis* strains have been isolated but, following the tendency of the past years seen in *A. baumannii* and taking into consideration the increased used of colistin to treat infections caused by MDR *K. pneumoniae, P. aeruginosa* and *A.baumannii*, some resistant strains will be isolated in the near future. As we did not have this colistin-resistant *A. nosocomialis* strains we decided to generate these mutants. The same procedure was done for ATCC 19606 *A. baumannii*, and 2 clinical strains, one *A. pittii* and one *A. nosocomialis*, however we are just going to focus in *A. nosocomialis*.

The strains used were subjected to serial passages on plates with increasing concentrations of colistin. The selected *A. nosocomialis* wild-type strain had an initial MIC < 1 mg/L. During the mutant production, there was an inflection point (16 mg/L of colistin in the plate for selection) where the strain showed a drastic change in the MIC of colistin (from < 1mg/L to 128 mg/L). The strains isolated previous to this inflection point tolerate up to 8 mg/L of colistin. Other characteristics seen in this high colistin-resistant mutant compared to the wild type strain were that the mutant had a change in colony morphology with tiny colonies observed and it did not grow on MacConkey agar. The strains, recovered after the initial passages on plates with colistin, were tolerant to this antibiotic (up to 8 mg/L of colistin) since they grew on increasing concentrations of this antibiotic but their initial MIC was not modified. At a given point, two different morphologies were observed which corresponded to a colistin-susceptible and a colistin-resistant strains (Fig 2.1). After this inflection point, the strains were not able to grow on selective MacConkey media and the MIC for the resistant strain went up to ≥ 128 mg/L (Table 2.1). To our knowledge, this is the first description of a colistin-resistant mutant with a decreased ability to grow on MacConkey selective medium.

STRAINS	COLISTIN IN PLATE*(mg/L)	MIC (mg/L)	GROWTH ON McConkey
Acinetobacter nosocomialis	0	≤1	YES
	0.25	≤1	YES
	0.5	≤1	YES
	1	≤1	YES
	2	≤1	YES
	4	≤1	YES
	8	≤1	YES
	16	128	NO
	32	128	NO
	64	256	NO
	128	512	NO
	256	512	NO

Table 2.1. MIC of colistin and growth on McConkey plates for the Acinetobacter nosocomialis.
* Colistin in plate represents the colistin concentration used in the agar plate to select for colistin resistance.

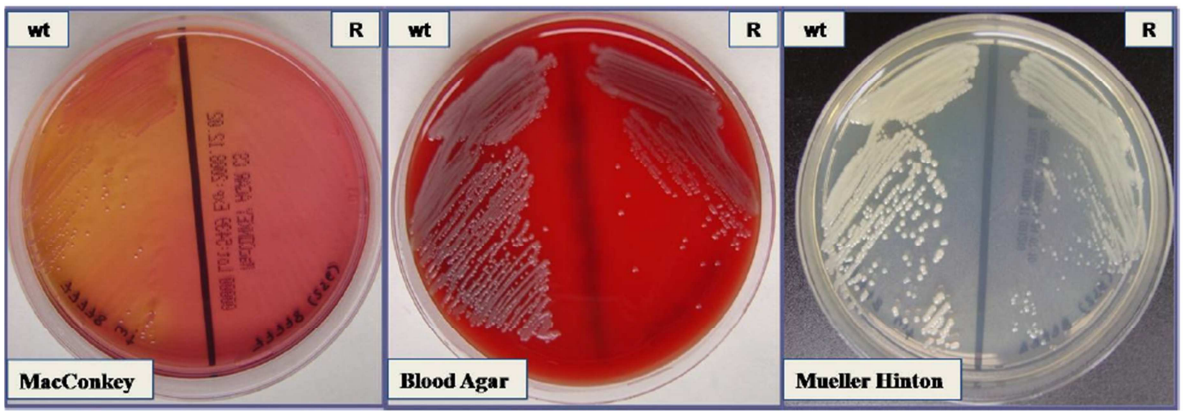


Figure 2.1 Morphological changes observed when the *Acinetobacter nosocomialis* clinical isolate became resistant to colistin. Before becoming resistant to colistin the wild-type strain grew in all the plates. Afterwards, the resistant mutant did not grow on McConkey media.

2.2. Antimicrobial resistance profiles of the generated mutants.

The mutants generated were colistin-resistant but it is important to know if they also showed changes in the MIC of other types of antibiotics. In table 2.2, the MIC of different strains against different antimicrobial agents is showed.

MIC (mg/L)			Antimicrobial	Agents					
COLISTIN IN PLATE(mg/L)	FX	VA	AZ	CI	TM	IP	TZ	ETP	CO
0,125	24	64	0,38	0,125	0,38	0,5	2	3	≤1
2	24	64	0,38	0,125	0,38	0,5	2	3	≤1
8	24	64	0,38	0,125	0,38	0,5	2	3	≤1
16	1.5	0,75	0,047	0,064	0,19	0,064	0,75	0,016	128
32	1,5	0,75	0,047	0,064	0,19	0,064	0,75	0,016	128
512	1,5	0,75	0,047	0,064	0,19	0,064	0,75	0,016	512

FX (cefoxitime) VA (vancomycin) AZ (azitromicin) CI (ciprofloxacin) TM (tobramycin) IP (imipenem) TZ (ceftazidime) ETP (ertapenem) CO (colistin)

Table 2.2. MIC of differensts strains generated against several antibiotics.

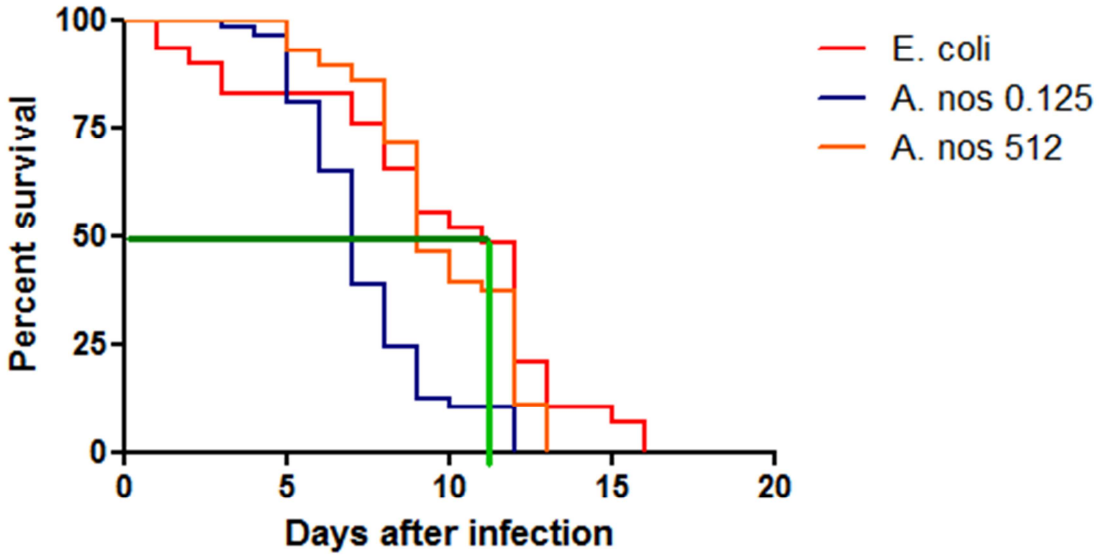
In the table 2.2 is it possible to observe that the mutants generated with resistance to colistin had lower MICs for most of the antibiomicrobial agents tested. For ciprofloxacin and tobramycin the MIC just decreased one dilution and for ceftazidime

slightly more, however for the rest of the antibitoics the number of dilutions that the MIC decreases is higher, like the case of cefoxitime and vancomycin, that the MIC went from 24 mg/L to 1.5 mg/L and from 64 mg/L to 0.75 mg/L, respectively. For the carbapenems, imipenem and ertapenem, the decreases were from 0.5 mg/L to 0.064 mg/L and 3 mg/L to 0.016 mg/L, respectively. The MIC of azitromycin for the colistin-resistant mutants decreased four dilutions respect to the initial strain.

2.3. *In vivo* virulence using the *C. elegans* model.

It was demonstrated that the new strains generated are highly resistant to colistin, however their ability to be resistant to the other antibiotic families decreases dramatically. One important factor apart from the resistance profile of the new strains is their ability to cause virulence, this ability sometimes is even more important than the resistant profile, therefore some experiments to test the virulence of the new strains were performed.

In collaboration with Dr. Paula Espinal some experiments using *Caenorhabditis elegans* were performed. These experiments allow us to investigate the virulence of the strains selected. The results are showed in Figure 2. 2.



A. nosocomialis 0.125 vs *A. nosocomialis* 512: p<0.0001

E. coli vs *A. nosocomialis* 0.125: p<0.0001

E. coli vs *A. nosocomialis* 512: p=0.1647

Figure 2.2. Percentatge of survival after the infection in three different strains. (red *E. coli* as a control) (Blue *A. nosocomialis* colistin-susceptible) (Orange *A. nosocomialis* colistin-resistant)

The figure 2.2 showed a significantly different virulence of both strains being more virulent the initial strain colistin-susceptible compared to the colistin-resistant. The LT_{50} for the colistin-susceptible (blue line) is 7 days compared to the 9 days for the colistin-resistant (orange line). For the control strain of *E. coli* the LT_{50} is 11 days which is showed in the green line.

2.4. Sequence of the genome of the colistin-susceptible and colistin-resistant *A. nosocomialis* strains.

This part was done in collaboration with the Prof. Marc Rolain (University of Marseille, Marseille, France). The genomes of these two strains were sequenced and analyzed following the methodology described in the Material and Methods Section. The initial strain colistin-susceptible and the colistin-resistant mutant (512) *A. nosocomialis* were sequenced. On comparing the two sequences of the genomes of both strains several

differences were observed in those genes potentially related to colistin resistance and which have been previously reported in *A. baumannii.*

- The ***pmrABC*** operon.

The *pmrABC* operon encodes two component regulatory system (genes *pmrA* and pmrB) that regulates the expression of the *pmrC* gene which encodes a phosphoethanolamine transferase enzyme involved in lipid A modification.

```
col-S_A.nosocomialis_pmrA       MTKILMIEDDFMIAESTTTLLQYHQFEVEWVNNGLDGLAQLAKNKFDLIL 50
col-R_A.nosocomialis_pmrA       MTKILMIEDDFMIAESTTTLLQYHQFEVEWVNNGLDGLAQLAKNKFDLIL 50
col-S_A.baumannii_pmrA          MTKILMIEDDFMIAESTITLLQYHQFEVEWVNNGLDGLAQLAKTKFDLIL 50
                                ********** ********** ********** .******

col-S_A.nosocomialis_pmrA       LDLGLPMMDGMQVLKQIRQRAATPVLIISARDQLQNRVDGLNLGADDYLI 100
col-R_A.nosocomialis_pmrA       LDLGLPMMDGMQVLKQIRQRAATPVLIISARDQLQNRVDGLNLGADDYLI 100
col-S_A.baumannii_pmrA          LDLGLPMMDGMQVLKQIRQRAATPVLIISARDQLQNRVDGLNLGADDYLI 100
                                **************************************************

col-S_A.nosocomialis_pmrA       KPYEFDELLARIHALLRRSGVEAQLASQDQLLESGDLVLNVEQHIATFKG 150
col-R_A.nosocomialis_pmrA       KPYEFDELLARIHALLRRSGVEAQLASQDQLLESGDLVLNVEQHIATFKG 150
col-S_A.baumannii_pmrA          KPYEFDELLARIHALLRRSGVEAQLASQDQLLESGDLVLNVEQHIATFKG 150
                                **************************************************

col-S_A.nosocomialis_pmrA       QRIDLSNREWAILIPLMSHPNKIFSKANLEDKLYDFDSDVTSNTIEVYVH 200
col-R_A.nosocomialis_pmrA       QRIDLSNREWAILIPLMSHPNKIFSKANLEDKLYDFDSDVTSNTIEVYVH 200
col-S_A.baumannii_pmrA          QRIDLSNREWAILIPLMTHPNKIFSKANLEDKLYDFDSDVTSNTIEVYVH 200
                                ****************:*********************************

col-S_A.nosocomialis_pmrA       HLRAKLGKDFIRTIRGLGYRLGQS 224
col-R_A.nosocomialis_pmrA       HLRAKLGKDFIRTIRGLGYRLGQS 224
col-S_A.baumannii_pmrA          HLRAKLGKDFIRTIRGLGYRLGQS 224
                                ************************
```

Figure 2.3. Comparative analysis of pmrA protein in colistin-resistant and colistin-susceptible *A. nosocomialis* and *A. baumannii*.

```
col-S_A.nosocomialis_pmrB      VHYSLKKRLIWGTSIFSVILGCILIFSAYKVALQEVDEILDTQMQYLAER 50
col-R_A.nosocomialis_pmrB      VHYSLKKRLIWGTSIFSVILGCILIFSAYKVALQEVDEILDTQMQYLAER 50
col-S_A.baumannii_pmrB         VHYSLKKRLIWGTSIFSVILGCILIFSAYKVALQEVDEILDTQMKYLAER 50
                               **************************************:*****

col-S_A.nosocomialis_pmrB      TAEYPLKTVSSKFDLHRTYHEEDLFLDIWAYKDQANLSHHLHLLVPPVQQ 100
col-R_A.nosocomialis_pmrB      TAEYPLKTVSSKFDLHRTYHEEDLFLDIWAYKDQANLSHHLHLLVPPVQQ 100
col-S_A.baumannii_pmrB         TAEHPLKTVSSKFDFHKTYHEEDLFIDIWAYKDQAHLSHHLHLLVPPVEQ 100
                               ***:**********:*:********:*********:************:*

col-S_A.nosocomialis_pmrB      AGFYSHKTAEGVVRTYVLPLKDYQIQVSQQERVREAFAWELAGSMFIPYL 150
col-R_A.nosocomialis_pmrB      AGFYSHKTAEGVVRTYVLPLKDYQIQVSQQERVREAFAWELAGSMFIPYL 150
col-S_A.baumannii_pmrB         AGFYSHKTAQGIVRTHVLPLKDYQIQVSQQERVREAFAWELAGSMFIPYL 150
                               *********:*:***:**********************************

col-S_A.nosocomialis_pmrB      IILPFAIFALAAIISRGLKPIDDFKNELMERDSEELTPIEVHDYPQELLP 200
col-R_A.nosocomialis_pmrB      IILPFAIFALAAIISRGLKPIDDFKNELMERDSEELTPIEVHDYPQELLP 200
col-S_A.baumannii_pmrB         IILPFAIFALAAIIRRGLKPIDDFKNELKERDSEELTPIEVHDYPQELLP 200
                               ************* ************ ********************

col-S_A.nosocomialis_pmrB      TIDEMNRLFDRISKAQNEQKQFIADAAHELRTPVTALNLQ---------- 240
col-R_A.nosocomialis_pmrB      TIDEMNRLFDRISKAQNEQKQFIADAAHELRTPVTALNLQ---------- 240
col-S_A.baumannii_pmrB         TIDEMNRLFERISKAQNEQKQFIADAAHELRTPVTALNLQTKILLSQFPE 250
                               *********:******************************

col-S_A.nosocomialis_pmrB      ----------LARIQHLVTQLLALAKQDVTLSMVEPTGYFQLNDVALNCV 280
col-R_A.nosocomialis_pmrB      ----------LARIQHLVTQLLALAKQDVTLSMVEPTGYFQLNDVALNCV 280
col-S_A.baumannii_pmrB         HESLQNLSKGLARIQHLVTQLLALAKQDVTLSMVEPTGYFQLNDVALNCV 300
                                         ************************************

col-S_A.nosocomialis_pmrB      EQLVNLAMQKEIDLGFVRNDPVEMHSIEPTVHSIIFNLIDNAIKYTPHQG 330
col-R_A.nosocomialis_pmrB      EQLVNLAMQKEIDLGFVRNDPVEMHSIEPTVHSIIFNLIDNAIKYTPHQG 330
col-S_A.baumannii_pmrB         EQLVNLAMQKEIDLGFVRNEPIEMHSIEPTVHSIIFNLIDNAIKYTPHQG 350
                               ******************:*:**************************

col-S_A.nosocomialis_pmrB      VINISVYTDPDNFACIQIEDSGAGIDPENYDKVLKRFYRVHHHLEVGSGL 380
col-R_A.nosocomialis_pmrB      VINISVYTDPDNFACIQIEDSGAGIDPENYDKVLKRFYRVHHHLEVGSGL 380
col-S_A.baumannii_pmrB         VINISVYTDQDHYACIQIEDSGAGIDPENYDKVLKRFYRVHHHLEVGSGL 400
                               ******** *::************************************

col-S_A.nosocomialis_pmrB      GLSIVDRATQRLGGTLTLDKSLELGGLSVLVKLPKVLHLHETRA 424
col-R_A.nosocomialis_pmrB      GLSIVDRATQRLGGTLTLDKSLELGGLSVLVKLPKVLHLHETRA 424
col-S_A.baumannii_pmrB         GLSIVDRATQRLGGTLTLDKSLELGGLSVLVKLPKVLHLHETRA 444
                               *********************************************
```

Figure 2.4. Comparative analysis of pmrB protein in colistin-resistant and colistin-susceptible *A. nosocomialis* and *A. baumannii*.

No mutations were found in any of the three genes that conformed pmrAB, however several mutations and deletions are observed when comparing with *A. nosocomialis* and A. *baumannii*.

- The ***lpxACD* operon**.

The *Lpx*ACD operon is constituted by genes involved in the synthesis of lipid A, which is one of the parts of the LPS, which its modification is involved in the resistance to colistin. The biosynthetic pathway of the synthesis of lipid A, in which the proteins lpxACD are involved, is observed in the Figure 2.5.

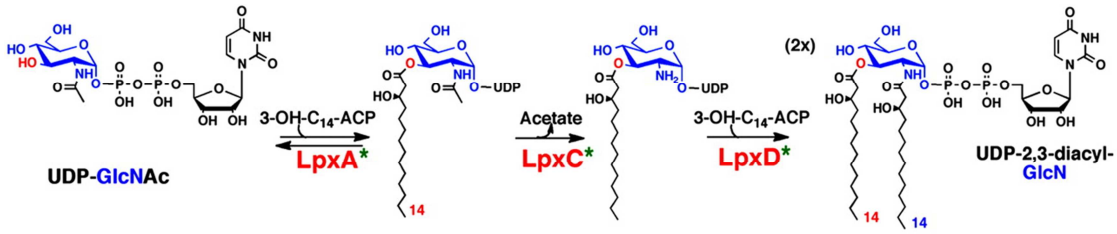


Figure 2.5. Biosynthetic pathway of the synthesis of lipid A.

Several mutations, deletions or insertions have been reported for *A. baumannii* .The three genes were analyzed in both strains sequenced and the results are showed in figure 2.6 for lpxA, figure 2.7 for lpxC and figure 2.8 for lpxD.

```
colS            MLRCSIGPYCVIGPQVTIGAGTKLHSHVVVGGFTRIGQNNEIFQFASVGEVCQDLKYKGE
colR            MLRCSIGPYCVIGPQVTIGAGTKLHSHVVVGGFTRIGQNNEIFQFASVGEVCQDLKYKGE
                ************************************************************

colS            ETWLEIGNNNLIREHCSLHRGTVQDNSLTKIGSHNLLMVNTHIAHDCIVGDHNIFANNVG
colR            ETWLEIGNNNLIREHCSLHRGTVQDNSLTKIGSHNLLMVNTHIAHDCIVGDHNIFANNVG
                ************************************************************

colS            VAGHVHIGDHVIVGGNSGIHQFCKIDSYSMIGGASLILKDVPAYVMASGNPAHAFGINIE
colR            VAGHVHIGDHVIVGGNSGIHQFCKIDSYSMIGGASLILKDVPAYVMASGNPAHAFGINIE
                ************************************************************

colS            GMRRKGWSKNTIQGLREAYKLIFKSGLTSVQAVEQIKSEILPSVPEAQLLIDSLEQSE
colR            GMRRKGWSKNTIQGLREAYKLIFKSGLTSVQAVEQIKSEILPSVPEAQLLIDSLEQSE
                ************************************************************
```

Figure 2.6. Comparative lpxA protein from colistin-susceptible and colistin-resistant *A. nosocomialis*. Col S (Colsitin susceptible). ColR (Colistin resistant)

```
colS            FIPHTVDGGIVFRRIDLDPPVDIPANALLIQEAFMCSNLVTGDIKVGTIEHVMSAIAGLG
colR            FIPHTVDGGIVFRRIDLDPPVDIPANALLIQEAFMCSNLVTGDIKVGTIEHVMSAIAGLG
                ************************************************************

colS            IDNLIVEVSASEVPIMDGSAGPFIYLLMQGGLREQDAPKKFIRILKPVEALIDDKKAIFR
colR            IDNLIVEVSASEVPIMDGSAGPFIYLLMQGGLREQDAPKKFIRILKPVEALIDDKKAIFR
                ************************************************************

colS            PHNGFQLNFTIDFDHPAFAKEYQSATIDFSTETFVYEVSEARTFGFMKDLDYLKANNLAL
colR            PHNGFQLNFTIDFDHPAFAKEYQSATIDFSTETFVYEVSEARTFGFMKDLDYLKANNLAL
                ************************************************************

colS            GASLDNAIGVDDIGVVNEEGLRFADEFVRHKILDAVGDLYLLGHQIIAKFDGYKSGHALN
colR            GASLDNAIGVDDIGVVNEEGLRFADEFVRHKILDAVGDLYLLGHQIIAKFDGYKSGHALN
                ************************************************************

colS            NQLLRNVQSDPSNYEIVTFNDEKDYF
colR            NQLLRNVQSDPSNYEIVTFNDEKDYF
                *************************
```

Figure 2.7. Comparative lpxC protein from colistin-susceptible and colistin-resistant *A. nosocomialis*. Col S (Colsitin susceptible). ColR (Colistin resistant)

```
colS            MKVQQFRLDELAHLVKGELIGEGSLQFSNLASLENAEVNHLTFVNGDKYLEQAKASRAGA
colR            MKVQQFRLDELAHLVKGELIGEGSLQFSNLASLENAEVNHLTFVNGDKYLEQAKASRAGA
                ************************************************************

colS            FIVTATLKEYLPEKDNFIIVDNPYLAFAILTHVFDKKLTSKGIESTAQIHPSAVISEAAY
colR            FIVTATLKEYLPEKDNFIIVDNPYLAFAILTHVFDKKLTSKGIESTAQIHPSAVISEAAY
                ************************************************************

colS            IGHYVVIGENCVVGDNTIIQSHTKLDDDVEVGKDCFIDSHVTITGSSKLGDRVRVHSNTV
colR            IGHYVVIGENCVVGDNTIIQSHTKLDDDVEVGKDCFIDSHVTITGSSKLGDRVRVHSNTV
                ************************************************************

colS            IGSEGFGFAPYQGKWHRIAQLGSVLIGNDVRIGSNCSIDRGALDNTILEDGVIIDNLVQI
colR            IGSEGFGFAPYQGKWPRIVQLGSVLFGNDVGIGSNCSIDGGVLDNPILEDGVIIDTLVQI
                *************** **.******:**** ******** *.***.*********.****

colS            AHNVHIGSNTAIAATCGIAGSTKIGKNCILAGACGVAGHLSIADNVTLTGMSMVTKNISE
colR            AHNVHIGSNTVIAVTGAIAGSPKLAKTVF----------------------------
                **********.**.* .****.*:.*. :

colS            AGTYSSGIGLFENSQWKKTIVRLRQLADVPLTQITKRLDHIQAQIESLESTFNLRK
colR            ---------------------------------------------------------
```

Figure 2.8. Comparative lpxD protein from colistin-susceptible and colistin-resistant *A. nosocomialis*. Col S (Colsitin susceptible). ColR (Colistin resistant)

Figures 2.6, 2.7 and 2.8 show the amino acid sequences of the lpxA, lpxC and lpxD proteins of the wild-type and the colistin-resistant *A. nosocomialis* mutant, and several mutations have been observed in all the three genes, but it is important to highlight the stop codon present in lpxD.

Next step was to know if the mutations and the stop codon were associated with colistin resistance, therefore PCRs to amplify the all the lpx*ACD* genes of the different colistin-tolerant and resistant A. nosocomialis mutants were performed, these mutants covered before and after the inflection point.

The PCR products confirmed that all the wild-type strains (0.125/2/8) showed the same sequence, and the same sequence was also observed in all the colistin-tolerant strains (16/32/512).

2.5. LPS production of the wild-type and mutants *A. nosocomialis* strains.

In previous assays it was observed the stop codon present in the *lpxD* gene, the hypothesis is that this stop codon together with other mutations in the *lpxAC* genes, did not allow the bacteria to form the LPS and the strain did not have LPS in their membrane, therefore, the amount of LPS present in two colistin-susceptible *A. nosocomialis* and two colistin-resistant *A. nosocomialis* strains was calculated. The method used is a commercial kit that allow us to calculate the amount of LPS or endotoxin present in the bacteria. The results are showed in table Z.6

***A. nosocomialis* strains**	**LPS (EU/mL)**
0.125	1.74×10^5
2	6.24×10^4
16	59.9
512	143

Table. 2.3. Concentration of LPS in four different strains of *A. nosocomialis.*

2.6. Proteomic approach of the different strains generated.

This work was done in collaboration with Dr. Mario Ferrer-Navarro.
After the genomic approach, proteomic studies were performed in order to know if there were potential proteins involved in the mechanism of resistance of colistin to *A. nosocomialis.*

Comparative studies of the most susceptible and resistant strain of *A. nosocomialis*, were performed using DIGE experiments with the OMPs of the two strains. Prior to perform the DIGE experiments, one-dimensional SDS-PAGE experiments as an initial approach were performed in order to have an initial idea about the expression of the proteins. The results of the SDS-PAGE are showed in the Figure 2.9.

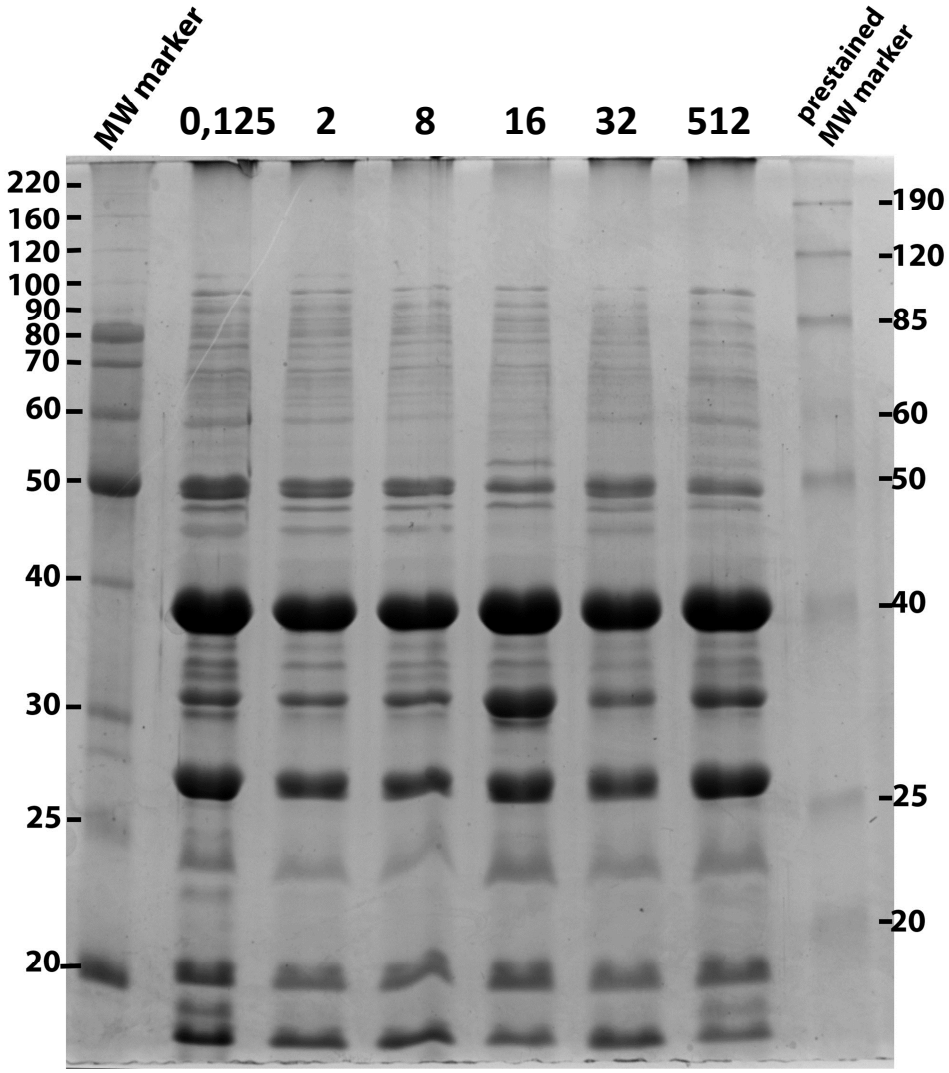


Figure 2.9. SDS-PAGE of six of the generated mutants. Each column is named by the amount of colistin used when the mutants were generated.

In the Figure 2.9 the OMP profiles of the colistin-susceptible strains (0.125/2/8) and the colistin-resistant strains (16/32/512) are presented. Due to the high amount of proteins present in the one-dimensional SDS-PAGE it was difficult to affirm which proteins were overexpressed or repressed, therefore 2D electrophoresis were carried out in order to observe better differences between the proteins expressed in the different strains.

Next step was to use the 2D electrophoresis technique for the same strains used for the one-dimensional SDS-PAGE. Figure 2.10.

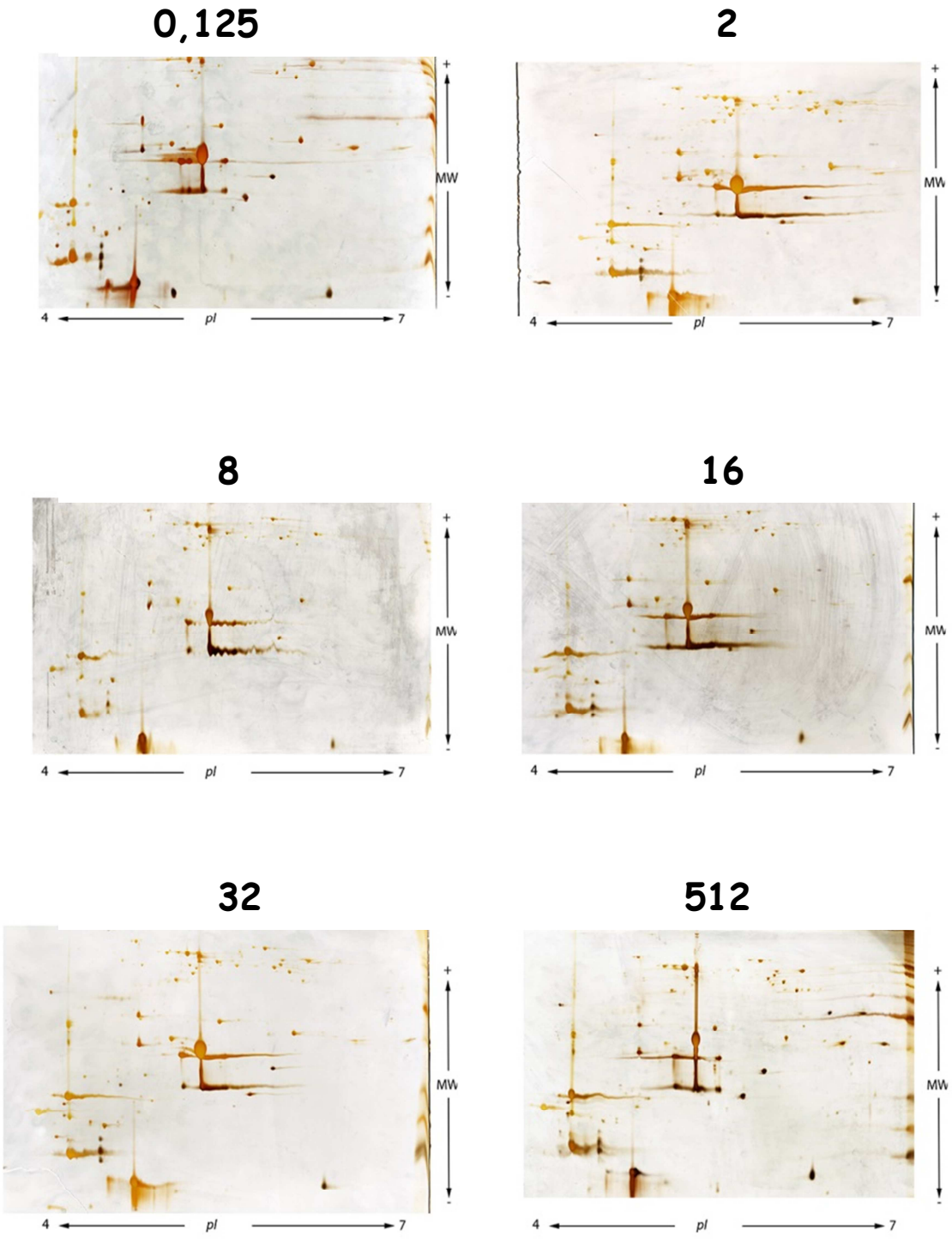


Figure 2.10. 2D electrophoresis gel of six *Acinetobacter nosocomialis* strains. Three colistin-susceptible (0.125/2/8) and three colistin-resistant (16/32/512).

Some differences between the outer-membrane protein expression in colistin-susceptible and colistin-resistant strains were observed. However with 2D electrophoresis is not possible to have a real quantification of the expression of the proteins, therefore DIGE experiments taking the initial strain (0.125) and the most resistant (512) were performed. The results are showed in Figure 2.11, and the proteins identified in the table 2.4.

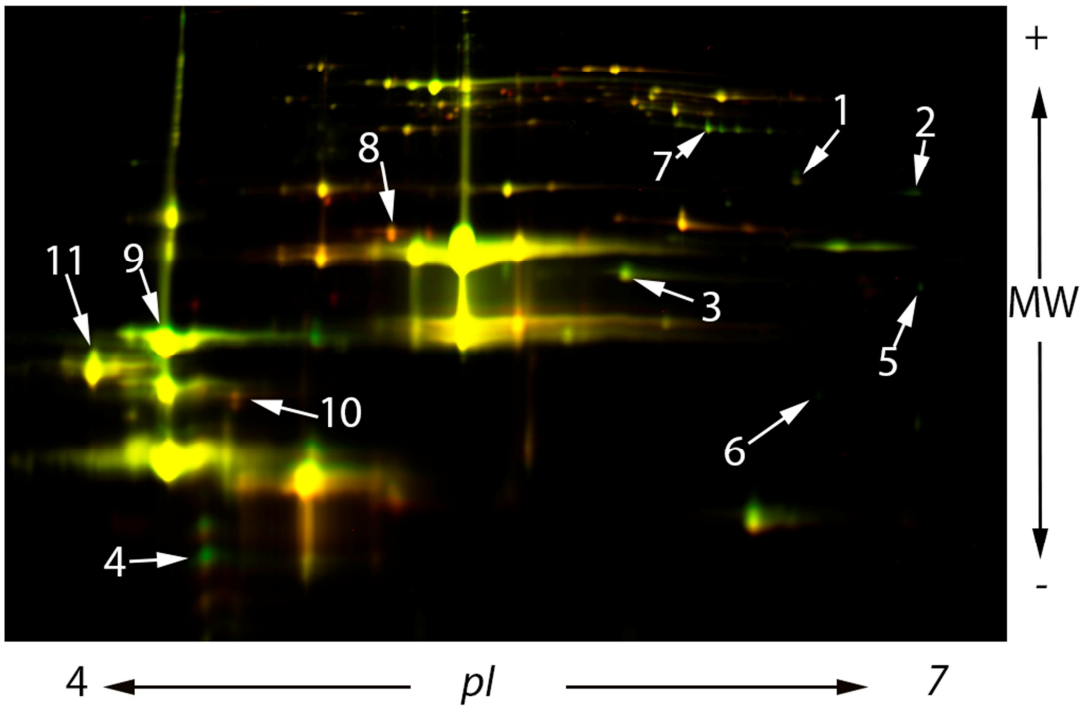


Figure 2.11. 2D electrophoresis resultant from the DIGE gel. The numbers show the proteins that have a different expression between 0.125 and 512 strains.

Spot number	Protein id	Protein	Fold change*	Anova P-value
1	gi|407441282	OmpA_C-like	1.3	0.0239
2	gi|490848590	hypothetical protein F984_02367 (NodT family RND efflux system)	2.1	0.00911
3	gi|497190250	hypothetical protein (tetratricopeptide repeat family protein)	1.5	0.0167
4	gi|593656836	putative carbapenem-associated resistance protein (CarO)	1.7	0.00914
5	gi|446899273	OmpW-like protein	1.4	0.0274
6	gi|354459714	OmpA (Isoform)	2	0.0233
7	gi|487978330	succinate dehydrogenase flavoprotein subunit	1.7	0.000768
8	gi|487978520	Porin (OprB) Carbohydrate-selective porin	-1.9	0.00533
9	gi|493628869	membrane protein (outer membrane beta-barrel domain protein)	1.4	0.0774
10	gi|487981035	signal peptide protein	-1.2	0.0237
11	gi|587819016	putative porin	1.1	0.0613

*Positive values indicate an increased presence in the resistant strain. Negative values indicate a decreased presence in the resistant strain.

Table. 2.4. Proteins identified by DIGE with a significative expression level.

.

Eleven different proteins were identified with different expression rates, just two proteins were down-regulated in the resistant strains, these two proteins were OprB with -1.9 fold, and a signal peptide protein with -1.2 fold protein, the rest of the proteins found were overexpressed in the colistin-resistant *A. nosocomialis* strain. The role of ompA has been commented previously, and the resistant strain has 2 fold the protein overexpressed compared with the wild-type. Another outer membrane protein, OmpW, it is up-regulated 1.4 fold. Several efflux systems together with some unknown proteins are overexpressed in the resistant strain.

In summary we can conclude that the colistin-resistant A. nosocomialis mutants generated by step-wise method had a certain rate of colistin-tolerance during the first steps, and an inflection point was observed, becoming highly resistant. The A. nosocomialis with high level of colistin resistance lost the virulence and showed an increase susceptibility top other antimicrobial agents. The resistance to colistin is mainly due to mutations and a stop codon in lpxD, that lead to a complete loss of the LPS. The down and up-regulation of outer membrane proteins could be related to both colistin-resistant or the decrease in the MIC of several antibiotics as previously commented.

Chapter 3

Peptides and peptidomimetics active against colistin-resistant *Acinetobacter* species

Context.

There is an urgent need of new antibiotics, especially against the ESKAPE group of bacteria. In particular we are going to focus mainly in *Acinetobacter* species, that together with *K. pneumoniae* and *P. aeruginosa* are the only bacteria isolated from patients resistant to colistin, that as commented previously, is the last option to treat infections caused by strains with a high resistance profile.

There are not many works published in the literature about active compounds against colistin-resistant strains. In fact, there is a real need of new active compounds that could substitute, or be used at the same time as colistin or polymyxin B.

In this chapter of the thesis we have tackled the search of new antimicrobials by using compounds from different sources, some of them natural compounds, others optimized natural compounds and some others fully synthetic.

3.1. Active peptides against colistin-resistant *Acinetobacter species*

3.1.1. Screening of antimicrobial peptides against colistin-susceptible and colistin-resistant *A. baumannii* strains

Some years ago, and due to the difficulty to have access to *Acinetobacter* strains resistant to colistin, (there were just a few worldwide), some *Acinetobacter* colistin mutants were generated using a multi-step method in the laboratory of Prof. Vila. Mutants for *Acinetobacter baumannii, Acinetobacter pitti* and *Acinetobacter nosocomialis* were generated as explained before. (Chapter 2.1)

The main purpose of this study was to perform an initial screening of peptides already known, but never tested before against colistin-resistant *A. baumannii* strains, in order to obtain a hit to be optimized.

Some commercially available peptides (Table 3.1) were tested against both colistin-resistant and colistin-susceptible *A. baumannii* strains, the results are shown in Table 3.2.

Name	Sequence	Source	Structure
Mastoparan	INLKALAALAKKIL	*Vespula lewisii*	Helix
Indolicidin	ILPWKWPWWPWRR	Bovine neutrophils	Extended
Melittin	GIGAVLKVLTTGLPALISWIKRK RQQ	Honeybee venom	Helix
Cecropin B	KWKIFKKIEKVGRNIRNGIIKAGP AVAVLGEAKAL	Chinsese oak silk mouth	Unknown
Cecropin A	KWKLFKKIEKVGQNIRDGIIKAG PAVAVVGQATQIAK	*Hyalophora cecropia*	Helix
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAI AIQGGPR	*Sus scrofa*	Helix
Magainin II	GIGKFLHSAKKFGKAFVGEIMNS	*Xenopus laevis*	Helix
Magainin I	GIGKFLHSAGKFGKAFVGEIMKS	*Xenopus laevis*	Helix
β-Defensin	DHYNCVSSGGQCLYSACPIFTKI QGTCYRGKAKCCK	Homo sapiens	3 (S-S)
Buforin I	AGRGKQGGKVRAKAKTRSSRAG LQFPVGRVHRLLRKGNY	*Bufo bufo gargarizans*	Helix
HNP-1	ACYCRIPACIAGERRYGTCIYQG RLWAFCC	Homo sapiens	3 (S-S)
HNP-2	CYCRIPACIAGERRYGTCIYQGRL WAFCC	Homo sapiens	3 (S-S)
Histatin 5	DSHAKRHHGYKRKFHEKHHSHR GY	Homo sapiens	Helix
Histatin 8	KFHEKHHSHRGY	Homo sapiens	Fragment of Histatin 5
Bactenicin	RLCRIVVIRVCR	Bovine neutrophils	Extended

Table 3.1 Peptides used in the initial screening

Antimicrobial peptide **(mg/L) [μM]**	**Colistin susceptible** ***A. baumannii* ATCC 19606 wt**	**Colistin resistant** **A. baumannii ATCC 19606 mutant**
Histatin 5	>256 [>84.3 μM]	>256 [>84.3 μM]
Buforin I	>256 [>60 μM]	>256 [>60 μM]
Magainin II	256 [103.8 μM]	256 [103.8 μM]
β-defensin	256 [65.2 μM]	256 [65.2 μM]
Magainin I	64 [23.6 μM]	64 [23.6 μM]
Histatin 8	32 [20.5 μM]	32 [20.5 μM]
HNP-2	50 [14.8 μM]	50 [14.8 μM]
Cecropin A	32 [>8 μM]	256 [63.9 μM]
Cecropin B	32 [8.3 μM]	128 [33.4 μM]
Bactenicin	64 [43.1 μM]	16 [10.8 μM]
Cecropin P1	1,6 [0.48 μM]	>25 [> 7.5 μM]
HNP-1	50 [14.5 μM]	3,25 [0.95 μM]
Indolicidin	8 [4.2 μM]	16 [8.4 μM]
Melittin	4 [1.4 μM]	2 [0.7 μM]
Mastoparan	4 [2.7 μM]	1 [0.68 μM]
Colistin	0,5 [0.43 μM]	256 [221.6 μM]

Table 3.2 MICs of different peptides against colistin-susceptible and colistin-resistant *A. baumannii* strains.

According to the results obtained, it was possible to classify the peptides into four different groups. In the first group all of the peptides were inactive against both colistin-susceptible and colistin-resistant *A. baumannii* strains. **Buforin** and **histatin 5** had a high MIC, with more than 256 mg/L against both strains, **magainin II** and **β-defensin** had also a high MIC with 256 mg/L for both strains. The results for **magainin I** were slightly better with a MIC of 64 mg/L for both strains, in addition **histatin 8** and **HNP-2** had also the same MIC for both colistin-susceptible and colistin-resistant *A. baumannii* strains, with 32 and 50 mg/L respectively. The results for **cecropin A** and **B** were similar, with a MIC of 32 mg/L for colistin-susceptible, and higher MICs of 256 and 128 mg/L for colistin-resistant respectively. The case of **bactenicin** was strange, obtaining a MIC (16 mg/L) lower for colistin-susceptible compared to 64 mg/L for colistin-resistant *A. baumannii*.

The only compound present in the second group was **cecropin P1**, it had the same behaviour as colistin, having a low MIC (1.6 mg/L) for colistin-susceptible compared with a higher MIC (>25 mg/L) when was tested against colistin-resistant *A. baumannii*. The opposite behaviour was observed in the third group with **HNP-1**, with a low MIC (3.25 mg/L) for colistin-resistant, compared to high MIC (50 mg/L) when was tested against colistin-susceptible.

The last group was formed by three peptides, which showed good activity against both strains used. The less active peptide of this group was **indolicidin**, with a MIC of 8 and 16 mg/L against colistin-susceptible and colistin-resistant strains respectively. **Melittin** had a MIC of 4 mg/L when tested against colistin-susceptible and 2 mg/L when tested against colistin-resistant, and **mastoparan**, had similar behaviour, having the same MIC as melittin (4 mg/L) for colistin-susceptible and a MIC (1 mg/L) for colistin-resistant.

After analyzing the results, it was found that melittin was a really active peptide against both colistin-susceptible and colistin-resistant *A. baumannii*, however it had a high cytotoxicity whats makes it impossible to be used as a future antimicrobial (175). For indolicidin, a lot of optimizing studies have been performed (176), however the analogs synthesized were never tested against colistin-resistant *A. baumannii*. In order to confirm the results obtained in the first screening, mastoparan and indolicidin, (melittin was discarded due to its high lytic activity), were tested against more strains of both colistin-susceptible (14 strains) and colistin-resistant (13 strains) *A.* baumannii. The results are shown in the table 3.3.

(mg/L) [μM] **Strains**	**Antimicrobial peptides**	**MIC_{50}**	**MIC_{90}**	**Range**
Colistin-susceptible	Mastoparan	4[2.7]	8[5.4]	1-16[0.68-10.8]
A. baumannii (14)	Indolicidin	16[8.4]	32[16.8]	4-32[4.2-16.8]
	Colistin	<0,5[<0.4]	<0,5[<0.4]	<0,5[<0.4]
Colistin-resistant	Mastoparan	8[5.4]	8[5.4]	2-8[1.4-5.4]
A. baumannii (13)	Indolicidin	16[8.4]	16[8.4]	16 [8.4]
	Colistin	>512[>443.3]	>512[>443.3]	32->512[27.7->443.3]

Table 3.3 MIC_{50} and MIC_{90} of different peptides for colistin-susceptible and colistin-resistant *A. baumannii* clinical isolates.

The results from table 3.3 shows that MICs of mastoparan and indolicidin slightly increase. In the case of mastoparan, they are below two digit numbers with a MIC_{50} and MIC_{90} of 4 and 8 mg/L respectively and a range of 1-16 mg/L in colistin-susceptible *A. baumannii*. For colistin-resistant, the results were similar, showing the same MIC_{50} and MIC_{90} (8 mg/L), and a range of 2-8 mg/L. The results for indolicidin for colistin-susceptible MIC_{50} and MIC_{90} were 16 and 32 mg/L respectively, and a range between 4 and 32 mg/L, and all the MICs for indolicidin when tested against colistin-resistant strains were 16 mg/L. If we compare these peptides in front of colistin, it is possible to observe that for the colistin-susceptible strains, the MIC of colistin was better than indolicidin and mastoparan, however colistin-resistant *A. baumannii* strains were tested, the values of MIC_{50} and MIC_{90} of colistin were >512 mg/L and a range between 32 and >512, so these results confirmed a good *in vitro* activity of mastoparan. Also confirmed our initial hypothesis that mastoparan was more active than indolicidin, therefore we will focus only in mastoparan.

3.1.2. Killing curves of mastoparan against colistin-susceptible and colistin-resistant *A. baumannii* strains.

In order to have an idea of the behaviour of this peptide in front of both colistin-susceptible and colistin-resistant *A. baumannii* strains, killing curve experiments were performed. The two strains of the initial screening were used for these experiments. The results are shown in the figure 3.1.

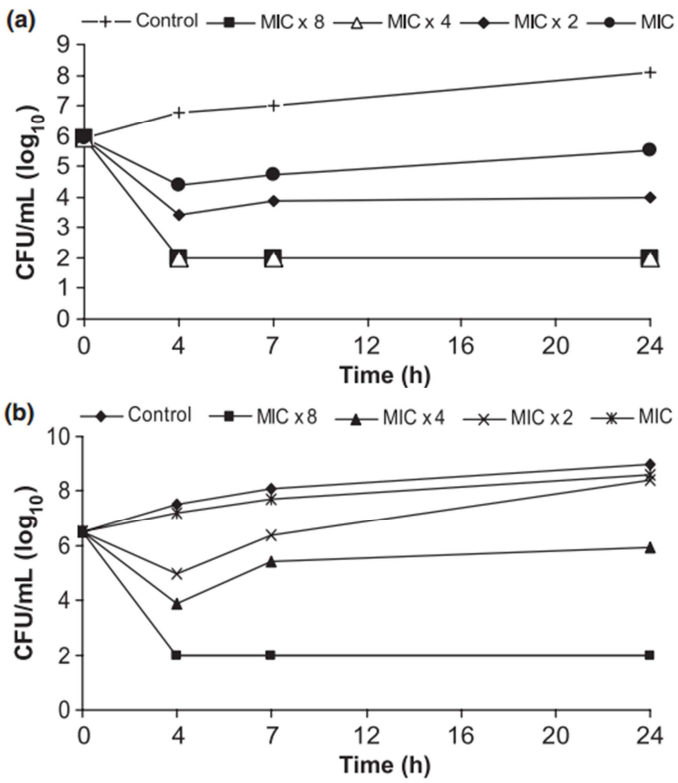


Figure 3.1 Results of time-killing experiments. a) colistin-resistant ATCC 19606 *A. baumannii* incubated with mastoparan (MIC 1mg/L). b) colistin-susceptible ATCC 19606 *A. baumannii* incubated with mastoparan (MIC 4mg/L).

When comparing the values of mastoparan against the two strains, it was possible to observe a better activity against colistin-resistant *A. baumannii*, were the effect of both 8xMIC and 4xMIC was bactericidal by decreasing from 6 log cfu/mL to 2 log cfu/mL aproximately in four hours. At the same concentrations against colistin-susceptible the same bactericidal effect was observed at 8xMIC, however no bactericidal behaviour was observed at concentration 4xMIC and a regrowth was observed after four hours. At the rest of the concentrations no bactericidal effect was observed neither in the colistin-susceptible nor in the colistin-resistant, however it was important to point out the fact that at MIC concentration, in the colistin-resistant *A. baumannii* strain, a small decrease of the number of bacteria was observed, however in the colistin-susceptible the curve was almost similar to the control.

3.1.3. Stability assay and design of mastoparan analogs

As commented previously, one of the most important drawbacks of the antimicrobial peptides is their poor *in vivo* stability. Most of them have a very short half-life, therefore stability is one of the most important properties that need to be improved when working with peptides.

Stability assays incubating mastoparan with human serum were performed in order to check the stability of the peptide, and to found out the most susceptible positions of the peptide.

A preliminary stability assay incubating mastoparan with human serum was performed in order to evaluate the stability of the peptide and, specially, to identify those peptide bonds more susceptible to hydrolytic cleavage. Samples of mastoparan were analyzed after different incubation times both by reverse-phase HPLC and MALDI-TOF. A precised determination of the half-life of mastoparan from this experiment was not possible due to the fact that mastoparan, in the analytical conditions used, co-eluted with one of the degradation products.

After analyzing all the data obtained from mass-spectrometry, it was observed that the stability of mastoparan was surprisingly high for being a lineal peptide with L-amino acids (appearing the mass until 6 hours), and the only compound resulting from the action of the proteases was [2-14]-mastoparan, the product of hydrolysis of the Leu^{1}-Asn^{2} peptide bond (figure 3.2 and 3.3).

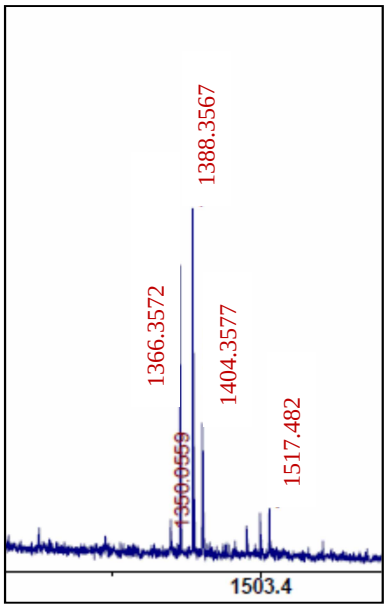


Figure 3.2. MALDI-TOF spectra of mastoparan after incubation with human serum at 24 hours. The main compound is [2-14]-mastoparan. Molecular weight: 1366 Da (+H). Molecular weight (+Na): 1388. Molecular weight (+K): 1404 Da

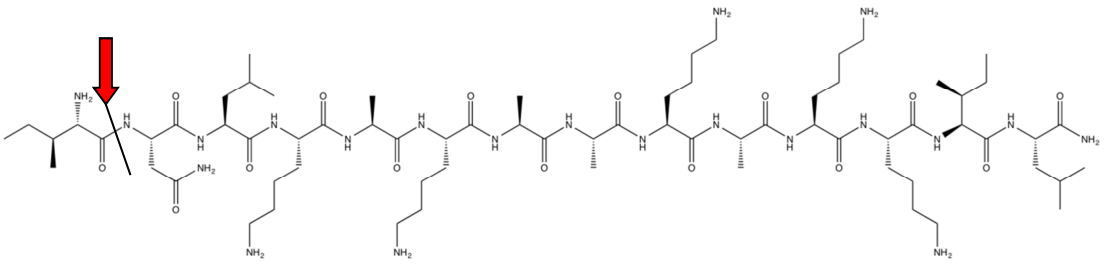
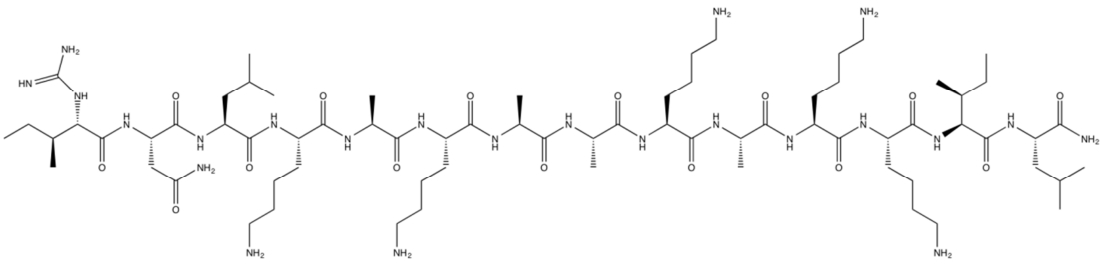


Figure 3.3. Chemical structure of mastoparan, showing the hydrolysis susceptible point.

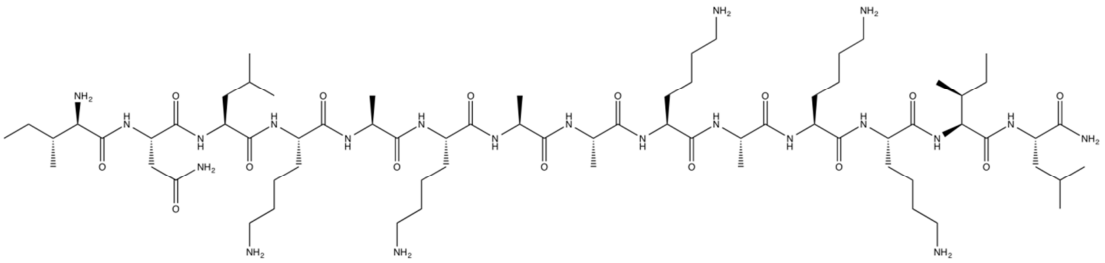
Taking into account the information obtained in the stability assay, a library of mastoparan analogs were designed and synthesized with the purpose of increasing the stability without losing activity. The analogs that were designed are shown in figure 3.4.

Peptide **1**



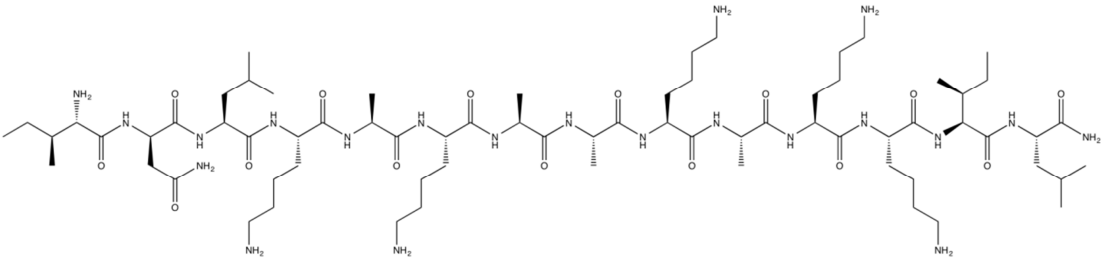
Gu-Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-NH_2

Peptide **2**



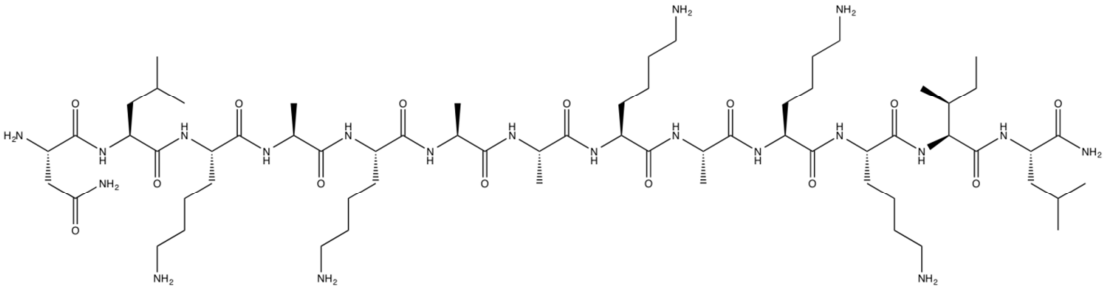
D-Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-NH_2

Peptide **3**



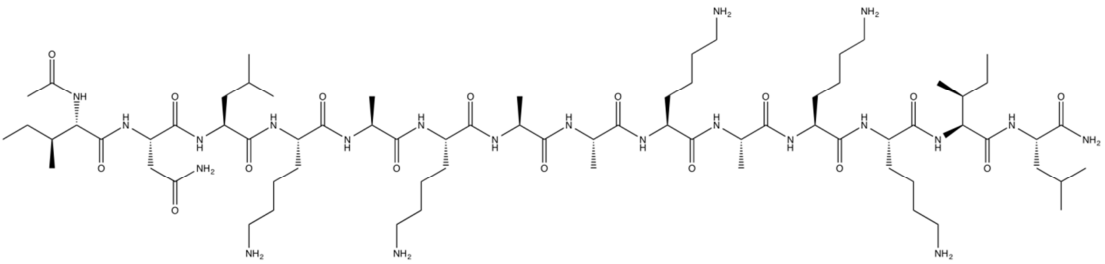
Ile-D-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-NH_2

Peptide **4**



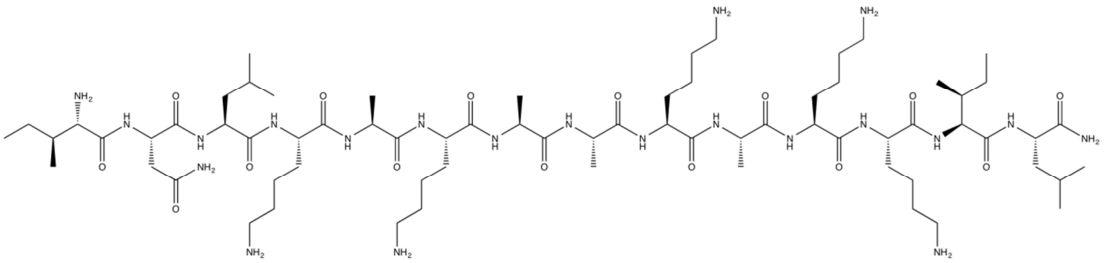
Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-NH_2

Peptide **5**



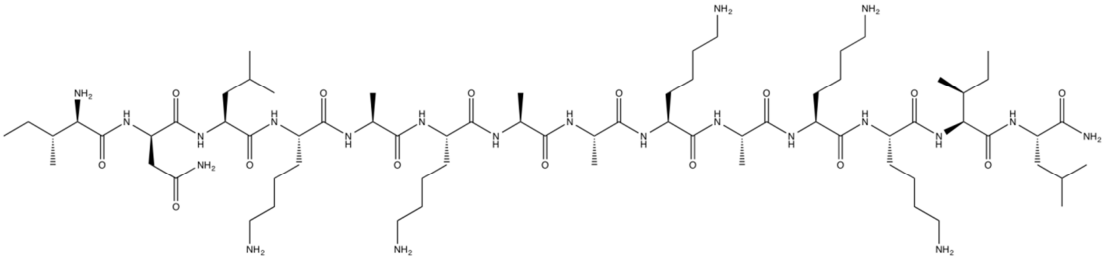
Ac-Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-NH_2

Peptide **6** (Mastoparan)



Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-NH_2

Peptide **7**



D-Ile-D-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-NH_2

Figure 3.4. Chemical structure of all the mastoparan analogs synthesized.

Peptide **1** had only an extra guanidinium group in the N-terminal, this allow the last residue (isoleucine) to be more protected, due to its steric hindrance produced by this group, moreover it keeps the positive charge in the N-terminal. The last isoleucine from peptide **2** and asparagine from peptide **3** are D-amino acids. Peptide **4** is the species observed after the action of the enzymes present in the human serum,[2-14]-mastoparan . The design of Peptide **5** followed the same strategy as peptide **1**. The N-terminal was protected with an acetyl group, this protection had also a steric hindrance, however the positive charged of the N-terminal was removed. Peptide **6** was the original mastoparan. Finally, peptide **7** had both D-isoleucine and D-asparagine at positions 1 and 2 respectively.

The synthesis of these peptides was carried out using a Fmoc/tBu solid phase peptide synthesis approach. As most of the peptides had common amino acids at the C-terminal, the strategy was to synthesize first the common part and then to split the resin in different batches to synthesize all the different analogs. figure 3.5

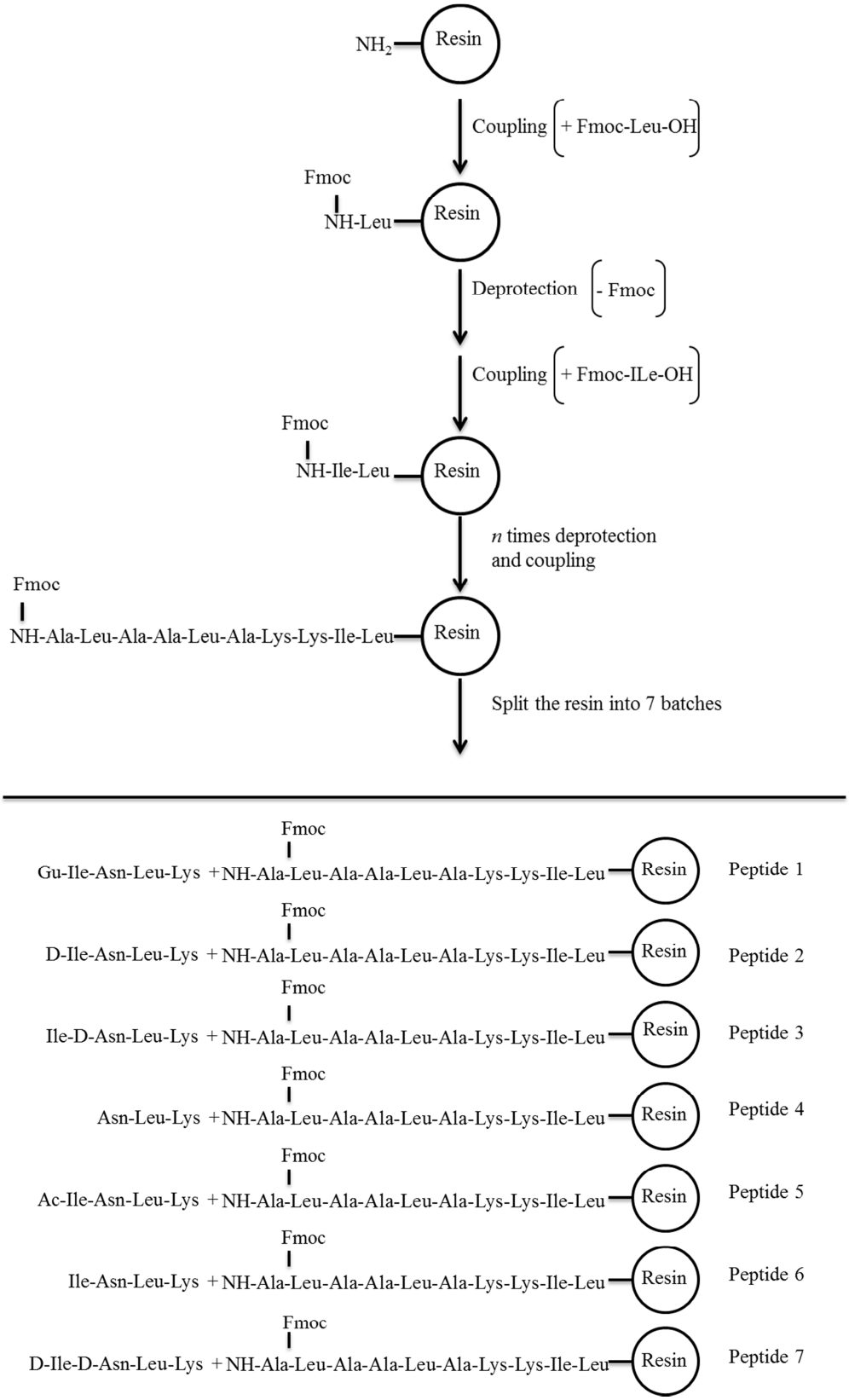
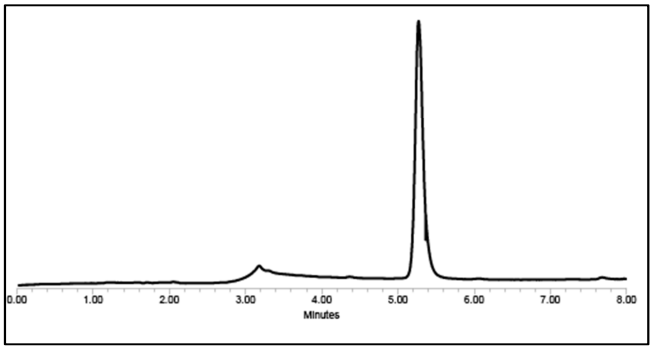
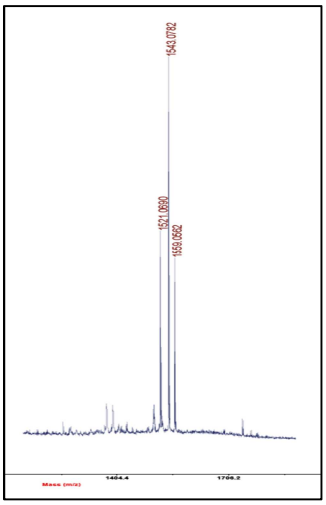


Figure 3.5. Scheme of the synthesis of mastoparan analogs

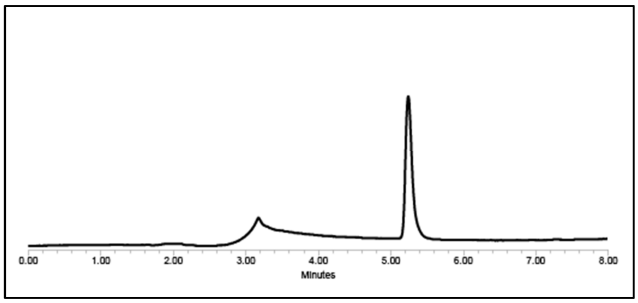
The peptides were synthesized and purified as explained in the materials and methods part. The HPLC and MALDI-TOF spectra are showed in the Figure 3.6

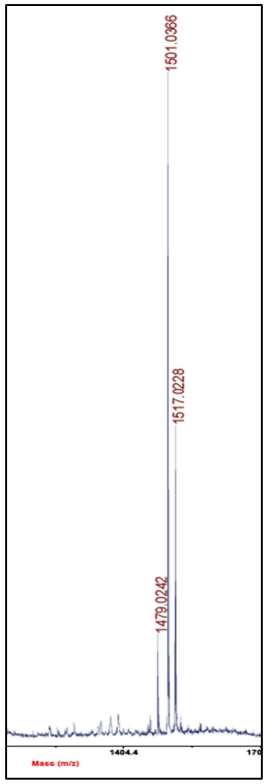
Peptide 1 (Gu-INLKALAALAKKIL-NH_2)



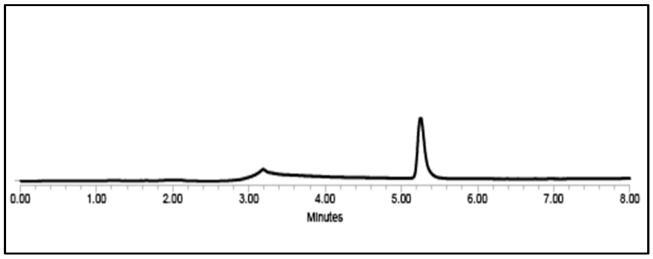


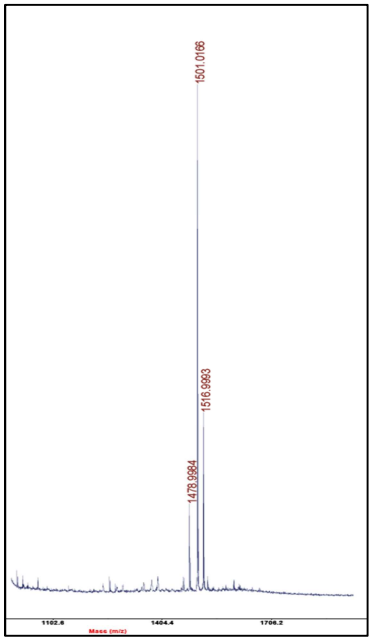
Peptide 2 (iNLKALAALAKKIL-NH_2)



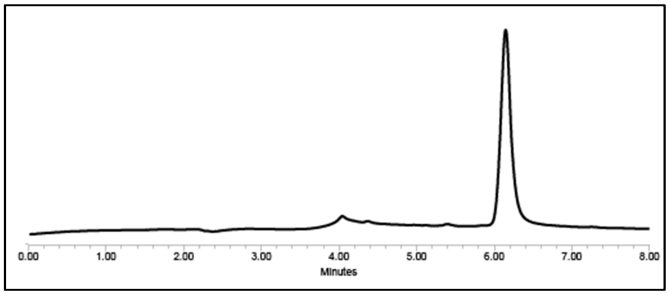


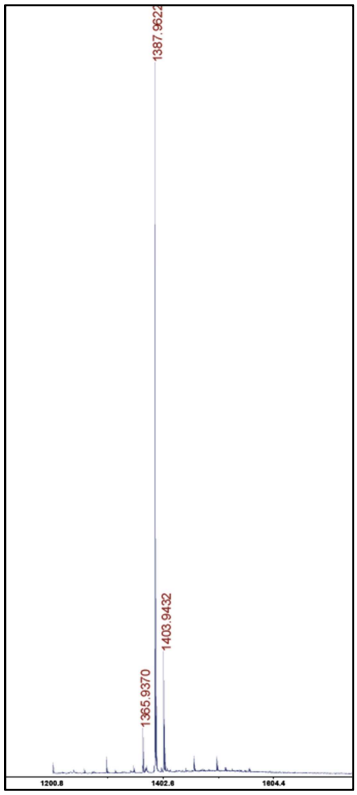
Peptide 3 (InLKALAALAKKIL-NH_2)



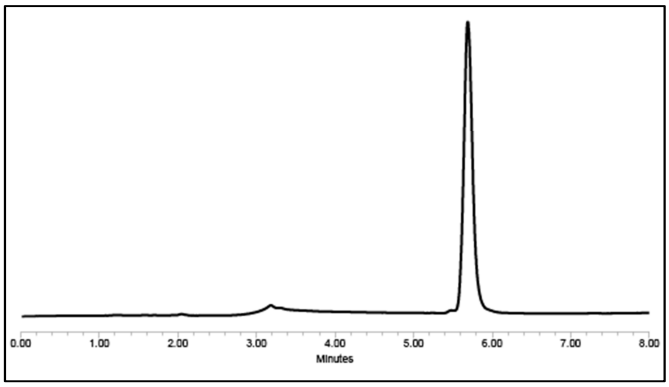


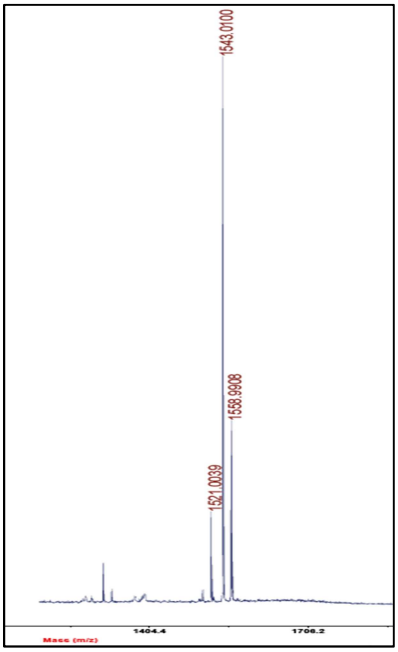
Peptide 4 (NLKALAALAKKIL-NH_2)



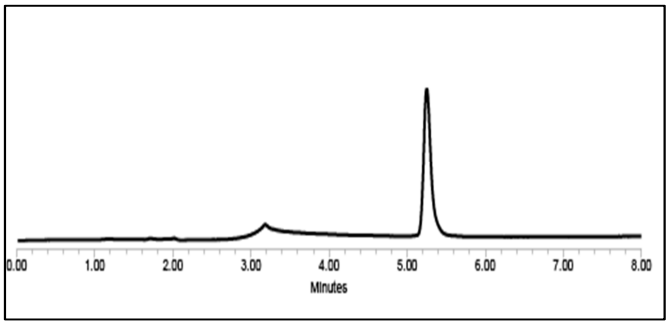


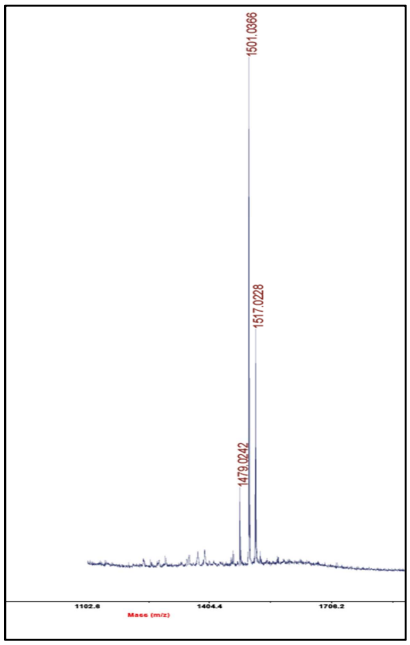
Peptide 5 (Ac-INLKALAALAKKIL-NH_2)





Mastoparan (INLKALAALAKKIL-NH_2)





Peptide 7 (inLKALAALAKKIL-NH_2)

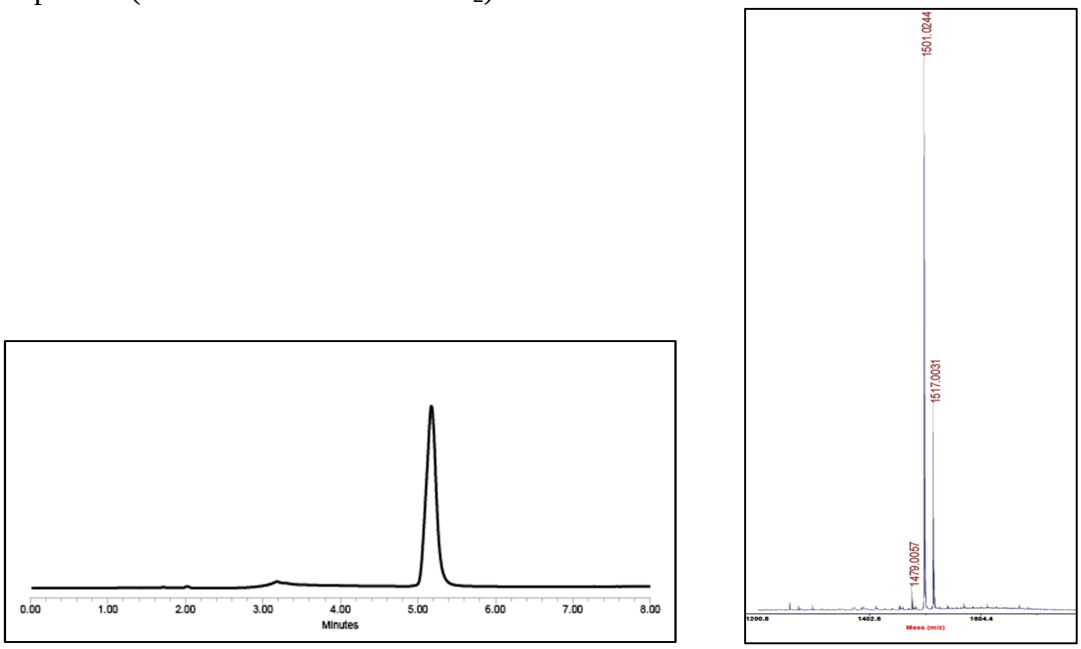


Figure 3.6. HPLC spectra and MALDI-TOF of the mastoparan analogs synthesized.

3.1.4. Evaluation of activity, stability and toxicity of the mastoparan analogs.

All the peptides synthesized were tested against four different strains of *A. baumannii*. All of them were colistin-resistant clinical isolates with a high resistance profile. The cytotoxicity of mastoparan and all the analogs synthesized was also tested. The evaluation of stability of the new compounds in order to know if our aim, increase the stability, was achived was also performed. The results are showed in Table X.4

		Minimal inhibitory concentration mg/L (μM)					
	Antimicrobial peptide	**CR17**	**CR86**	**Ab11**	**Ab113**	**Stability in human serum**	**Toxicity MTT IC_{50} (HeLa)**
1	Gu-INLKALAALAKKIL	4 (2,6)	4 (2,6)	4 (2,6)	4 (2,6)	> 24h	13 μM
2	iNLKALAALAKKIL	32 (21,6)	32 (21,6)	64 (43,3)	64 (43,3)	> 24h	41 μM
3	InLKALAALAKKIL	32 (21,6)	64 (43,3)	128 (86,5)	256 (173)	> 24h	73 μM
4	NLKALAALAKKIL	32 (19,2)	64 (38,4)	128 (76,8)	128 (76,8)	> 24h	109 μM
5	Ac-INLKALAALAKKIL	16 (10,5)	32 (21)	64 (42)	64 (42)	> 24h	29 μM
6	**Mastoparan**	**4 (2,7)**	**4 (2,7)**	**4 (2,7)**	**4 (2,7)**	**6h**	**32 μM**
7	inLKALAALAKKIL	128 (86,5)	128 (86,5)	128 (86,5)	256 (173)	> 24h	21 μM

Table 3.4. MIC against colistin-resistant *A. baumannii* clinical isolates, stability in human serum and toxicity in HeLa cells of mastoparan analogs. D- amino acids are represented in lower case.

As it was possible to observe in the MIC results of the analogs, the only peptide that keeps the same level of activity was peptide **1**, with the same values as the original mastoparan peptide (4 mg/L). The rest of the peptides had really high values compared with the two previous peptides commented.
It was important to highlight that this high MICs obtained by most of the analogs, gave us also very useful information. When comparing the results obtained by peptide **5** and **1** it was possible to observe how important was the positive charge present in the N-terminal residue. It also gave us an idea of how important was to have all L-amino acids, and the importance of having a high life-time, because the resulting peptide after the incubation of human serum (peptide **4**) does not have any activity, therefore it is important to have a high half-life time in order to have the active species more time present in blood.

In terms of cytotoxicity, HeLa cells were selected in order to be tested against all the analogs synthesized. HeLa cells are cervical cancer isolated from a patient in 1951. The most important feature of this cell line is that cells are remarkably durable and prolific, therefore is one of the most common type of cells used when cytotoxicity want to be tested, in this case MTT was they assay used. The initial assay performed was to have an idea of how toxic was mastoparan, and the result obtained was an IC_{50} of 32 μM, this value is in the middle range of toxicity. Some of the compounds synthesized had approximately the same toxicity, like peptide **5** and **7** with an IC_{50} of 29 μM and 21 μM, respectively. Peptide **2** was slightly less toxic with IC_{50} of 41 μM. Peptide **3** and **4** were, significantly less toxic with IC_{50} of 73 μM and 109 μM. The only peptide that seems to be more toxic than mastoparan was the active peptide **1**, with an IC_{50} of 13 μM.

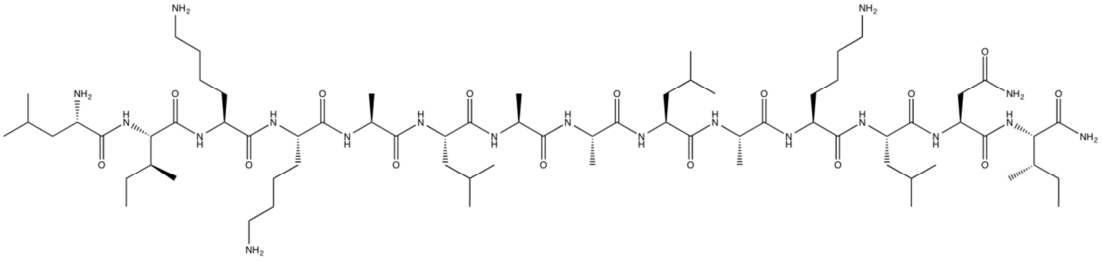
3.1.5. New mastoparan analogs: synthesis, activity, stability and toxicity.

Before to start with the design of a second serie of mastoparan analogs a careful analysis of the literature was performed.
Three peptides were synthesized following the article published by Jones and Howl, were they claimed that the enantiomeric version of mastoparan (peptide **10**), together with the retro version (peptide **8**) and the retroenantiomeric version (peptide **9**) were less cytotoxic than mastoparan, therefore these three peptides were synthesize in order

to test their activity (177). Peptide **11** was synthesized for the same reason, in the article published by Lozano *et al*., demonstrate that just changing the leucine present in position 9, for a lysine, it decreases the cytotoxicity, and add a positive charge in the peptide (178). Peptide **12** was synthesized in order to observe the behaviour of a non-natural amino acid (D-ornithine) in the peptide, together with an extra positive charge. The last two peptides were synthesized according to the information obtained during the synthesis of the first analogs. As it was important to keep the positive charge in the N-terminal, and changing the sequence was dangerous, due to the fact that by changing one residue the activity could decrease a lot. The idea was to introduce another extra positive charge in the C-terminal. Peptide **13** and peptide **14** were the original mastoparan and retro sequence with a positive charge in the C-terminal.

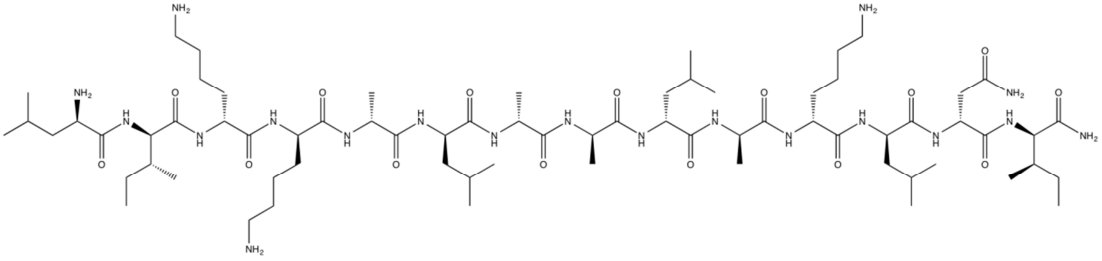
Putting together these precedents and our own previous results we undertook the synthesis of peptides 8-14 (figure 3.7)

Peptide 8



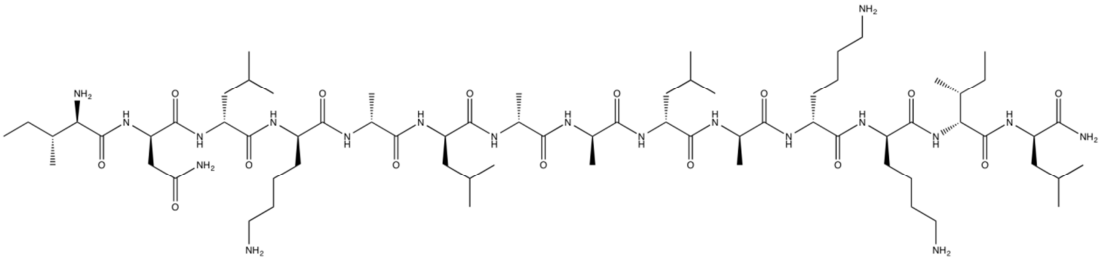
Leu-Ile-Lys-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Leu-Asn-Ile-NH_2

Peptide 9



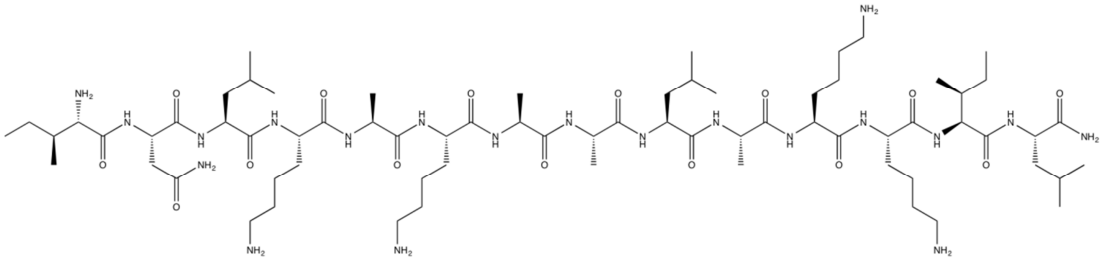
D-Leu-D-Ile-D-Lys-D-Lys-D-Ala-D-Leu-D-Ala-D-Ala-D-Leu-D-Ala-D-Lys-D-Leu-D-Asn-D-Ile-NH_2

Peptide 10



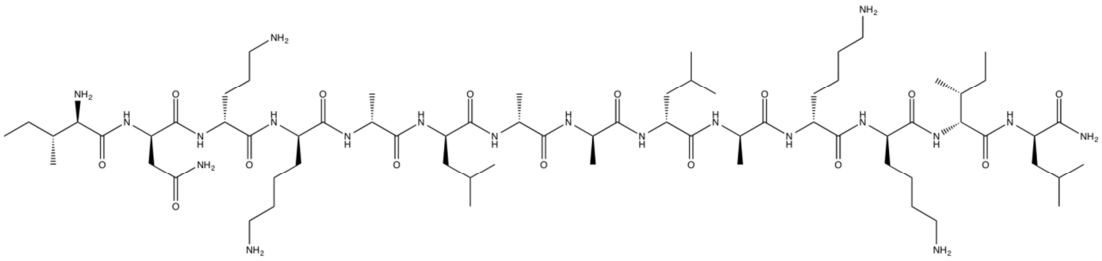
D-Ile-D-Asn-D-Leu-D-Lys-D-Ala-D-Leu-D-Ala-D-Ala-D-Leu-D-Ala-D-Lys-D-Lys-D-Ile-D-Leu-NH_2

Peptide 11



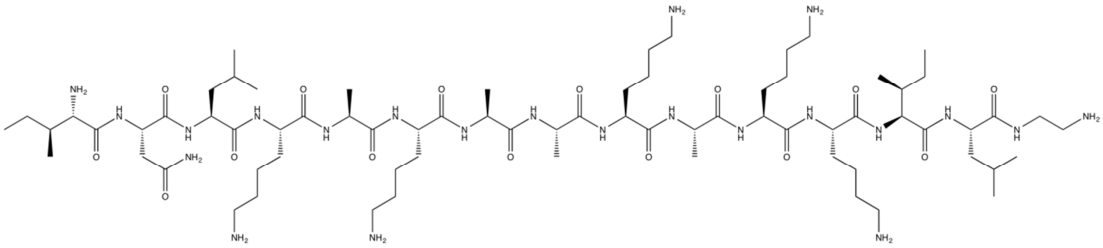
Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2

Peptide 12



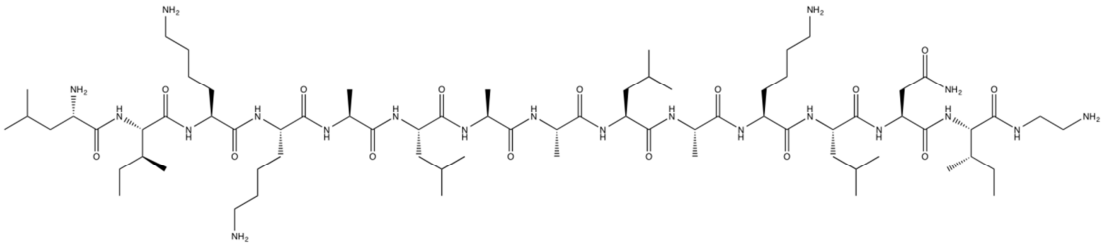
D-Ile-D-Asn-D-Orn-D-Lys-D-Ala-D-Leu-D-Ala-D-Ala-D-Leu-D-Ala-D-Lys-D-Lys-D-Ile-D-Leu-NH_2

Peptide 13



Ile-Asn-Leu-Lys-Ala-Lys-Ala-Ala-Lys-Ala-Lys-Lys-Ile-Leu-CH_2-CH_2-NH_2

Peptide 14

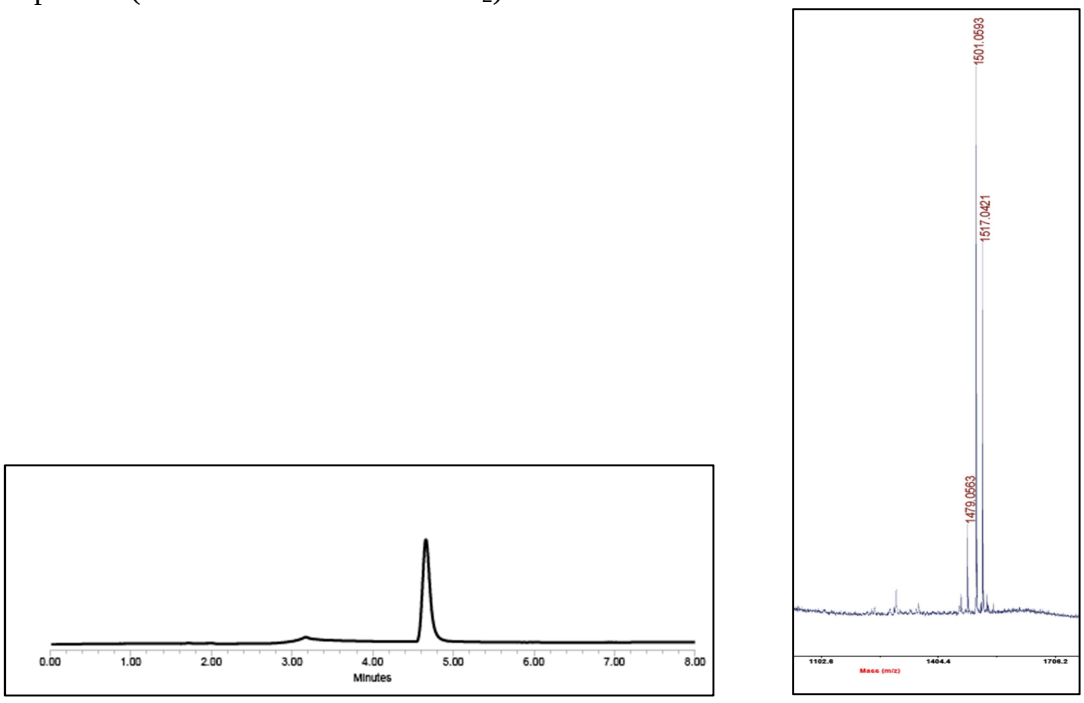


Leu-Ile-Lys-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Leu-Asn-Ile-CH_2-CH_2-NH_2

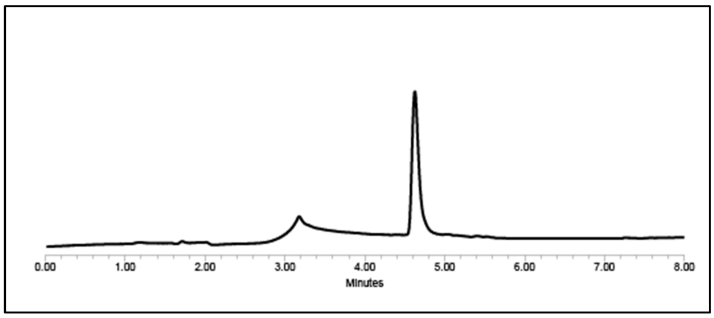
Figure 3.7. Chemical structure of all the mastoparan analogs synthesized.

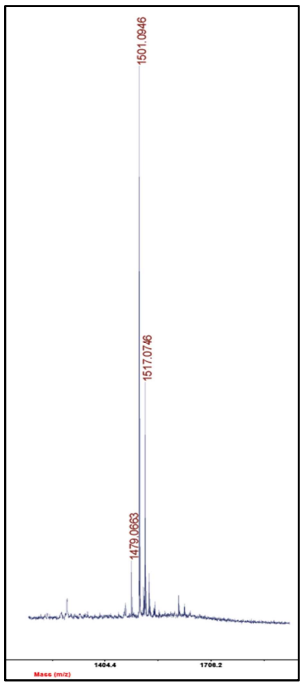
The synthesis of the new analogs was carried out using the same procedure as has been commented previously, however the resin used for the peptides **13** and **14** was different due to the fact that they have a different C-terminal. The procedures used are explained in detail in the materials and methods part. The HPLC and MALDI-TOF spectra are showed in the (figure 3.8)

Peptide 8 (LIKKALAALAKLNI-NH_2)

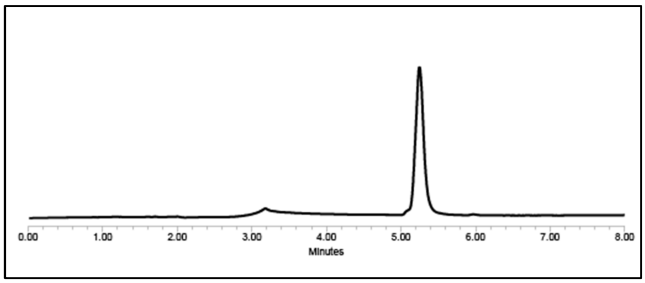


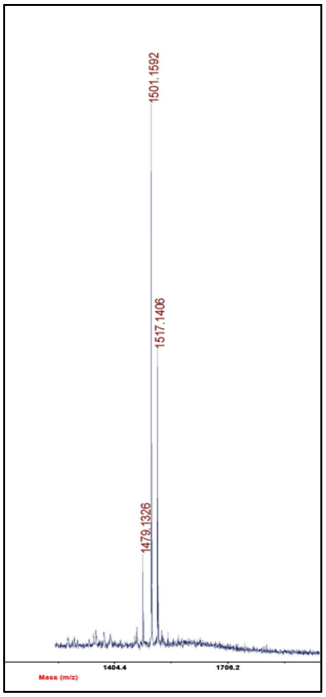
Peptide 9 (likkalaalaklni-NH_2)



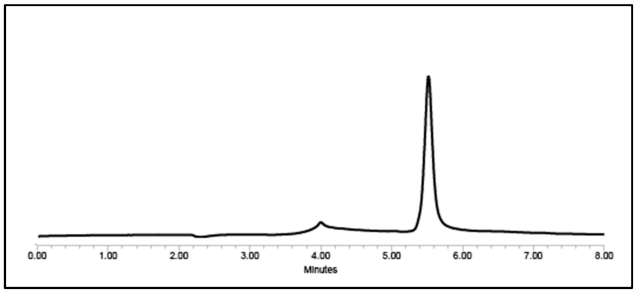


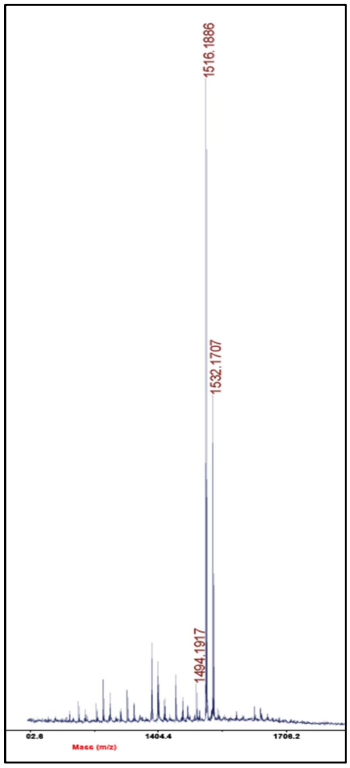
Peptide 10 (inlkalalakkil-NH_2)



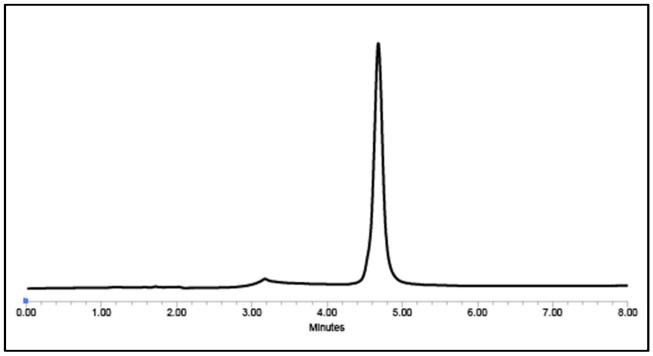


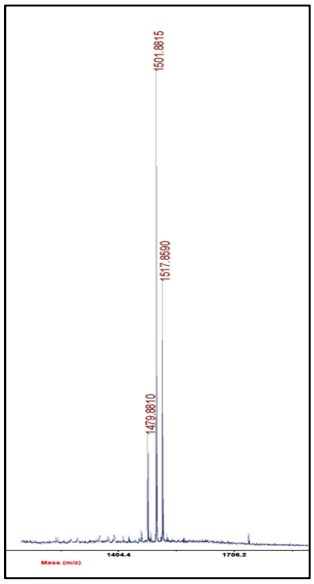
Peptide 11 (INLKAKAALAKKIL-NH_2)



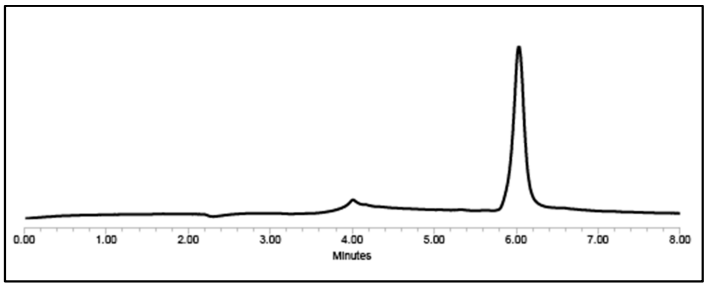


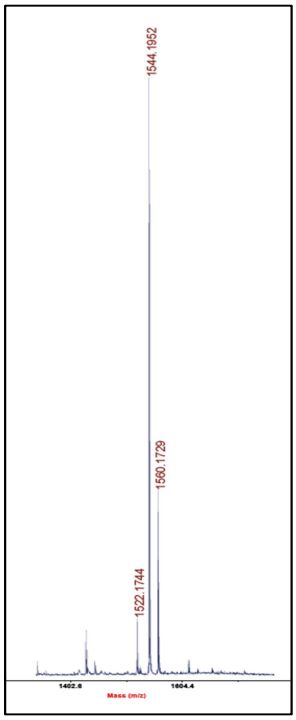
Peptide 12 (in(orn)kalaalakkil-NH_2)



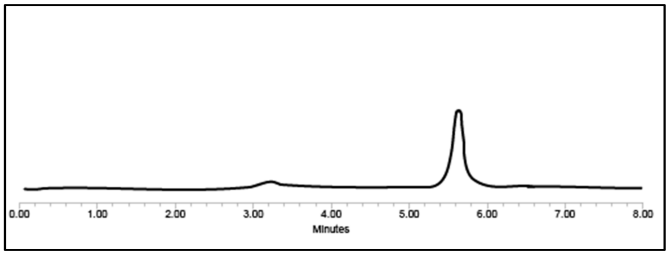


Peptide 13 (INLKALAALAKKIL-CH_2-CH_2-NH_2)





Peptide 14 (LIKKALAALAKLNI-CH_2-CH_2-NH_2)



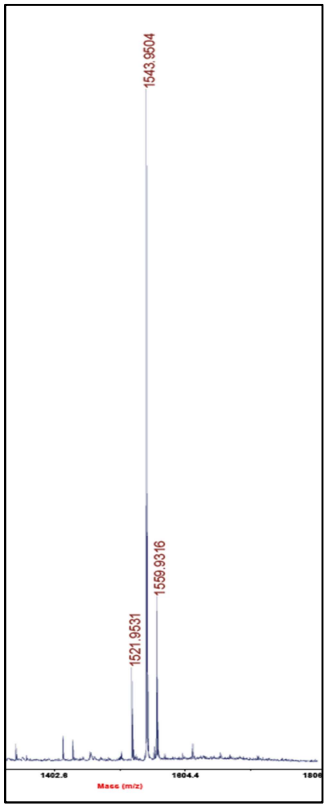


Figure 3.8. HPLC spectra and MALDI-TOF of the mastoparan analogs synthesized.

All the peptides synthesized were tested against the same strains used to test the activity of the first mastoparan analogs. In the table 3.5 it is possible to observe the results, as well as the toxicity and the stability in human serum.

		Minimal inhibitory concentration mg/L (μM)					
	Antimicrobial peptide	**CR17**	**CR86**	**Ab11**	**Ab113**	**Stability in human serum**	**Toxicity MTT IC_{50} (HeLa)**
8	LIKKALAALAKLNI	256 (173)	256 (173)	256 (173)	256 (173)	2h	192 μM
9	likkalaalaklni	256 (173)	256 (173)	256 (173)	256 (173)	> 24h	200 μM
10	inlkalaalakkil	4 (2,7)	4 (2,7)	4 (2,7)	4 (2,7)	> 24h	10 μM
11	INLKAKAALAKKIL	256 (171,5)	256 (171,5)	256 (171,5)	256 (171,5)	6h	290 μM
12	in(or)kalaalakkil	128 (86,5)	64 (43,2)	64 (43,2)	128 (86,5)	>24h	200 μM
13	INLKALAALAKKIL-$CH_2CH_2NH_2$	4 (2,6)	4 (2,6)	2 (1,3)	4 (2,6)	6h	5 μM
14	LIKKALAALAKLNI-$CH_2CH_2NH_2$	64 (42)	128 (84,1)	128 (84,1)	64 (42)	2h	150 μM

Table 3.5. MIC against colistin-resistant *A. baumannii* clinical isolates, stability in human serum and toxicity in HeLa cells of mastoparan analogs. D- amino acids are represented in lower case.

In terms of activity just two peptides maintain the same activity as the best compounds from the previous series, these compounds were peptide **10**, and peptide **13**, with the same activity as peptide **1** and mastoparan, the rest of the peptides synthesized had really high MICs.

About the cytotoxicity and stability in human serum, it was possible to observe, that the peptides that had D-amino acids in their sequence were highly stable compared to peptides with just L-amino acids. The first three peptides synthesized followed the same behaviour as commented previously, however peptide **8**, had even lower stability than mastoparan. It was also important to mention that both retro versions of mastoparan (peptides **8** and **9**) were less cytotoxic with IC_{50} of 192 and 200 μM respectively, compared to mastoparan (IC_{50} of 32 μM) and its enantiomer (peptide **10**) with a IC_{50} of 5 μM. The aim of synthesizing these peptides was due to the low cytoxicity observed in the manuscript published by Jones and Howl, however only peptides **8** and **9** showed less cytotoxicity.

Peptide **11**, as following the manuscript published by Lozano *et al*., had a really low cytotoxicity, although it has no activity and the same stability as mastoparan.

For peptide **12**, it was important to mention that even though it has an extra positive charge it is less cytotoxic, this result break with the idea that positive charged residues introduce cytotoxicity in the peptide, however in some positions it probably make increase it. This cytotoxicity is not related to the global positive charge of the peptide, just associated with the positive charge in some key residues.
The last two peptides synthesized, with the extra positive charge in the C-terminal, had a low half-life due to the L-amino acids that had in their sequence, noticing that they both had the same stability as their analogs without the positive charge in C-terminal. The activity of peptide **13** was the same as the active peptides, however was the most cytotoxic peptide with a IC_{50} of 5 μM. Peptide **14** was slightly more active than peptide **8**, however it was not enough for considering good activity, and was also more cytotoxic.

3.2. *In vivo* studies of the best compounds.

After analyzing all the data obtained from all the peptides synthesized we decided to move into the *in vivo* model, therefore we had to select which were the best candidates to be tested *in vivo*. Three peptides (peptides **1**, **10** and **13**) and mastoparan were active against colistin-resistant *A. baumannii* strains. Mastoparan was the first candidate selected because it was the less toxic compound, although it was also the less stable. When talking about the other three peptides, peptide **13** was the first discarded, due to its high toxicity and the most expensive and difficult in terms of large-scale. When comparing peptides **1** and **10** it was possible to observe that both had the same activity and stability, and similar cytotoxicity, however peptide **1** is made by L-amino acids and peptide **10** is made by D-amino acids. Even though they had the same stability *in vitro* we though peptide **10** would be more stable when administering it into mice. So we decided to study *in vivo* both peptide **10** and mastoparan.
The *in vivo* studies were performed in a stage in the laboratory of Professor Jerónimo Pachón from the Hospital Virgen del Rocío.

The first experiment that was performed was to calculate the hemolytic activity of these two peptides. figure 3.9

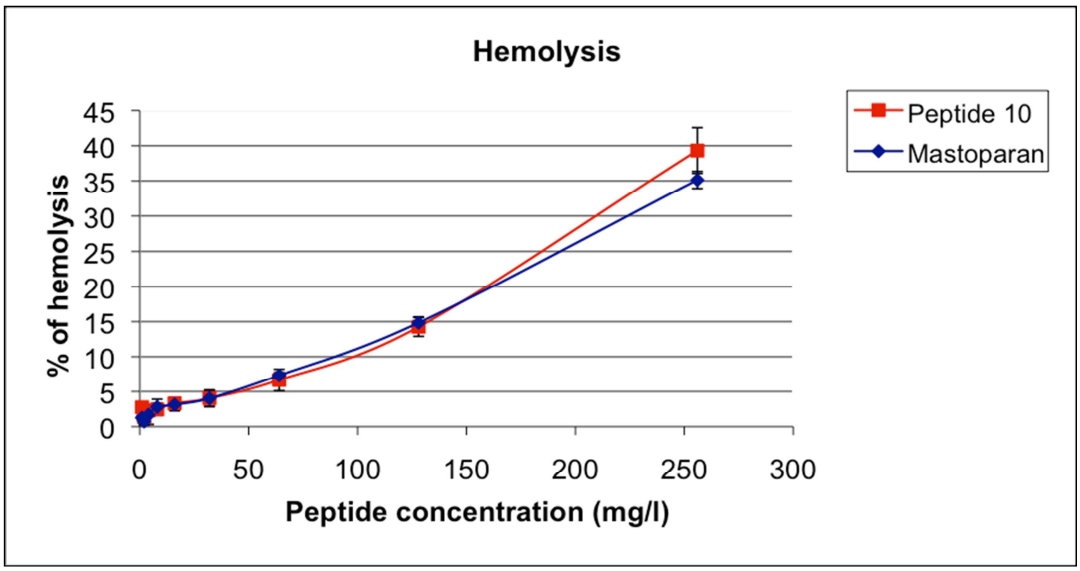


Figure 3.9. Percentatge of hemolysis of mastoparan and peptide 10 at different concentrations.

The figure X.9 shows that before 100 mg/L of the peptide concentration almost no hemolysis was observed, however if the concentration increases some hemolysis was observed with 35% and 39% of hemolysis for mastoparan and peptide **10** respectively. These results were promising because the percentage of hemolysis at the active concentration of the peptides (4 mg/L) was almost negligible.

Next step was to calculate the *in vivo* toxicity of the two peptides. 32 mg/kg was taken as the initial dose taking into account previous assays performed by Dr. Pachón group with antimicrobial peptides (179). 32 mg/kg of each peptide were injected into six mice respectively, after 24 hours the mice injected with mastoparan were alife, however the mice treated with peptide **10** were dead. Taking into account this information we increased the amount of peptide for mastoparan up to 64 mg/kg and decreased to 16 mg/kg for peptide **10**, after 24 hours the 12 treated mice were dead, with that data it was possible to knew the maximum dose for mastoparan which was 32 mg/kg. Next dose tested for peptide **10** was 8 mg/Kg, and after 24 hours all the treated mice were alife, so the maximum dose for peptide **10** was 8 mg/kg.

The results obtained fitted really good with the *in vitro* data, The higher *in vivo* toxicity of peptide **10** correlates well with its higher cytotoxicity, compared with mastoparan, and its higher stability in human serum. This high stability in human serum allow the peptide to be more time inside of the mice causing also more damage.

After calculating the lethal dose of the peptides it was time to calculate the lethal dose of the strain that will be used in further experiments. The strain used was a pan-resistant strain. Three groups of four mice each were inoculated. The amount of bacteria injected per group was 10^5, 10^6 and 10^7 cfu/mL. From all the groups, the only survival mice were the ones inoculated with 10^5 cfu/mL, therefore the lethal dose of bacteria, and the concentration used for the experiments will be 10^6 cfu/mL.

Once the toxicity of the peptides in mice and the lethal dose were calculated it was time for calculating the ED_{50}, this parameter is the effective dose that keeps alife the 50% of the mice. table 3.6

(4h) Administration Doses	**Peptide 10 (10 mice)**	**Mastoparan (10 mice)**	**Control (3 mice)**
16 mg/Kg	-	7 exitus	3 exitus
8 mg/Kg	9 exitus	10 exitus	-
4 mg/Kg	9 exitus	-	-

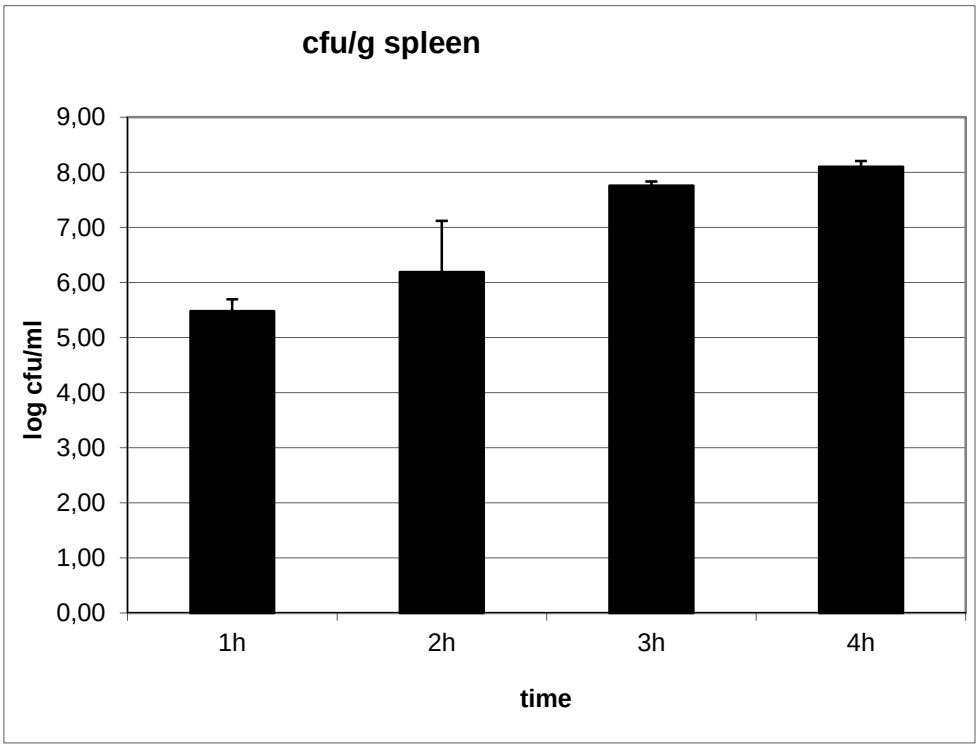
Table 3.6. ED_{50} of peptide 10 and mastoparan.

Analyzing the data obtained in the first assay, no ED_{50} was obtained, none of the groups had 5 mice alife (50%). The closest dose to ED_{50} was 16 mg/kg in mastoparan with a 30% of survival, the other dose of mastoparan, 16 mg/kg, had no survivals, and both doses for peptide **10**, 8 and 4 mg/kg, had just a 10% of survival.

Taking into account that we did not have any ED_{50} for any of the peptides, we took a look into the procedure of performing the ED_{50} assay. The mice were inoculated intraperitoneally with the lethal dose of bacteria, in this case 10^6 cfu/mL, and after 4 hours the dose of peptide was administered intraperiteoneally. The time of administration of the peptide could be too long, therefore it was decided to monitor the infection after 1, 2, 3 and 4 hours.

3 mice per group were selected, each group belongs to a period of time. The 12 mice were inoculated with the lethal dose at the same time, and after each period of time, 3 of them were sacrificed using sodium thiopental. Lungs and spleen were extracted from each mice. Once all the spleens and lungs were isolated, each one was inserted into a sterile bag and 1 mL of LB media was added, all of it was homogenized and after several dilutions, the amount of bacteria in each organ was calculated, the results are showed in figure 3.10.

a)



b)

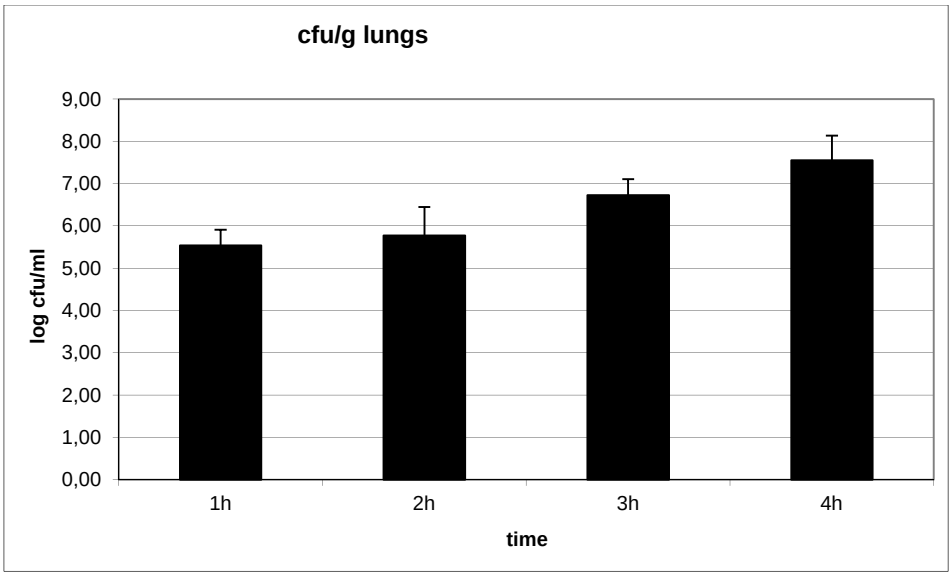


Figure 3.10. a) Number of bacteria in lungs at a different time points. b) Number of bacteria in spleen at a different time points.

The Figure 3.10 shows the amount of bacteria in each organ at different periods of time. At 1 hour the amount of bacteria in lungs and spleen was similar with $10^{5,5}$ cfu/mL, this information allows us to have an idea how fast does bacteria disperses. It would be normal to have $10^{5,5}$ cfu/mL in the spleen due to the intraperitoneally injection. However the same amount of bacteria was observed in lungs were bacteria need to achieve the blood vessels in order to arrive into the lungs.

In the second point time, 2 hours, the amount of bacteria continue increasing reaching more than 10^{6} cfu/mL in the spleen and slightly less than 10^{6} cfu/mL in lungs. The differences between both organs were not so high but at 3 and 4 hours time the differences reached were higher with at around 10^{8} in the spleen for both times compared to the $10^{6,7}$ and 10^{7} cfu/mL in lungs for 3 and 4 hours respectively.

Apart from the counting of bacteria in these organs, hemocultures were performed in each mice. Hemocultures consist in extracting blood from the heart while is pumping, this blood is spread into a blood agar plate in order know if there were bacteria in the blood torrent, all of the samples collected were positive.

After analyzing the data obtained after monitorizing the infection process, the decision was to reduce the time of administration of the peptides to two hours, this should allow the peptide to be more effective.

The same procedure was performed, with the same doses only decreasing the time of administration of the peptide in 2 hours, the results are shown in table 3.7

(2h) Administration Doses	**Peptide 10 (10 mice)**	**Mastoparan (10 mice)**	**Control (3 mice)**
16 mg/Kg	-	10 exitus	3 exitus
8 mg/Kg	10 exitus	10 exitus	-
4 mg/Kg	10 exitus	-	-

Table 3.7. ED_{50} of peptide 10 and mastoparan.

The results obtained when reducing the time of administration of the peptide were surprising, instead of having best results, worst results were obtained with any survivals in any of the groups, that means that the mice that survive during the first experiment was due to an administration failure.

In a third experiment a new set of doses of peptides was used 32, 4 and 2 mg/Kg for mastoparan; and 2 and 1 mg/Kg for peptide **10**. Only three mice were used for this experiment with the idea to repeat with higher mice if two or three mice survived, however there was just one survival with 32 mg/kg for mastoparan. Table 3.8

(2h) Administration Doses	**Mastoparan (3 mice)**	**Peptide 10 (3 mice)**	**Control (3 mice)**
32 mg/Kg	2 exitus	-	3 exitus
4 mg/Kg	3 exitus	-	-
2 mg/Kg	3 exitus	3 exitus	-
1 mg/Kg	-	3 exitus	-

Table 3.8. ED_{50} of peptide 10 and mastoparan.

Eventhough we did not have any ED_{50}, a pneumonia model was performed in order to know if there were some systemic activity with n=3 in each time point. Doses used were 8 mg/kg for peptide **10** and 32 mg/kg for mastoparan. Peptides were administered after 2 hours of infection. Time points were 2 hours and 24 hours. Table 3.9

Log cfu/mL	**0h**	**2h control**	**2h mastoparan**	**2h peptide 10**	**24h control**	**24h mastoparan**	**24h peptide 10**
Average	6.99	7.50	7.51	7.51	7.05	7.04	7.09
SD	0.38	0.61	0.42	0.30	0.13	0.18	0.21

Table 3.9. Number of bacteria in lungs at different times in the treatment with mastoparan and peptide 10.

No systemic effect was observed at any time nor peptide. In order to find any activity in the peptides we decided to change the strain into a more susceptible strain and also reduce the administration of the peptide to 1 hour. The strain selected was the ATCC 19606 mutant colistin-resistant, which was used for the screening of the first peptides. This strain is less virulent than the pan-resistant and does not have a high resistant profile against all the antibiotic families. The number of mice used in each group was three and the set doses used were 32 mg/kg for mastoparan and 8 mg/kg for peptide **10**. None of the mice survived the infection.

The same model of peritoneal sepsis was used to analyze and count the amount of bacteria in the peritoneal in order to observe a local effect of the peptide. In this case the doses used were 8 mg/kg for peptide **10** and 16 mg/kg for mastoparan. The procedure used was, infection followed by a treatment with peptide after 1 hour, and sacrifice them after 1h. 10 mice for each group were used. Results are showed in table 3.10

Log cfu/mL	Control	Mastoparan	Peptide 10
1	8.43	9.06	8.49
2	8.32	7.70	8.58
3	8.67	8.75	8.26
4	8.20	8.99	8.20
5	8.59	9.03	7.33
6	8.15	8.83	8.61
7	8.71	8.74	8.73
8	8.75	8.67	8.59
9	8.52	8.96	8.72
10	8.61	8.95	8.77
Average	**8.50**	**8.77**	**8.43**
SD	0.21	0.40	0.43

Table 3.10. Number of bacteria in the peritoneal after 2h of treatment with mastoparan and peptide 10.

When comparing the average of log cfu/mL in all three groups no significance is found. After all the experiments performed have been proved to be unsuccessful, we started thinking about the reasons of the lack of *in vivo* antimicrobial activity and the first hypothesis was that a possible binding to any of the proteins or compounds present in the serum reducing the effective concentration of the peptide. Furthermore this could be the reason why a linear peptide with L-amino acids will have this high stability in human serum.

Taking into account the first hypothesis a MIC assay in presence of serum was performed in order to see if the MICs were higher or at the same level. The results of the MIC values are showed in table 3.11.

MIC mg /L(μM)	CR17				Ab14			
% of Serum	**0%**	**25%**	**50%**	**75%**	**0%**	**25%**	**50%**	**75%**
Mastoparan	4(2.7)	16(10.8)	64(43.3)	128(86.5)	4(2.7)	32(21.6)	128(86.5)	128(86.5)
Peptide 10	4(2.7)	16(10.8)	64(43.3)	128(86.5)	2(1.35)	32(21.6)	64(43.3)	128(86.5)

Table 3.11. MICs of mastoparan and peptide 10 against colistin-resistant *A. baumannii* in the presence of different percentage of human serum.

The information obtained with this assay showed that the MIC of the two peptides increase progressively when more human serum is added. This fact support the hypothesis suggested previously. The amount of different proteins present in the serum is very high therefore it will be almost impossible to test all of them in order to know which is the one involved in the interaction with the peptide.
We just tested albumin (human serum concentration is 40 g/L) because is the main protein present in the human serum, and the results obtained are showed in table 3.12

MIC mg/L (μM)	CR17				Ab14			
% of albumin	**0%**	**25%**	**50%**	**75%**	**0%**	**25%**	**50%**	**75%**
Mastoparan	4(2.7)	16(10.8)	32(21.6)	64(43.3)	4(2.7)	16(10.8)	32(21.6)	64(43.3)
Peptide 10	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)

Table 3.12. MICs of mastoparan and peptide 10 against colistin-resistant *A. baumannii* in the presence of different percentage of albumin.

The results represented in the table 3.12 showed that mastoparan had a high affinity for the albumin, having almost the same MIC values as when it is incubated with human serum, however the activity of peptide **10** remains the same, this information together with the one obtained when MICs are performed with human serum, suggest that it has a high affinity for a protein or compound present in the serum different than albumin.

3.3. Mechanism of action of mastoparan and analogs

Two different approaches were used in order to get insight in the mechanism of action of mastoparan and the analogs synthesized. The first approach was based on the use of a leakage assay, this experiment will allow us to have an idea of how lytic is the peptide tested quantifying the amount of carboxifluorescein released by each peptide synthesized, using different lipid compositions. The second approach was using transmission electron microscopy (TEM), to observe the effect of the peptides in the bacterial membrane.

3.3.1. Leakage

Leakage assays were performed using two different membrane mimetics, a negatively charge membrane, mimicking bacterial membranes and a neutral membrane. Negatively charged membranes are composed by phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidylglycerol (PG) (63:14:23), this composition was from *P. aeruginosa* but due to its high similarity with *A .baumannii* it can be used as well (180). 0.1, 0.25, 0.5, 1, 10 and 50 µM are the concentrations of peptides used to test the ability of them to release carboxyfluorescein from the liposome. (figure 3.11)

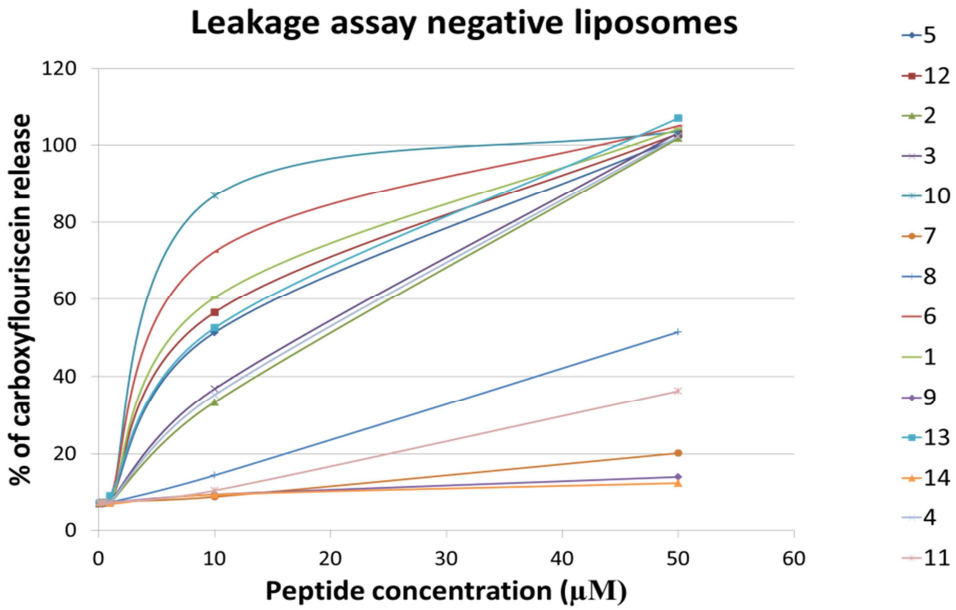


Figure 3.11. Percentage of carboxyfluoriscein released of all the mastoparan analogs at different concentrations using negatively charged liposomes.

The Figure 3.11 shows the percentage of release of each peptide at the concentrations commented below. No release is observed at concentrations below 1µM. At 10 µM it is possible to observe differences between all the peptides. Most of the active peptides against colistin-resistant strains of *A. baumannii* had a higher ability to release the fluorophore from the negative liposomes. Peptide **10** had a 87% of release followed by mastoparan and peptide **1** with 72 and 60 % respectively. The next peptide, the change of D-lysine for a D-ornithine (peptide **12**), had a MIC of 43-86 µM and a percentage of release of 57%, however the following peptides, both with 53% of release have a lower MIC, the peptide **13** (2.7 µM) and **5** (MICs between 10.5 and 42 µM). The next three peptides, **2**,**3** and **4**, had both high MICs and middle release values, with 33, 37 and 35%

respectively. All these peptides commented released all the carboxifluorescein present inside the liposome at 50 µM. The last four peptides were the less active in terms of both *in vitro* activity against *A. baumannii* and carboxyfluorescein release. For peptide **8**, the percentage of release at 10 µM was 14% and increased up to 51% at 50 µM, peptide **11** released 10% at 10µM and increased up to 38% when liposomes were incubated with 50µM of the peptide. The last three peptides, **7**, **9** and **14**, had high MICs and a very low release values at 10µM with 10%, at 50µM the only peptide in which it was possible to observe a slightly increase was in peptide **7** which increased up to 20%, the other two peptides maintain their values at around 10%.

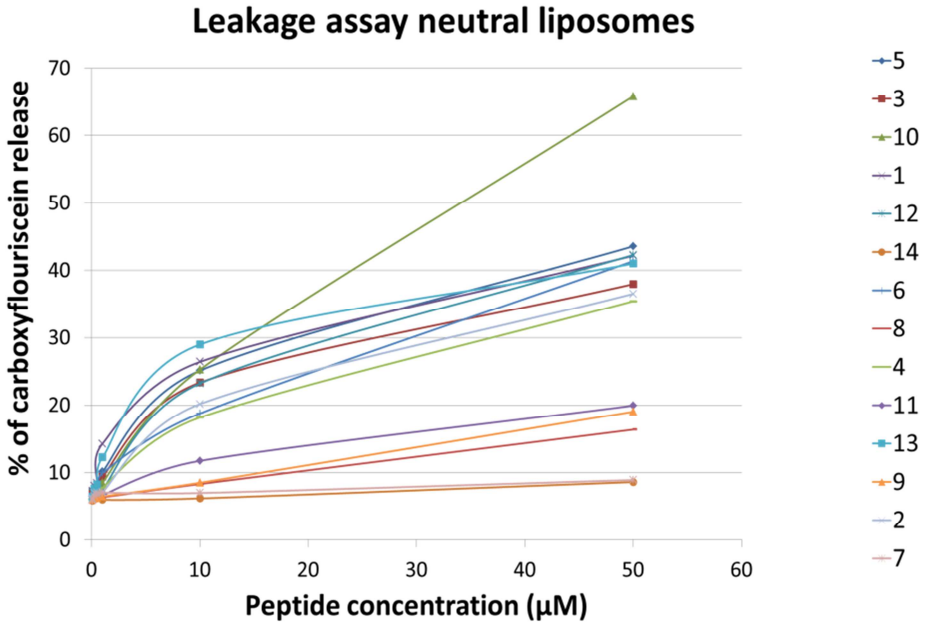


Figure 3.12. Percentatge of carboxyfluoriscein release of all the mastoparan analogs at different concentrations and using neutral liposomes.

The values observed for the neutral liposomes (EPC/Cholesterol)(5:1) are showed in figure 3.12. According to the results obtained they can be divided into four groups, the best compound was peptide **10**, with a 65% of carboxyfluorescein release at 50µM, the second group that was composed by peptides **13**, **1**, **5**, **12**, **3**, **2**, **6** and **5**, the percentage of release of all these peptides went from 44% to 36% at 50µM. The peptide **11** and both retro peptides, **8** and **9** belonged to the third group, the percentage of release were 20, 19 and 17% at 50µM respectively, and the last group with almost no activity observed neither at 10µM nor at 50µM with values less than 10%, these peptides were number **14** and **7**.

3.3.2. Transmission electron microscopy.

(Samples were prepared by Dr. Carmen López)

The effects of mastoparan and peptide **10** on the cell morphology of colistin-resistant *A. baumannii* were also investigated by transmission electron microscopy. Untreated cells of *A. baumannii* in LB medium showed a normal morphology with an undamaged structure of any of the membranes (figure 3.13). After 1 hour of incubation with mastoparan and the peptide **10** version (at MIC for each peptide), it was possible to observe that bacteria had a considerable damage by observing some fractures in the membrane in both bacteria incubated with mastoparan (figure 3.14) or incubated with peptide **10** (figure 3.15). We can not claimed with the information obtained in the TEM studies that the mechanism of action of this peptides is due to a membrane disruption or permeabilization but with the data obtained with the leakage experiments and also with all the information known about the mechanism of action of all the mastoparan peptides family, it seems that this is the mechanism in which the peptides might kill bacteria.

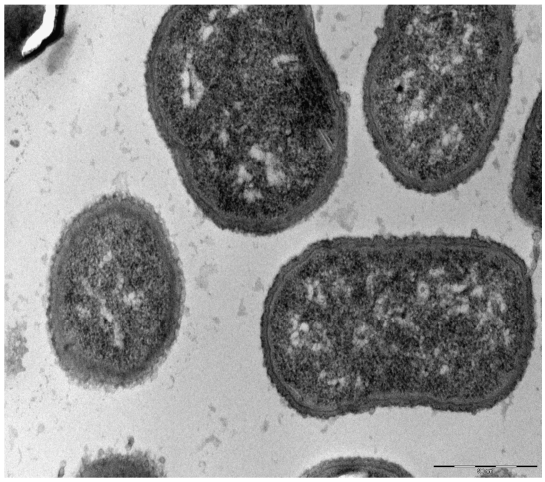


Figure 3.13. TEM of untreated colistin-resistant *A. baumannii* cells.

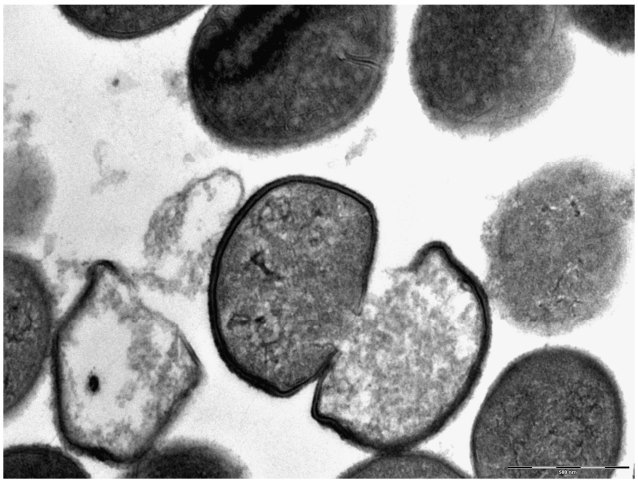


Figure 3.14. TEM of mastoparan incubated with colistin-resistant *A. baumannii* cells

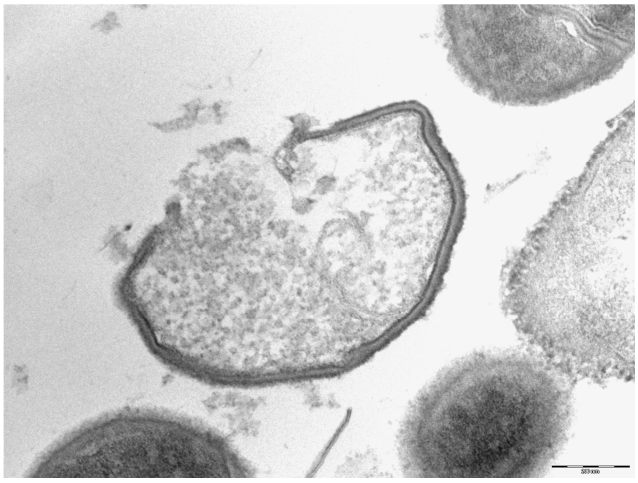


Figure 3.15. TEM of peptide 10 incubated with colistin-resistant *A. baumannii* cells

3.4. Frog-skin peptides against colistin-resistant *A. baumannii* species.

The approach used since now was to tested different commercial natural peptides, and the following optimization of the hit, however in this part of the chapter we are going to use a new approach by using a completely new peptides. In this case we tested six peptides isolated from frog-skin secretions, this work was done in collaboration with Prof. Conlon from United Arab Emirates University. The group of Prof. Conlon bought the crude secretions, and isolated the peptides by purifying it. The peptides isolated are showed in table 3.13.

Peptide	Primary structure	LC_{50} (µg/mL)
[G4K]XT-7	GLLKPLLKIAAKVGSNLL-NH_2	>1000
[E4K]alyteserin-1c	GLKEIFKAGLGSLVKGIAAHVAS-NH_2	>1000
PGLa-AM1	GMASKAGSVLGKVAKVALKAAL-NH_2	>1000
B2RP-Era	GVIKSVLKGVAKTVALGML-NH_2	560
CPF-AM1	GLGSVLGKALKIGANNLL-NH_2	300
[D4K]B2RP	GIWKTIKSMGKVFAGKILQNL-NH_2	250

LC_{50}. Mean concentration of peptide producing 50% haemolysis in three independent experiments.

Table.3.13. Primary structures and haemolytic activities (LC50) against human red blood cells of the frog skin-derived antimicrobial peptides used in this study.

Six peptides were isolated from different sources, CPF-AM1 and PGLa-AM1 are from *Xenopus amieti* (181), B2RP-ERa is from *Hylarana erythraea* (182), [E4K]alyteserin-1c is from *Alytes obstetricans* (183), [D4K]B2RP is from *Lithobates septentrionalis* (184) and [G4K]XT-7 is from *Silurana tropicalis* (185).

The first assays performed prior to be tested its antimicrobial activity was the hemolytic assay. In this assay it was possible to observe that three of the peptides had a low hemolytic activity, with a LC_{50} of more than 1000 µg/mL, the other three peptides, B2RP-ERa, CPF-AM1 and [D4K]B2RP had LC_{50} values of 560, 300 and 250 µg/mL respectively.

All six peptides were tested against both colistin-resistant and colistin-susceptible *Acinetobacter* species strains. The colistin-susceptible *Acinetobacter* strains used were the ATCC 19606, one strain of *Acinetobacter nosocomialis* and six strains of *A. baumannii* clinical isolates each one with different features. For colistin-resistant strains, three lab generated mutants (two *A. baumannii* and one *A. nosocomialis*), the other three strains used colistin-resistant were *A. baumannii* clinical isolates. The results of the MIC assays are showed in table 3.14 and table 3.15.

Peptide	***Acinetobacter* sp.Strains**							
mg/L (μM)	**19606**	**77778**	**G13**	**NM8**	**NM35**	**NM75**	**NM109**	**NM124**
[G4K]XT-7	32(17.3)	32(17.3)	4(2.2)	32(17.3)	32(17.3)	32(17.3)	16(8.7)	16(8.7)
[E4K]alyteserin-1c	8(3.5)	8(3.5)	8(3.5)	8(3.5)	16(7)	8(3.5)	4(1.8)	4(1.8)
PGLa-AM1	64(30.9)	64(30.9)	128(61.9)	16(7.7)	16(7.7)	16(7.7)	16(7.7)	16(7.7)
B2RP-Era	32(17)	32(17)	8(4.2)	32(17)	32(17)	16(8.5)	32(17)	32(17)
CPF-AM1	64(39.5)	64(39.5)	128(78.9)	16(9.9)	16(9.9)	16(9.9)	16(9.9)	16(9.9)
[D4K]B2RP	16(6.9)	8(3.4)	8(3.4)	8(3.4)	8(3.4)	8(3.4)	4(1.7)	4(1.7)
Colistin	0.5(0.43)	2(1.7)	<0.5(<0.43)	1(0.86)	1(0.86)	1(0.86)	<0.5(<0.43)	<0.5(<0.43)

Table 3.14. Minimum inhibitory concentrations (mg/L) of the frog skin-derived antimicrobial peptides against colistin-susceptible strains of *Acinetobacter baumannii* and *Acinetobacter nosocomialis*

Peptide	***Acinetobacter* sp.Strain**					
(mg/L) (μM)	**19606 mutant**	**77778 mutant**	**G13 mutant**	**Ab22P**	**Ab1**	**Ab113**
[G4K]XT-7	4(2.2.)	32(17.3)	4(2.2)	8(4.3)	32(17.3)	64(34.7)
[E4K]alyteserin-1c	4(1.8)	8(3.5)	8(3.5)	4(1.8)	8(3.5)	16(7)
PGLa-AM1	16(7.7)	64(30.9)	16(7.7)	16(7.7)	64(30.9)	128(61.9)
B2RP-ERa	8(4.2)	64(34)	8(4.2)	8(4.2)	32(17)	64(34)
CPF-AM1	4(2.5)	64(39.5)	8(4.9)	8(4.9)	64(39.5)	128(78.9)
[D4K]B2RP	4(1.7)	16(6.9)	16(6.9)	8(3.4)	16(6.9)	16(6.9)
Colistin	256 (221.6)	>256 (>221.6)	>256 (>221.6)	64(55.4)	>512 (>443.3)	256 (221.6)

Table 3.15. Minimum inhibitory concentrations (mg/L) of the frog skin-derived antimicrobial peptides against colistin-resistant strains of *Acinetobacter baumannii* and *Acinetobacter nosocomialis*.

Peptides [E4K]alyteserin-1c and [D4K]B2RP, were tested against multi-drug resistant *A. baumannii* previously (183, 184), and with these MIC assays performed confirmed that are the best two peptides with MICs between 4 and 16 mg/L, the rest of the peptides tested had higher MICs with doble digit values.

For colistin-resitant *Acinetobacter* strains, the values are similar or slightly better, some of the peptides that had high MICs for colistin-susceptible strains had good MIC values in colistin-resistant strains, however the best peptides were still the same, [E4K]alyteserin-1c and [D4K]B2RP, and the MIC values for both were also between 4 and 16 mg/L.

3.5. Active peptidomimetics against colistin-resistant *Acinetobacter baumanii*

This work have been done in collaboration with professor Paul Savage from (Brigham Young University).

3.5.1. Ceragenines used in the experiments

Ceragenines or also called CSA (Cationic steroid antibiotics), are antimicrobial compounds derived from the original structure of cholic acid. As shown in the figure 3.16, cholic acid has a large planar structure with a highly hydrophobic face (upper face in the figure); a more hydrophilic face (lower face in the figure) containing three polar hydroxyl groups; and a highly polar charged tail (a negatively charged carboxylate ion at physiological pH). The anphipaticity of the cholic acid molecule is even increased in the structure of ceragenins (figure 3.17), that keep the hydrophobic face of cholic acid and contain several cationic groups (at physiological pH) in the hydrophilic face. Ceragenins have no peptide bonds but due to this amphipathic structure, similar to that of several antimicrobial peptides, are classed as peptidomimetics.

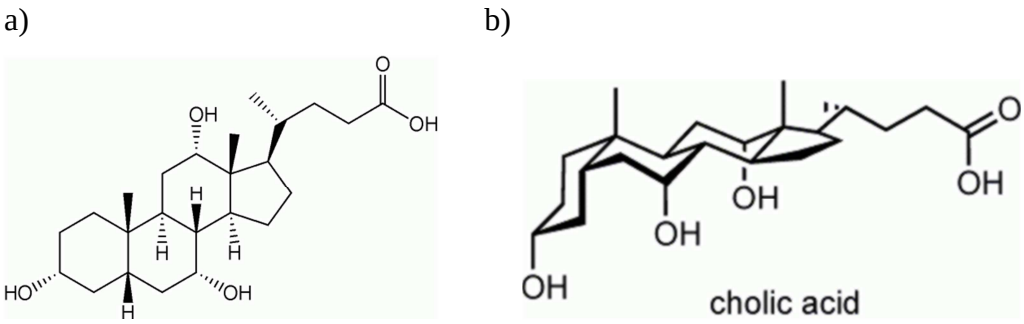
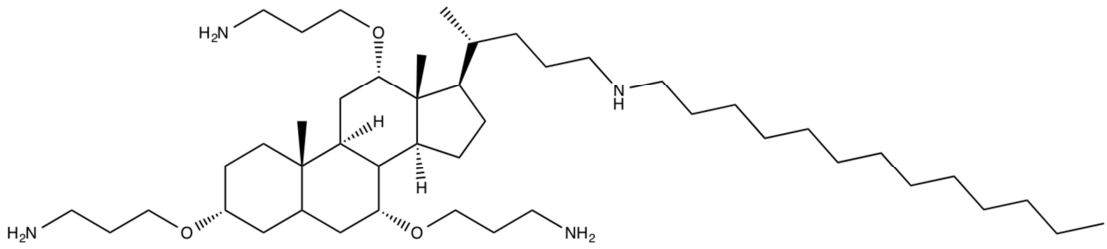
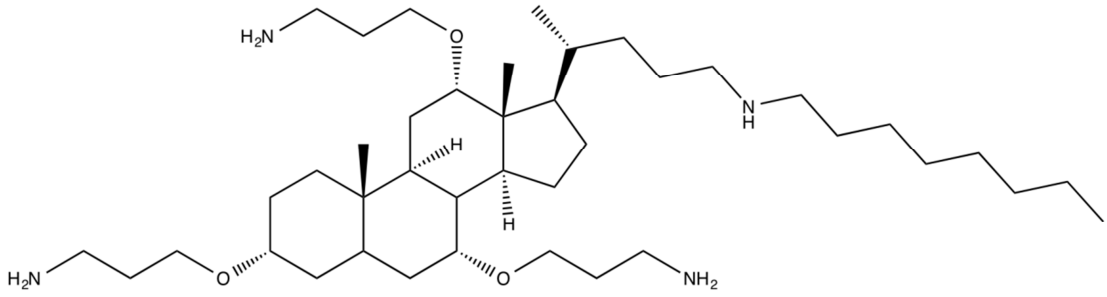


Figure 3.16. a) planar representation of cholic acid. b) chair representation of cholic acid

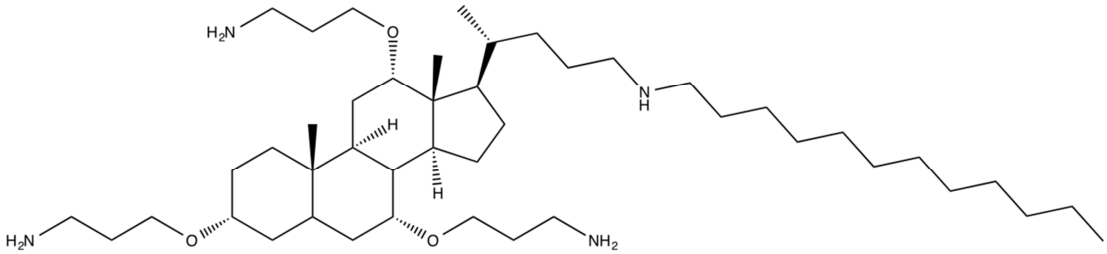
Ceragenins are promising compounds that could supersede antimicrobial peptides in terms of metabolic stability or toxicity. In our case, the peptidomimetics used for the experiments were supplied by Professor Paul Savage, and the structures of these compounds are showed in figure 3.17



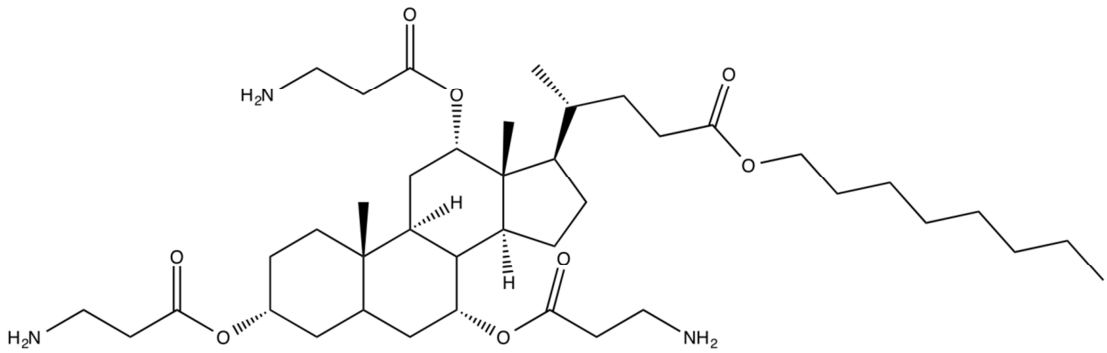
CSA-138



CSA-13



CSA-131



CSA-44

Figure 3.17. Chemical structure of ceragenins used.

The modification of the cholic acid for CSA-13, CSA-131 and CSA-138 in the hydrophilic face was by converting the three hydroxyl groups into three aminopropyl ethers (positively charged at physiological pH). For CSA-44 the modification introduced to cholic acid is an aminopropyl esther. The highly polar charged tail containing a carboxylate, was modified to obtain a secondary amine that was substituted by octyl, dodecyl and tridecyl in CSA-13, CSA-131 and CSA-138, respectively. The modification of the carboxylate group of the CSA-44 was performed introducing an octyl esther.

3.5.2. *In vitro* activity of Ceragenins against colistin-resistant and colistin-susceptible *A. baumannii, K. pneumoniae* and *P. aeruginosa*.

The initial aim was to test these ceragenins against colistin-resistant *A. baumannii*, however in order to know if these compounds were able to be active against several colistin-resistant bacteria, were also tested against both colistin-resistant *K. pneumoniae* and *P. aeruginosa* strains. Results are showed in table 3.16

MIC (mg/L) [μM]	*A. baumannii*		*K. pneumoniae*		*P .aeruginosa*	
ceragenins	**Col-S**	**col-R**	**col-S**	**col-R**	**col-S**	**col-R**
138	2 [2.7]	4[5.4]	16[21.4]	16[21.4]	1[1.3]	1[1.3]
13	4[5.9]	4[5.9]	8[11.8]	16[23.6]	<0,5[<0.7]	<0,5[<0.7]
131	2[2.7]	2[2.7]	8[10.9]	8[10.9]	<0,5[<0.7]	<0,5[<0.7]
44	8[10.9]	4[5.4]	4[5.4]	16[21.8]	1[1.4]	1[1.4]

TableX.16. MIC of the ceragenins against colistin-susceptible and colistn-resistant *A. baumannii*, *K. pneumonia* and *P. aeruginosa* clinical isolates.

Four diferent ceragenins were tested against both colistin susceptible and resistant strains of *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*. In *A. baumannii*, CSA-131 showed the best results with a MIC of 2 mg/L for both strains. For CSA-138 the MIC of colistin-susceptible was also 2 mg/L and 4 mg/L for the colistin-resistant strain, which was the same value for CSA-13 in both strains. CSA-44 had a MIC of 4 mg/L for the colistin-resistant strain and 8 mg/L for the colistin-susceptible.

For *K.pneumoniae* the best results correspond to CSA-131 with a MIC of 8 mg/L against both colistin-susceptible and colistin-resistant *K. pneumoniae* strains, CSA-13 and CSA-44 had the same values, with 8 mg/L and 16 mg/L for colistin-susceptible and colistin-resistant respectively. CSA-138 showed 16 mg/L for both strains.
CSA-131 and CSA-13 showed the best results for *P. aeruginosa* showing a MIC less than 0.5 mg/L for both colistin-susceptible and resistant. The MIC of CSA-138 and CSA-44 was slightly higher being 1 mg/L.

All the ceragenines had a good activity against all the strains, specially interesting was the activity against *P. aeruginosa*. It was also important to point out that ceragenins had a good activity independently of the grade of resistance to colistin of the strain, having approximately the same MIC in both strains, specially in *A. baumannii* and *P. aeruginosa*.

3.5.3. *In vitro* activity of ceragenins against a collection of *A. baumannii* and *P. aeruginosa*.

The best results were obtained when working with *A. baumannii* and *P. aeruginosa*, although the results against *K. pneumoniae* were relatively good, we discarted it due to its doble digit values in some MICs.
As it was done in the initial screening of the antimicrobial peptides, MIC assays were performed using more strains, all of them were colistin-susceptible, however the results of both colistin-resistant and colistin-susceptible are similar. The reuslts are showed in Table 3.17

MIC (mg/L) [μM]	***A. baumannii* (15)**		***P. aeruginosa* (15)**	
ceragenins	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**
138	2[2.7]	4[5.4]	2[2.7]	4[5.4]
13	2[2.9]	8[11.8]	4[5.9]	4[5.9]
131	2[2.7]	2[2.7]	1[1.4]	2[2.7]
44	4[5.4]	8[10.9]	4[5.4]	8[10.9]

Table 3.17. MIC_{50} and MIC_{90} of the ceragenins against colistin-susceptible and colistin-resistant *A. baumannii*, and *P. aeruginosa* clinical isolates

A collection of 15 diferents clinical isolates were tested against CSA-131, CSA-13, CSA-44 and CSA-138. In *A. baumannii*, CSA-13, CSA-131 and CSA-138 showed a MIC_{50} of 2 mg/L, and one dilution higher for CSA-44 with 4 mg/L. The MIC_{90} of CSA-131 and CSA-138 maintain the same value, 2 mg/L but for CSA-13 and CSA-138 increased up to 8 mg/L.

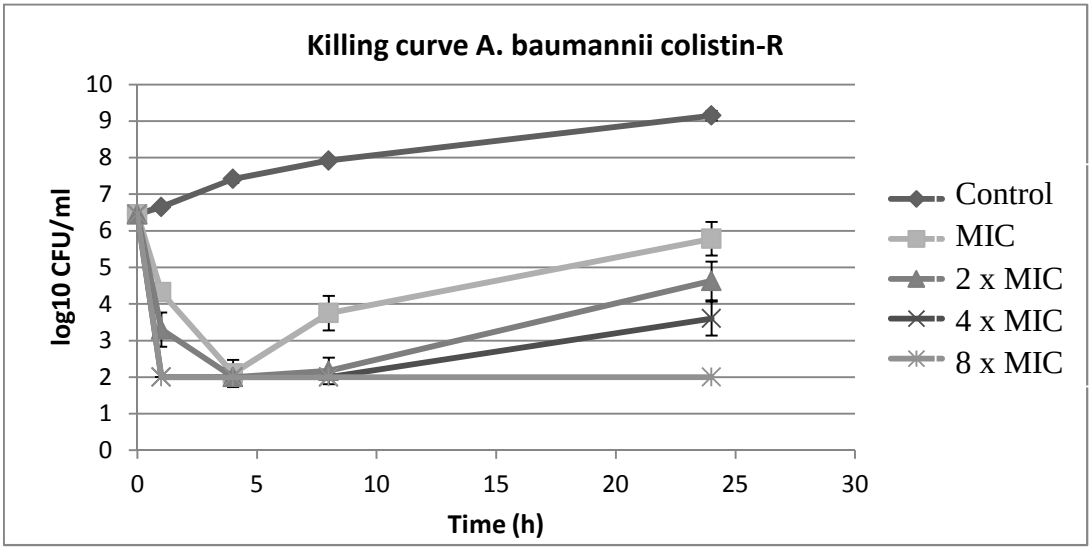
In the case of *P. aeruginosa*, the best results belonged to CSA-131 showing 1 mg/L and 2 mg/L for MIC_{50} and MIC_{90} respectively. The results for CSA-138 were 2 mg/L as MIC_{50} and 4 mg/L as MIC_{90}. Both CSA-13 and CSA-44 showed the same MIC_{50} of 4 mg/L, however in the MIC_{90} CSA-44 increased up to 8 mg/L while CSA-13 stayed with the same value.

The results obtained reaffirm the good activity of these compounds against *A. baumannii* and *P. aeruginosa*, highlighting CSA-131 as the best compound with lower MICs against all the strains.

3.5.4. Time-killing curves experiments.

Time-killing curves were performed for CSA-131 in order to observe the behaviour of this ceragenin against *A. baumannii* and *P. aeruginosa*. The results are showed in Figure 3.18.

a)



b)

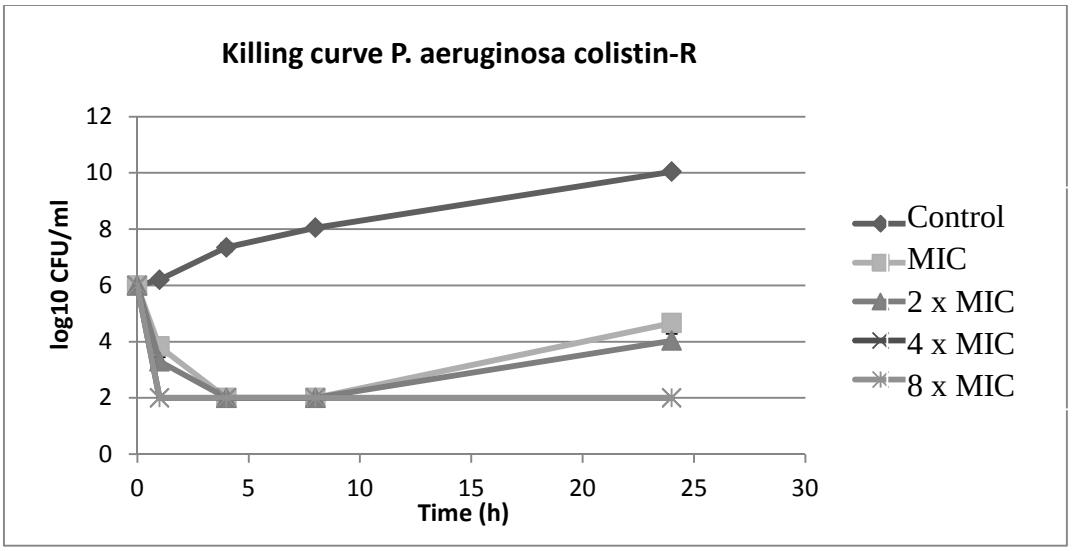


Figure 3.18. Results of time-killing experiments. a) colistin-resistant *A. baumannii* incubated with CSA-131. b) colistin-resistant *P. aeruginosa* incubated with CSA-131.

For *P. aeruginosa* both 4xMIC and 8xMIC were bactericidal throughout the curve, however MIC and 2xMIC were bactericidal only at 4 and 8h and observing a regrowth at 24h.

In the case of *A. baumannii*, only 8xMIC was bactericidal along the curve, both 2xMIC and 4xMIC were bactericidal at 1, 4 and 8h and a regrowth was observed at 24h, for the MIC only bactericidal activity was observed at 4h.

During these chapter, three different approaches have been presented. In the first approach, focused on mastoparan and analogs, the results obtained were not satisfactory, although alternatives are suggested in order to achieve better in vivo activities. Natural peptides tested from frog-skin secretions have shown good antimicrobial activity, however due to its sequence it might be a very susceptible peptide for the action of proteases and peptidases, so further optimization should be performed prior to in vivo studies. Finally, the ceragenins, due to its structure, should avoid all the problems commented for the frog-skin secretions peptides, and should be good candidates to be active in vivo.

Chapter 4

Development of hexacyclic peptides as ompA inhibitors

Context

OmpA is an important virulence factor, and is one of the most abundant proteins in the cell wall. OmpA is the most important protein involved in the adherence to eukaryotic cells. Together with the group of Dr. Jeromino Pachón we set up a project in which the approach was different from the typical antimicrobial agent discovery. In this case the idea was to find out a compound able to inhibit the adherence between ompA and the epithelial cells. Therefore, we design a cyclic peptide able to block the activity of the OmpA.

4.1. Protein homology, and virtual screening of EXORIS library

The initial hypothesis was to synthesize compounds able to block the adherence effect of ompA to the epithelial cells. OmpA is a highly conserved protein in most of the Gram-negative bacteria. This 325-residue protein was thought to contain two domains. The classic N-terminal domain, consisting of 171 amino acid residues, was shown to cross the membrane eight times in antiparallel β-strands with four relatively large and hydrophilic surface-exposed loops and short periplasmic turns. The C-terminal domain is located in the periplasm, and binds to the peptidoglycan thus connecting it to the outer membrane In the PDB (Protein Data Bank), some of the structures are reported, two from X-ray and other two from NMR, but all of them belong to *E. coli*. figure 4.1

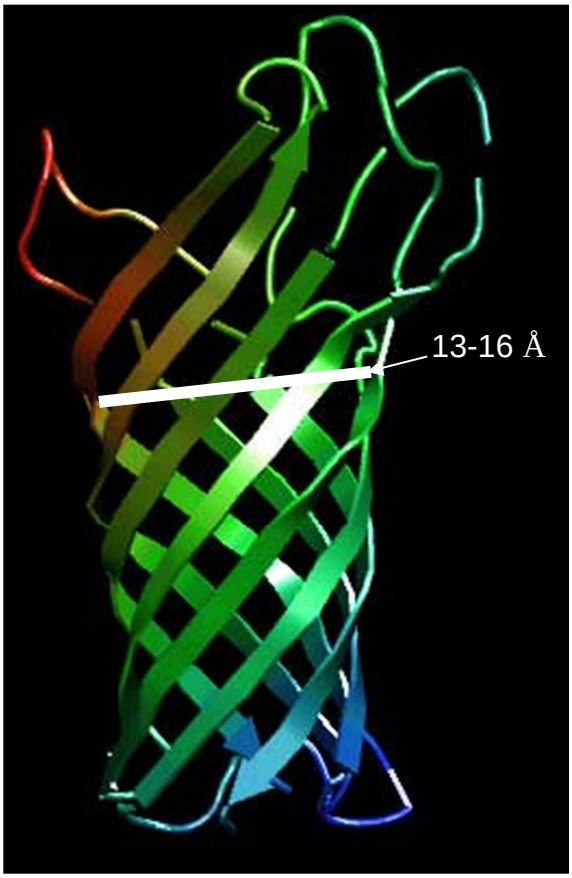


Figure 4.1. Chemical structure of OmpA.

The size of the pore of the trasmembrane protein is between 13 to 16 Å, this size fits quite good with the hexacyclic peptide library present in our lab called EXORIS. EXORIS library is a hexacyclic simetric peptide library containing all types of amino acids, but always with two prolines in it. EXORIS library cover all types of properties, polar, none polar, positively and negatively charged, aromatic.

After analyzing all the data from the PDB related to ompA, no structure of *A. baumannii* was found, however, and taking into account the high homology of ompA in all the Gram-negative bacteria, an homology model taking *E. coli* structure as a template was performed using I-TASSER program. Once the protein was obtained virtual screening of the EXORIS against the new ompA protein was launched. The results obtained are showed in the table. 4.1, and in the figure 4.2 to 4.8.

Cyclic peptide	Docking score
&Arg-D-Pro-Trp-Arg-D-Pro-Trp&	-10.8
&D-Trp-Pro-Arg-D-Trp-Pro-Arg&	-10.6
&Arg-Pro-Trp-Arg-Pro-Trp&	-10.3
&Trp-Pro-Arg-Trp-Pro-Arg&	-10.0
&Trp-Pro-D-Arg-Trp-Pro-D-Arg&	-9.2
&Arg-Pro-D-Trp-Arg-Pro-D-Trp&	-8.9
&Arg-D-Pro-D-Ile-Arg-D-Pro-Ile&	-8.5
&Trp-D-Pro-Arg-Trp-D-Pro-Arg&	-8.5
&Arg-D-Pro-Ile-Arg-D-Pro-Ile&	-8.2
&Ser-D-Pro-Trp-Ser-D-Pro-Trp&	-7.5
&Ser-D-Pro-D-Trp-Ser-D-Pro-Trp&	-7.4
&Ser-D-Pro-D-Ile-Ser-D-Pro-Ile&	-7.3
&Ser-D-Pro-Ile-Ser-D-Pro-Ile&	-7.2
&D-Ser-Pro-Ala-D-Ser-Pro-Ala&	-7.1
&Glu-D-Pro-Trp-Glu-D-Pro-Trp&	-7.0
&Ile-D-Pro-D-Trp-Ile-D-Pro-Trp&	-6.8
&Ser-D-Pro-Ala-Ser-D-Pro-Ala&	-6.8
&Ile-D-Pro-Trp-Ile-D-Pro-Trp&	-6.7
&D-Arg-Pro-Trp-D-Arg-Pro-Trp&	-6.1
&Ser-D-Pro-Glu-Ser-D-Pro-Glu&	-6.1
&Glu-D-Pro-D-Trp-Glu-D-Pro-Trp&	-6.1
&Ser-D-Pro-D-Glu-Ser-D-Pro-Glu&	-6.1
&Ser-Pro-Ala-Ser-Pro-Ala&	-5.6
&Glu-D-Pro-Ile-Glu-D-Pro-Ile&	-4.6
&Glu-D-Pro-D-Ile-Glu-D-Pro-Ile&	-4.4
&Gly-Pro-Ala-Gly-Pro-Ala&	-2.9

Table 4.1. Docking score of all the peptides from the EXORIS library.

The information that it is possible to extract from the *in silico* experiments have to be taken into account very carefully, due to the fact that the protein on which the virtual screening was performed is just a protein obtained from the homology model. In spite of the problems commented previously, the results obtained were really interesting, it is important to point out that the best six compounds had the same amino acids (Pro/Arg/Trp). The only difference between each other is just a change in the sequence and the different stereochemistry present in each compound.

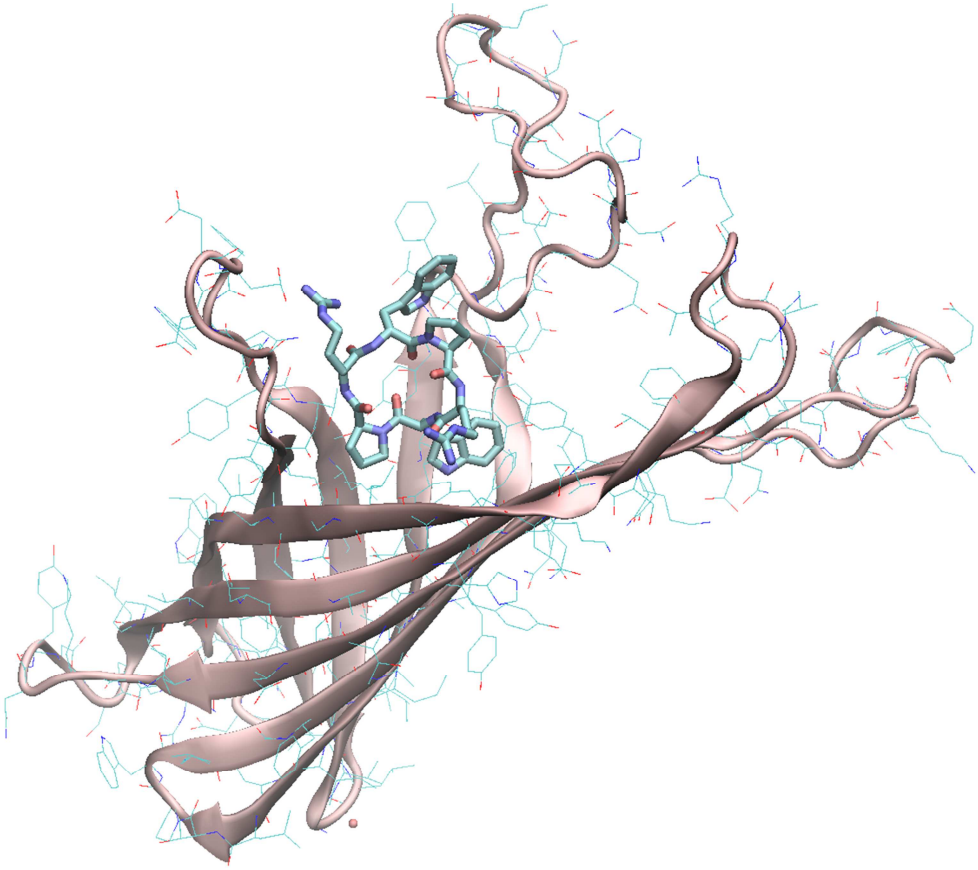


Figure 4.2 Different orientations and picture representations of a cyclic hexapeptide and ompA complex

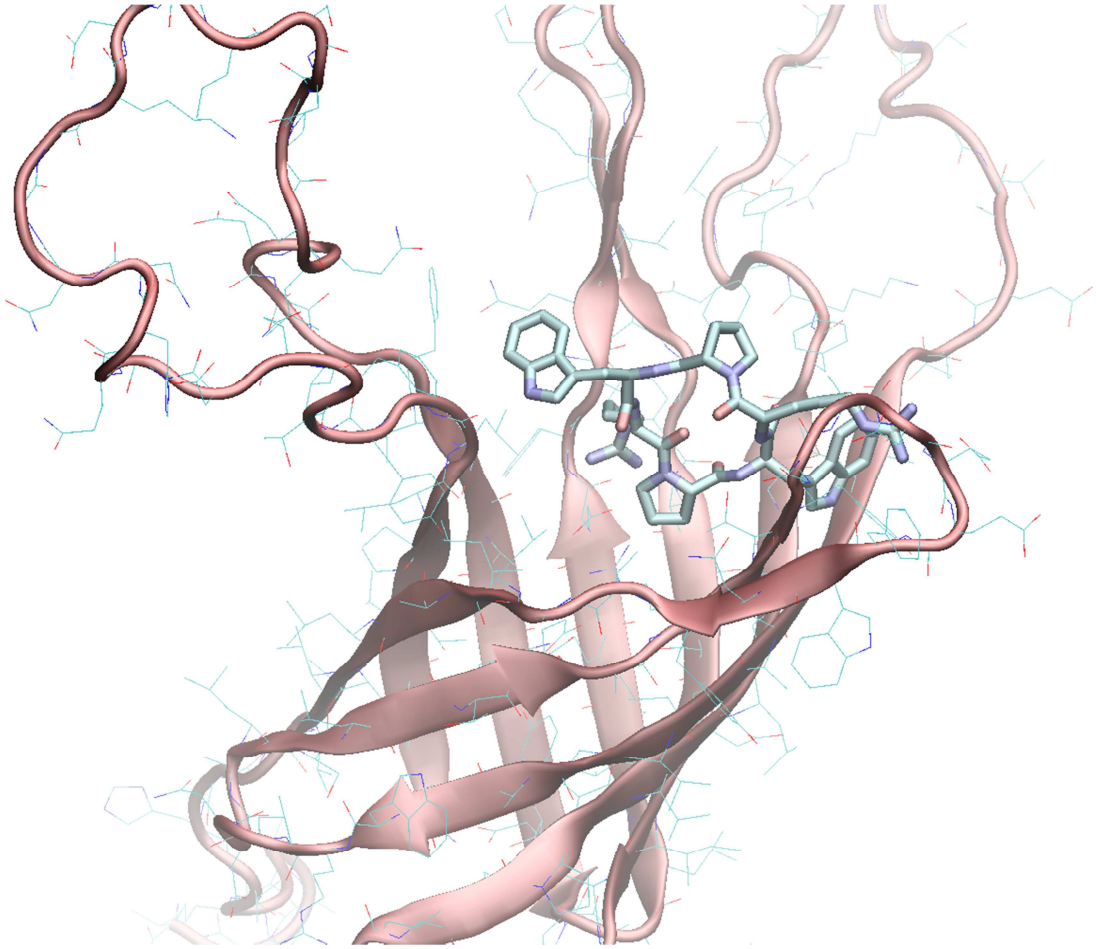


Figure 4.3 Different orientations and picture representations of a cyclic hexapeptide and ompA complex.

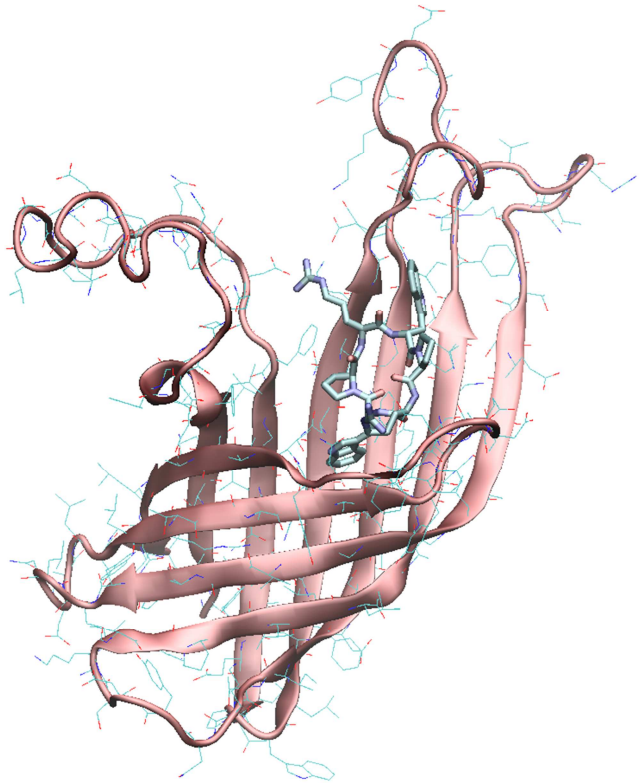


Figure 4.4 Different orientations and picture representations of a cyclic hexapeptide and ompA complex.

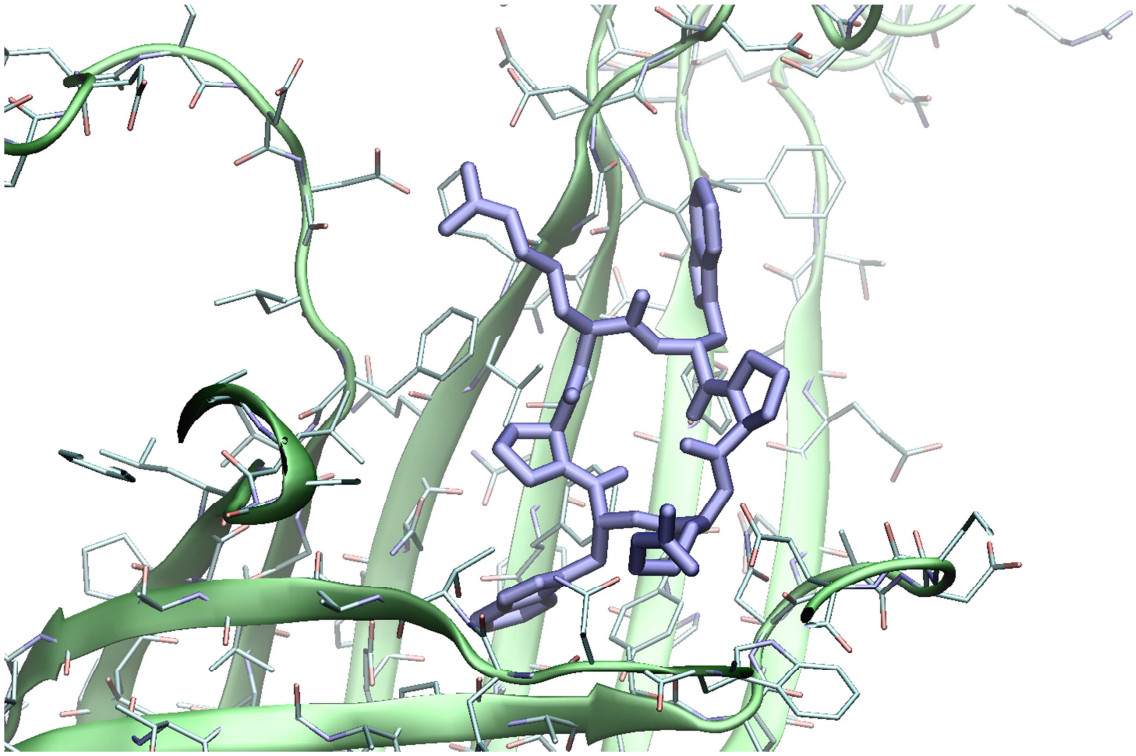


Figure 4.5 Different orientations and picture representations of a cyclic hexapeptide and ompA complex.

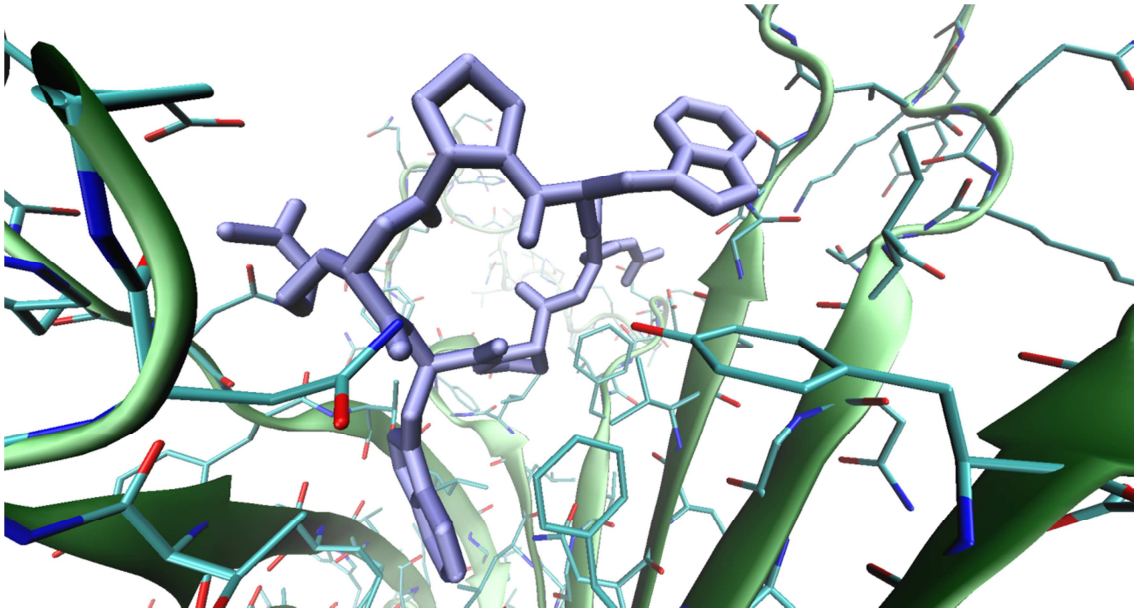


Figure 4.6 Different orientations and picture representations of a cyclic hexapeptide and ompA complex.

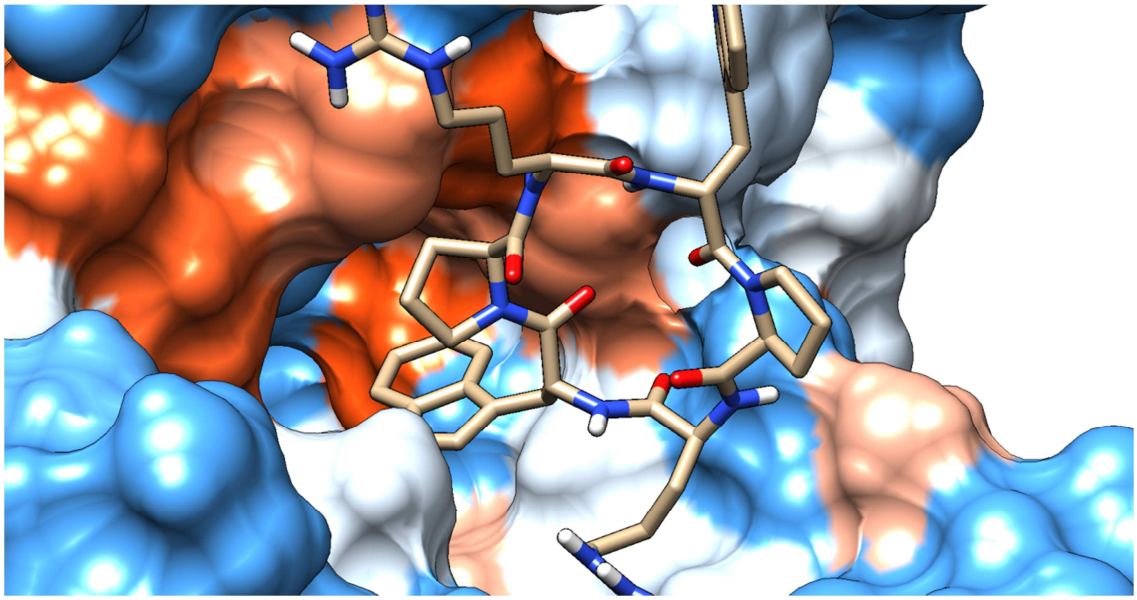


Figure 4.7 Different orientations and picture representations of a cyclic hexapeptide and ompA complex.

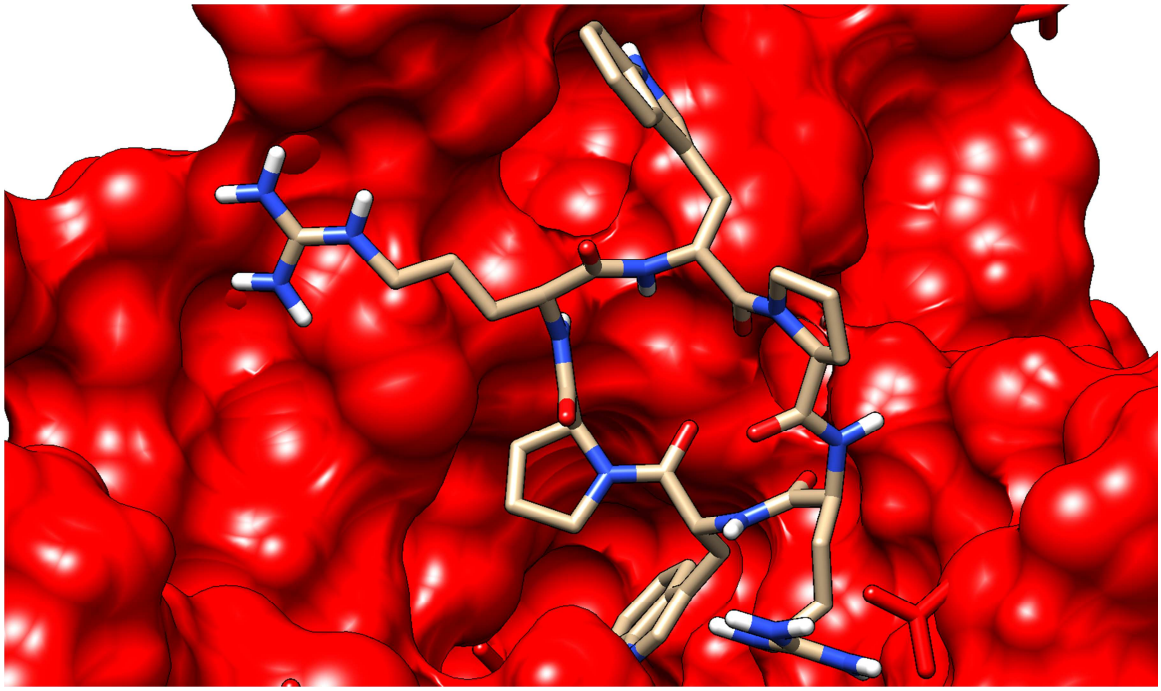


Figure 4.8 Different orientations and picture representations of a cyclic hexapeptide and ompA complex.

4.2. Synthesis of the hexacyclic peptides.

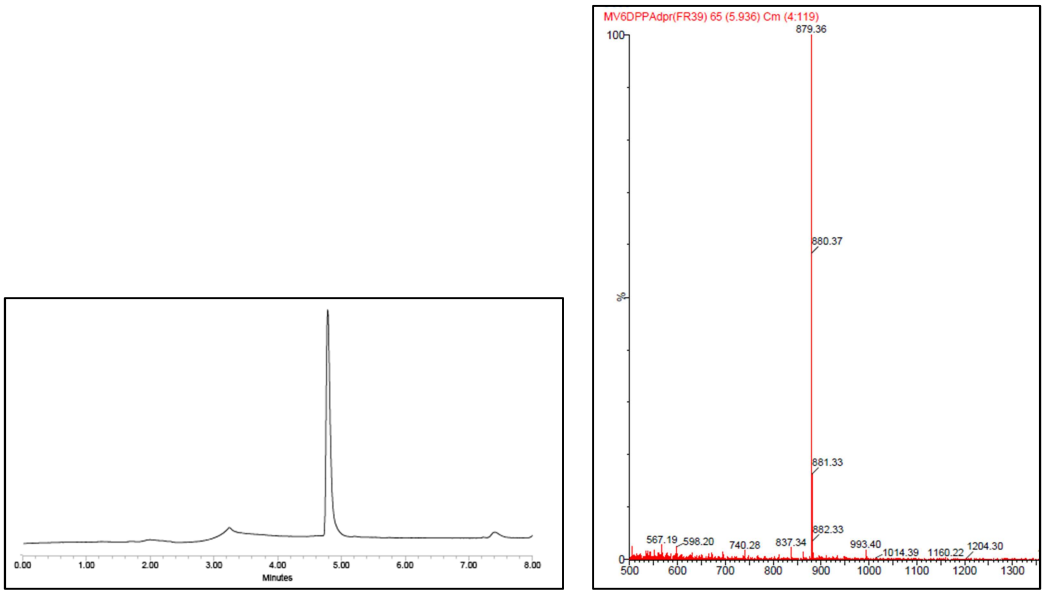
Several of the EXORIS library peptides were synthesized in order to be tested as ompA inhibitors. The peptides selected are showed in the table. 4.2.

n°	Cyclo peptide	Rational
MV6	&Arg-D-Pro-Trp-Arg-D-Pro-Trp&	Candidate (Trp,Arg)
MV5	&Trp-D-Pro-Arg-Trp-D-Pro-Arg&	Sequence change
MV3	&D-Arg-Pro-Trp-D-Arg-Pro-Trp&	Different stereosiomer
MV8	&Arg-Pro-D-Trp-Arg-Pro-D-Trp&	Different stereoisomer
MV9	&Ser-D-Pro-Trp-Ser-D-Pro-Trp&	Arg replaced by Ser
MV10	&Glu-D-Pro-Trp-Glu-D-Pro-Trp&	Arg replaced by Glu
NBA01011	&Ala-Ser-D-Pro-Ala-Ser-D-Pro&	No Trp
SXV4	Ac-Trp-D-Pro-Arg-Trp-D-Pro-Arg-OH	Negative control

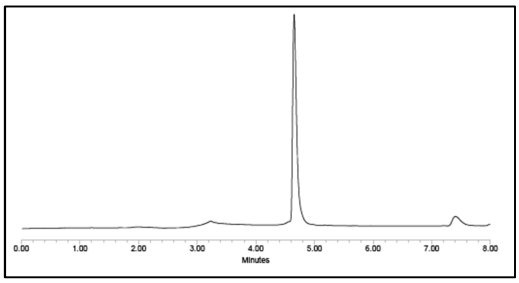
Table 4.2. Peptides selected from EXORIS library to be synthesized taking into account *in silico* results. & symbol means cyclic peptides.

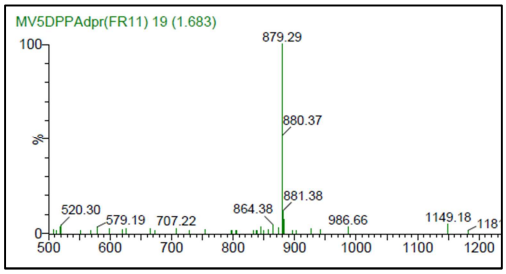
The peptides were synthesized using SPPS, the resin used was the 2-Chlorotrityl chloride. The procedure used is explained in material and methods section. In the Figure 4.9 it is possible to observe the HPLC and MALDI-TOF spectra of all the peptides synthesized.

MV6 (&Arg-D-Pro-Trp-Arg-D-Pro-Trp&)

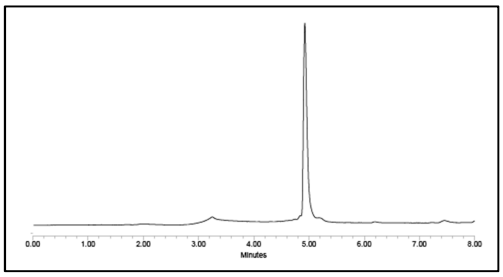


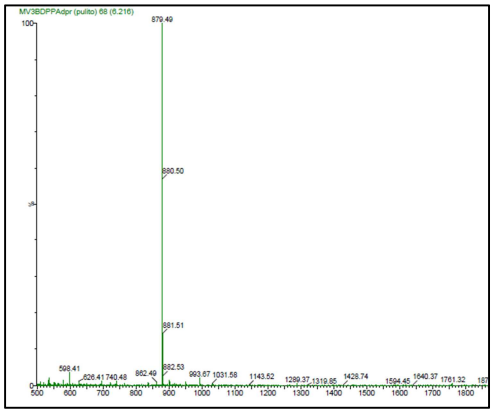
MV5 (&Trp-D-Pro-Arg-Trp-D-Pro-Arg&)



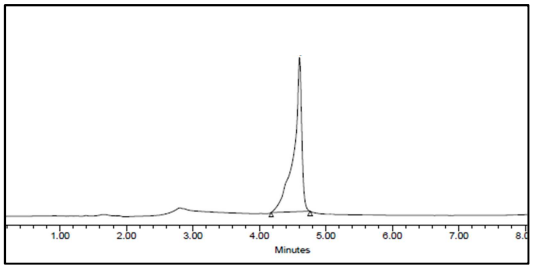


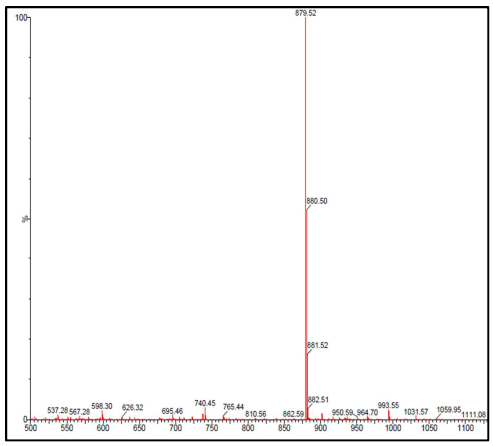
MV3 (&D-Arg-Pro-Trp-D-Arg-Pro-Trp&)



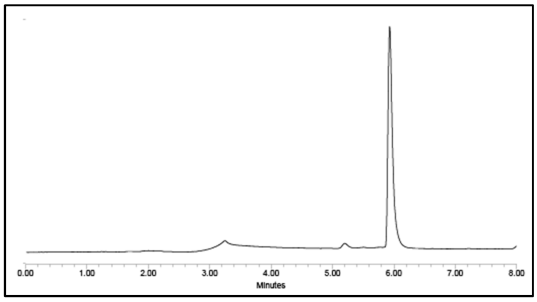


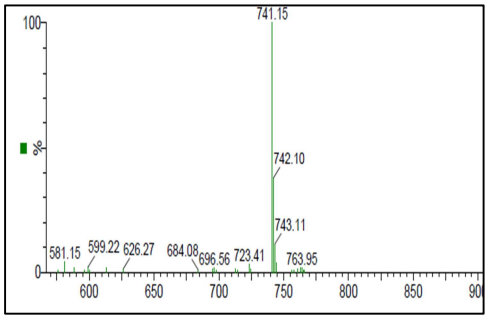
MV8 (&Arg-Pro-D-Trp-Arg-Pro-D-Trp&)



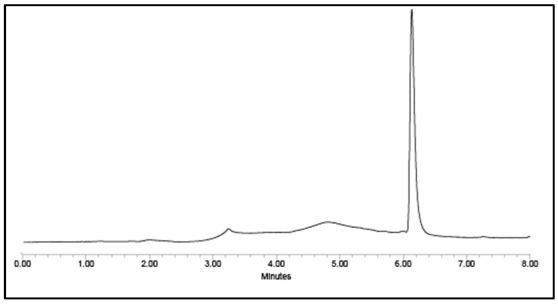


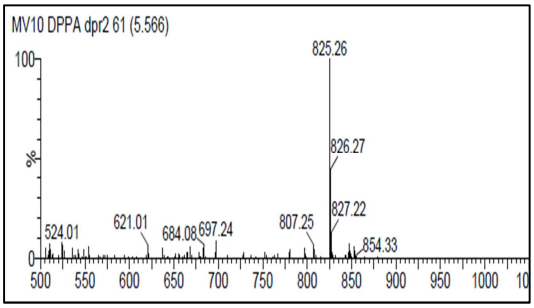
MV9 (&Ser-D-Pro-Trp-Ser-D-Pro-Trp&)



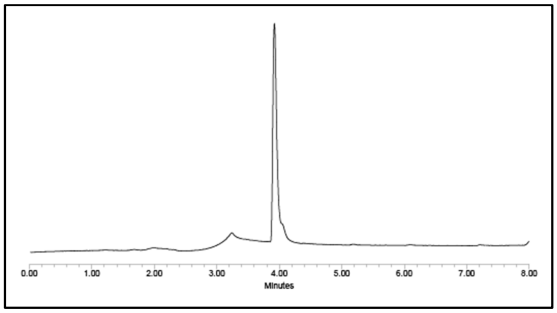


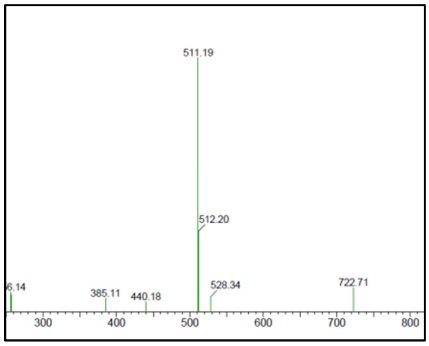
MV10 (&Glu-D-Pro-Trp-Glu-D-Pro-Trp&)



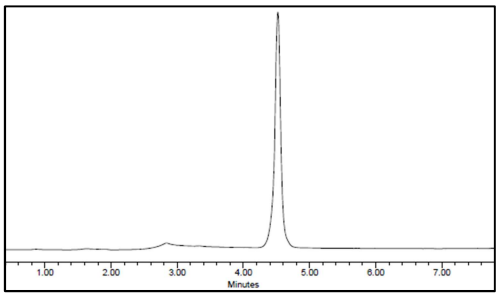


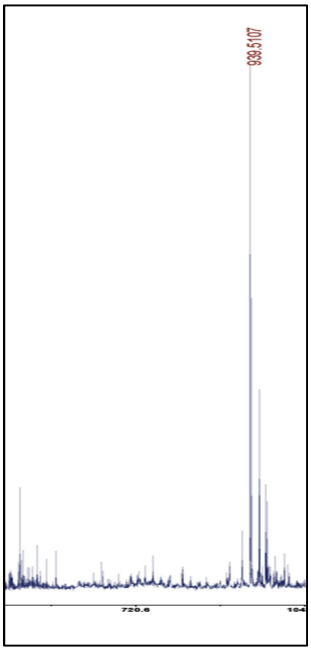
NBA01011 (&Ala-Ser-D-Pro-Ala-Ser-D-Pro&)





SXV4 (Ac-Trp-D-Pro-Arg-Trp-D-Pro-Arg-OH)





4.3. *In vitro* evaluation of the peptides.

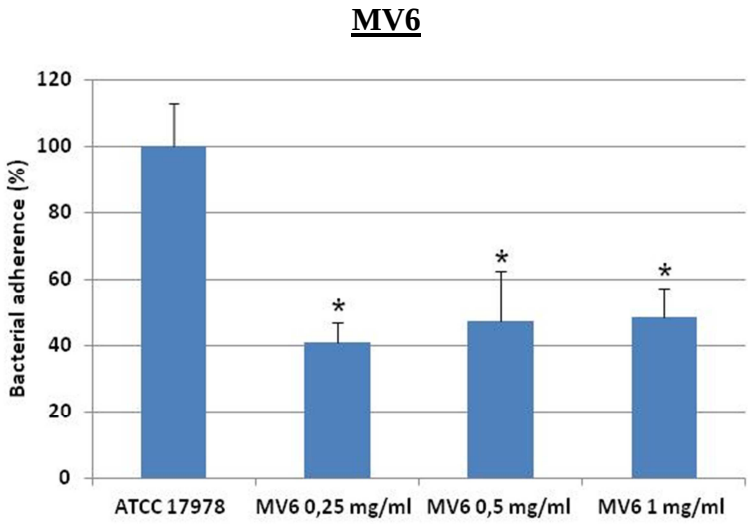
(This worked was performed by Dr. Smani in the IBIS from the Hospital Virgen del Rocío, Sevilla).

The aim of developing these compounds, as previously mentioned, was to inhibit the adherence between bacteria and eukaryotic cells, therefore two important features are necessary for these compounds, these are: i. not showing antibacterial activity by itself and ii. no cytotoxic activity. The first assays performed were the MTT assays in order to calculate the rate of toxicity of these peptides and the MIC against an *A. baumannii* strain, the results are showed in the table 4.3.

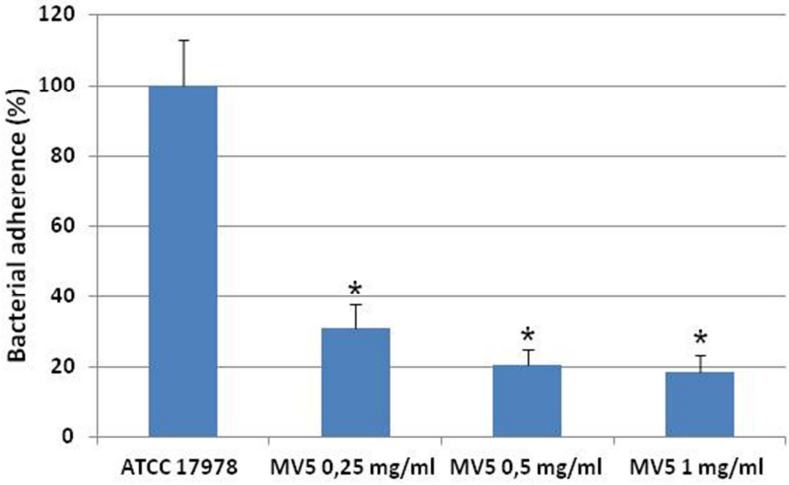
	Cell viability (%)			MIC (mg/L)
	Peptide concentrations			
Peptide	0,25 mg/mL	0,5 mg/mL	1 mg/mL	ATCC 17978
MV6	98,89±0,41	99,69±0,26	99,70±0,38	>500
MV5	98,09±0,27	98,33±0,40	98,48±0,55	>500
MV3B	99,47±0,39	99,77±0,17	100,13±0,18	>500
MV8	99,27±0,21	99,7±0,14	99,36±0,3	>500
MV9	98,86±0,08	100,05±0,39	98,89±0,43	>500
MV10	98,31±0,35	99,14±0,44	99,38±0,34	>500
NBA010011	98,17±0,60	98,79±0,28	98,55±0,21	>500
SXV4	98,9±0,45	99,51±0,26	100,02±0,37	>500

Table 4.3. Percentage of cell viability at different peptide concentrations and MIC of the different peptides.

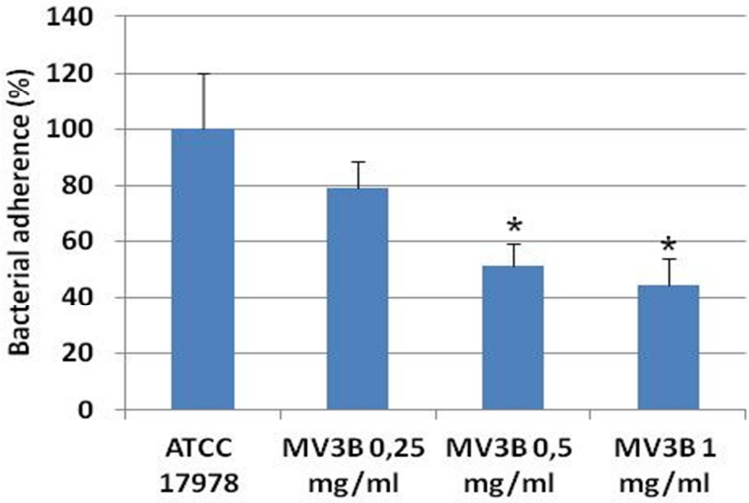
As it is possible to observe in the table above, any of the peptides tested showed antibacterial nor cytotoxic activities at the concentrations tested, therefore the next step was to test the ability of these peptides to block the interaction between eukaryotic cells and bacteria. The results obtained are showed in the Figure. 4.10



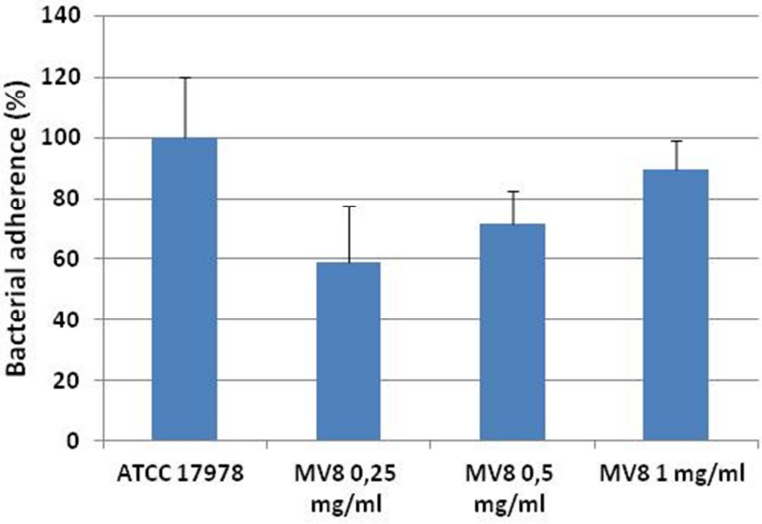
MV5


Bacterial adherence (%)
120
100
80
60
40
20
0
*
*
*
ATCC 17978
MV5 0,25 mg/ml
MV5 0,5 mg/ml
MV5 1 mg/ml

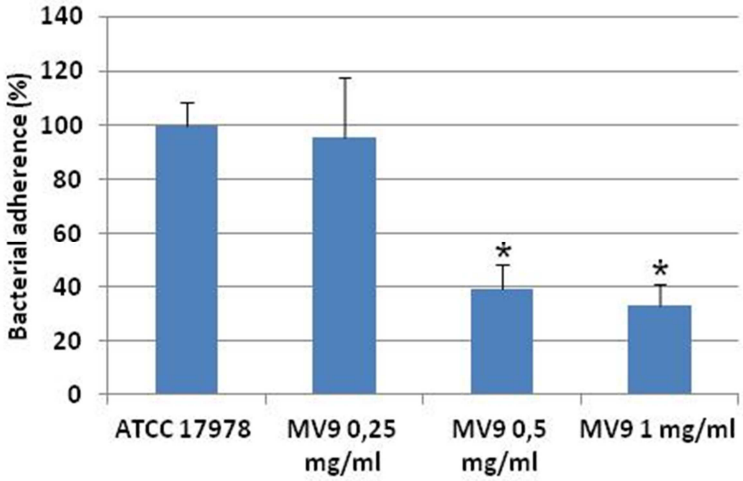
MV3B


Bacterial adherence (%)
140
120
100
80
60
40
20
0
*
*
ATCC 17978
MV3B 0,25 mg/ml
MV3B 0,5 mg/ml
MV3B 1 mg/ml

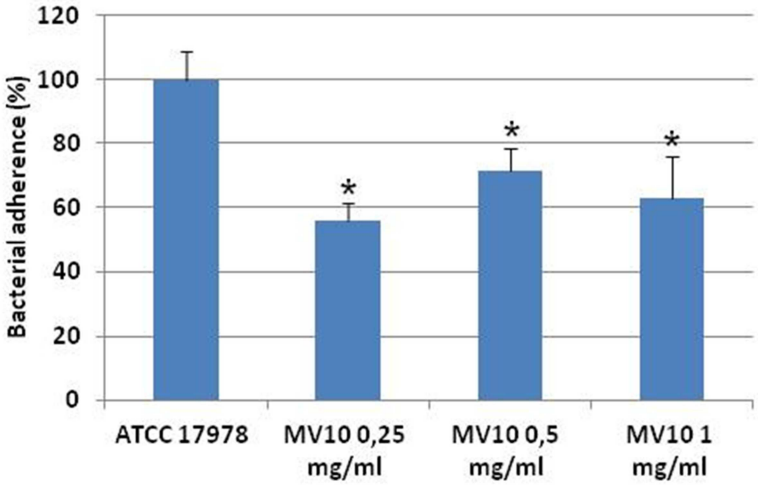
MV8


Bacterial adherence (%)
140
120
100
80
60
40
20
0
ATCC 17978
MV8 0,25 mg/ml
MV8 0,5 mg/ml
MV8 1 mg/ml

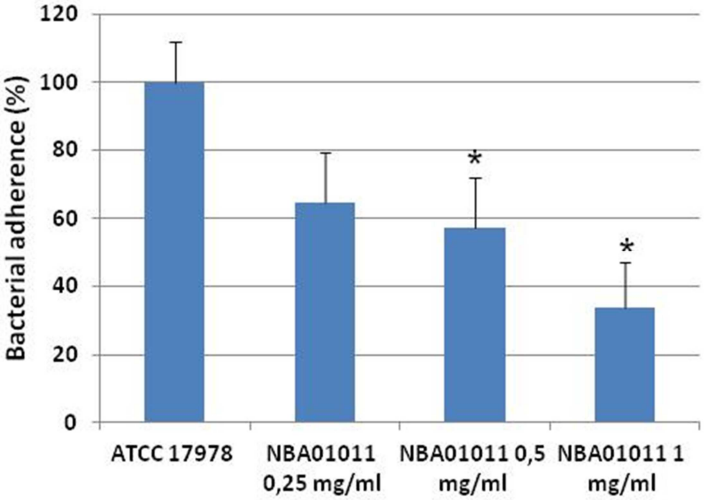
MV9



MV10



NBA01011



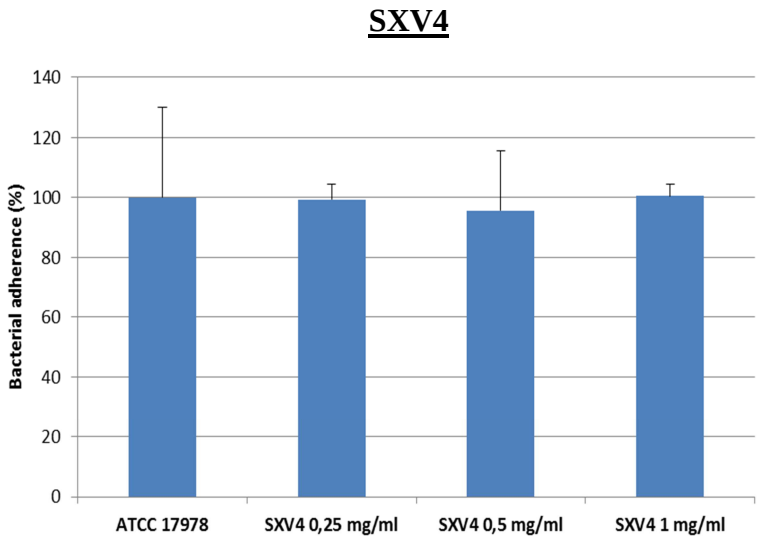


Figure 4.10. Percentatge of bacterial adherence at different peptide concentrations in different peptides. *p<0.05, treatment vs ATCC17978.

Analyzing the results of adherence, it was possible to observe that the best compound in the docking experiments (MV6) decrease the adherence around 60% in all the three concentrations tested, and with just changing the sequence for an Arg-Trp (MV5) instead of Trp-Arg the adherence decreases to 20 % at 1 mg/mL, and for this peptide the percentage of adherence was depending on the peptide concentration.

If we move the D-stereochemistry to the Arg (MV3B) compared to the best *in silico* compound (MV6), the activity was similar compared to MV6, with a 40 % of adherence at the highest concentration (1 mg/mL), the activity of this peptide was also concentration dependent. Next stereochemistry change was moving the D-stereochemistry to the Trp, the activity (MV8) was worse, and surprisingly the most active concentration is the lowest (0.25 mg/mL), in this case the activity of the peptide is reverse to the peptide concentration. The next two peptides are different than the ones tested previously, in this case the Arg was replaced by a Ser (MV9) and a Glu (MV10) in order to have an idea of the behavior of hexacyclic peptides containing other type of amino acids. The results for MV9 were peptide concentration dependent and the best result was achieved in the highest concentration used (1 mg/mL) with around 30 % of bacterial adherence, for the peptide with the Glu the results were worse with a bacterial adherence of around 60 % in all the three concentrations tested. Peptide NBA01011 was tested in order to check the behavior of the cyclic peptide without neither Arg nor Trp,

in this case Ala and Ser were used, and surprisingly, some activity was observed with an inhibition of the 70 % of the adherence at 1 mg/mL.

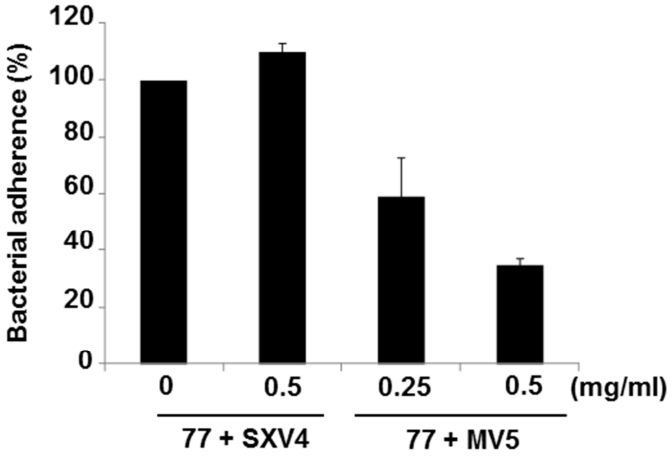
All the peptides, had at least some activity of inhibiting the adherence of bacteria to cells, however some demonstration that not all the peptides were able to bind was need, therefore a lineal peptide of the best *in vitro* compound MV5 was synthesized, with a carboxylic acid group as a C-terminal and acetylated in the N-terminal, and the results of this compound were the expected ones with no inhibition in the adherence (SXV4).

On comparing the data obtained in the docking experiments and in the *in vitro* assays it was possible to show that the initial *in silico* assays fitted really good with the results obtained, eventhough the protein used was not the real one, therefore after analyzing all the data MV5 was chosen as the candidate to continue performing all the assays initially planned.

A large quantity of this compound was synthesized following the same procedures as explained previously.

The inhibition of the bacterial adherence was just tested with a highly antibiotic susceptible *A. baumannii* strain (ATCC 17978), hence it was necessary to try the same procedure using a strain with highly resistant profile, therefore assays using a multi-drug resistant and pan-drug resistant *A. baumannii* strains were performed. The results are showed in figure 4.11.

a)



b)

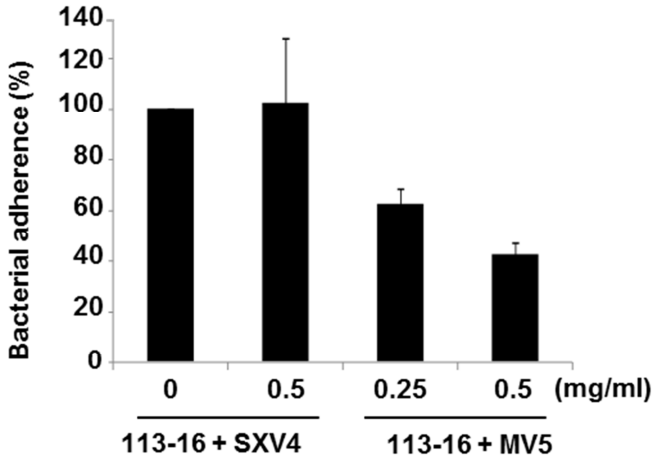


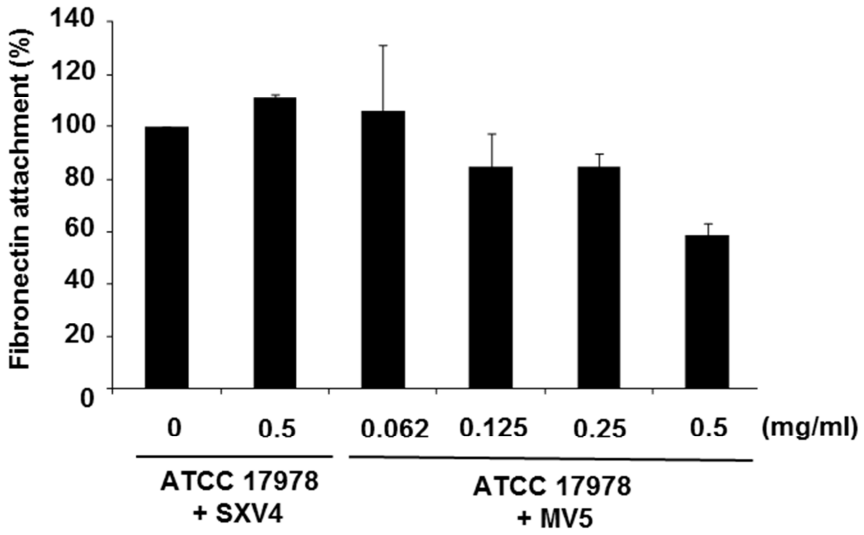
Figure 4.11. a) Percentage of inhibition assay between cells and a MDR at a different peptide concentrations. b) Percentage of inhibition assay between cells and a PDR at a different peptide concentrations.

The results for both strains were similar, however were slightly better as less resistant was the strain, thus it was possible to observe a range of activity starting with the ATCC 17978 with a 20 % of adherence at 0.5 mg/mL, for the same concentration the percentage of adherence in MDR strain increases up to 30 % and going further with a PDR strain, it doubles the percentage of bacterial adherence with 40 % at 0.5 mg/mL. Although the values increases when the resistance of the strain increases the percentage of inhibition was still really good, by inhibiting 60 % of adherence in a strain resistant to all the antibiotics.

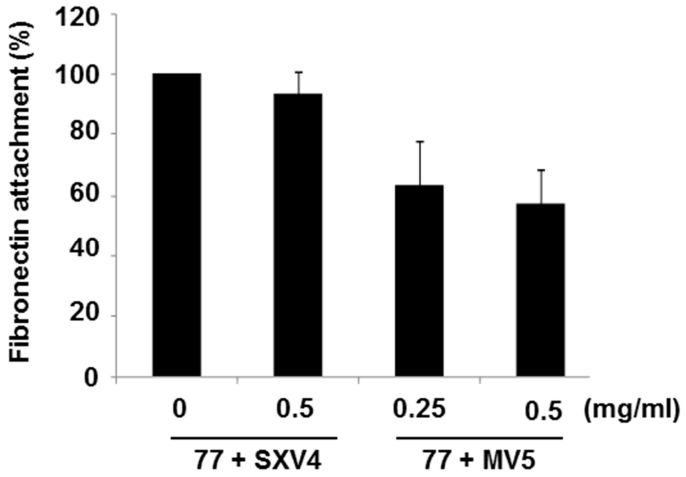
In the article published by Smani *et al*. (186), they demonstrate that the protein from the cells that was involved in the adherence to bacteria was fibronectin. Fibronectin is a high molecular weight protein (440 KDa) present in the extracellular matrix, it exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds. Fibronectin plays a major role in cell adhesion, growth, migration, and differentiation, and it is important for processes such as wound healing and embryonic development (187). Altered fibronectin expression, degradation, and organization has been associated with a number of pathologies, including cancer and fibrosis (188).

The idea was to quantify the percentage of bacteria attached to fibronectin in order to know that the real inhibition was by blocking this interaction. It was tested against the same three different strains previously used. The results are showed in the Figure 4.12.

a)



b)



c)

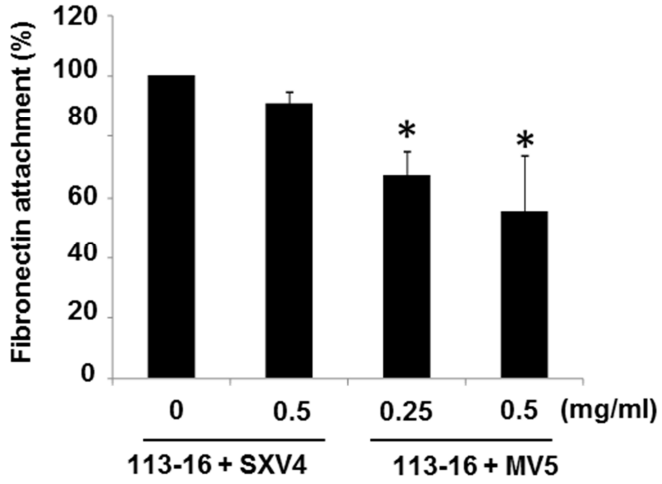
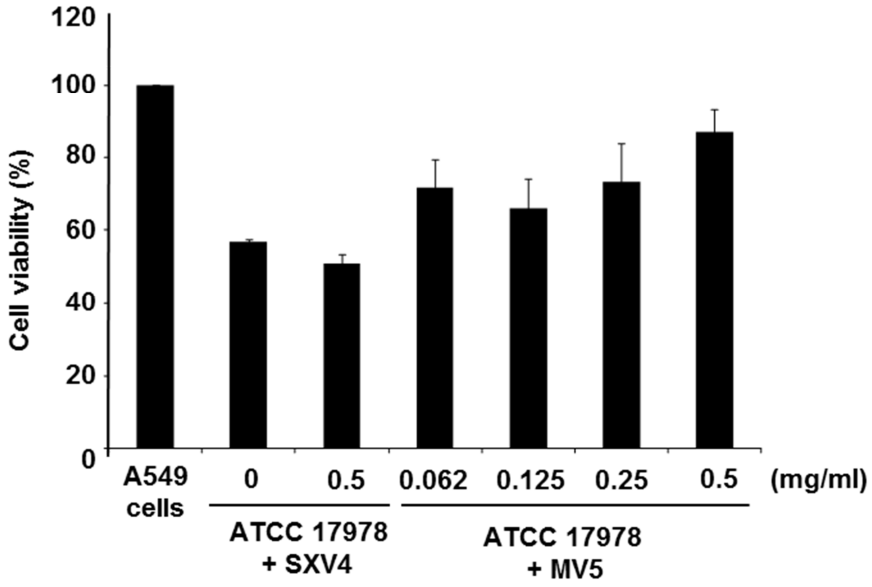


Figure 4.12. a) Percentage of adherence between fibronectin and a very susceptible strain at a different peptide concentrations. b) Percentage of adherence between fibronectin and a MDR strain at a different peptide concentrations. c) Percentage of adherence between fibronectin and a PDR strain at a different peptide concentrations. *$p<0.05$, treatment vs *A. baumannii*.

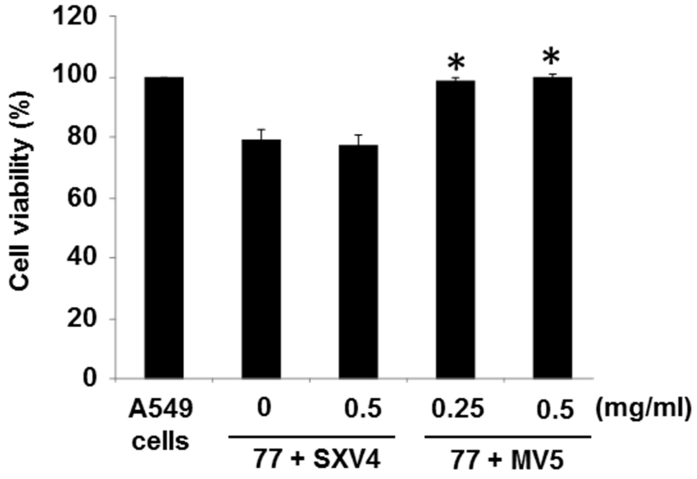
In the figures above it is possible to observe that the compound MV5 inhibit in between 50 and 60 % the adherence between fibronectin and bacteria. It is important to highlight that the amount of fibronectin attached was the same in all the strains used, however in the previous assays, the adherence was different in the different resistant strains, therefore this may suggest that MV5 could block other factors involved in the attachment of the bacteria.

Another way to measure the effectiveness of this compound was by calculating the cell viability in presence of the compound. Since the interaction between eukaryotic cell and bacteria produces the death of the cells, the role of the peptide should be to keep more cells alived. The results of the experiments carried out are showed in the figure 4. 13.

a)


120
100
80
60
40
20
0
Cell viability (%)
A549 cells
0
0.5
0.062
0.125
0.25
0.5
(mg/ml)
ATCC 17978 + SXV4
ATCC 17978 + MV5

b)


120
100
80
60
40
20
0
Cell viability (%)
*
*
A549 cells
0
0.5
0.25
0.5
(mg/ml)
77 + SXV4
77 + MV5

c)

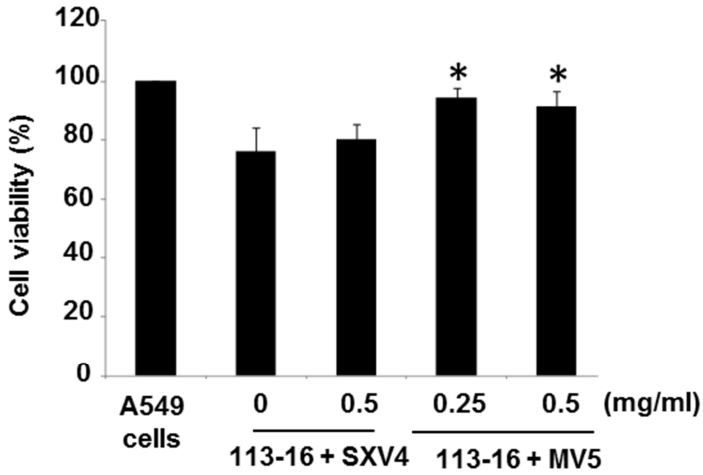


Figure 4.13. a) Percentage cell viability when incubated with a very susceptible strain and peptide at different concentrations. b) Percentage cell viability when incubated with MDR strain and peptide at a different concentrations. c) Percentage cell viability when incubated with PDR strain and peptide at a different concentrations. *p<0.05, treatment vs *A. baumannii*.

The results shown in figure 4.13 are very interesting. In the most susceptible strain, the percentage of cell viability increases from around 50% to more than 80 % when 0.5 mg/mL of MV5 was added, however when SXV4 is added at the same concentration, no effect is observed. When dealing with a more resistant strain such as a MDR, it increases from 80% to 100 % of cell viability, and as in the previous case the effect of SXV4 was negligible. In the case of PDR bacteria an increase from more than 70% to more than 90 % was observed. It is worthy of mention that the values used as a treatment were significantly different compared to the no treatment.

In all of these experiments performed we have observed that this cyclic peptide could be a good therapeutic option to treat *A. baumannii* infections, therefore we decided to carry out some *in vivo* experiments. The first experiments performed were the toxicity *in vivo*, the results obtained are showed in the Table 4.4.

	LD0	**LD50**	**LD100**
MV5 (mg/kg)	40	80	160

Table 4.4. Toxicity of MV5 *in vivo*.

The results obtained showed that a dose of 40 mg/Kg all of the mice were alive after 1 week, when a double dose was injected the percentage of survival mice was reduced to 50, and when 160 mg/kg was administered no survival was observed.
Next step was to determine the ED_{50}. The concentrations used were 10, 20 and 40 mg/Kg. The doses were administered 2h after the infection with a lethal dose, and the strain used for this experiment was *A. baumannii* ATCC 17978.

	MV5 (mg/kg)		
	10	**20**	**40**
Survival (%)	66,67	83,33	50

Table 4.5. ED_{50} of MV5 at different doses..

The three doses tested causes more than 50% of survival, therefore the best results were obtained with 20 mg/Kg, with which 83.33 % of survival is achieved. However going up in the doses with 40 mg/Kg the survival decreases to 50% probably due to the combination between the infection damage and the toxicity of the peptide. The percentage of survival when 10 mg/kg was administered was 66.67.

After calculating the ED_{50}, the local effectiveness of the peptide was determined, thus a murine sepsis peritoneal model was performed and 4 groups of mice were used. A group control one the other three with doses at 24, 48 and 72h respectively. In each group the amount of bacteria in lungs and spleen were calculated. The results obtained are showed in table 4.6.

Strain and treatment	n	Spleen bacterial concentration (Log CFU/g)	Lung bacterial concentration (Log CFU/g)
ATCC 17978 (control)	6	9.52 ± 0.17	9.78 ± 0.17
ATCC 17978 + MV5 10 mg/kg 24 h	6	8.79 ± 0.16	9.21 ± 0.28
ATCC 17978 + MV5 10 mg/kg 48 h	6	6.9 ± 1.32	6.81 ± 1.31
ATCC 17978 + MV5 10 mg/kg 72 h	6	5.48 ± 1.34	5.97 ± 1.46

Table 4.6. Log CFU/g in spleen in lungs in the four different groups.

The first group was sacrificed 24h after the infection and the log CFU/g was 9.52 and 9.78 for spleen and lungs, respectively, when a dose (10 mg/Kg) 2h after the infection was administered the amount of bacteria decreases to 8.79 and 9.21 log CFU/g in spleen and lungs, respectively. When 2 doses were administered, at 2h and 24h after the infection the amount of bacteria in lungs and spleen decreases considerably with values around 7 log CFU/g, and with 3 doses the values were even better achieving 5.48 and 5.97 log CFU/g in lungs and spleen, respectively.

The last experiments performed were the pharmacokinetic of this compound, 10 mg/Kg were injected to several mice and at different times, the concentration of peptide in the serum of the animal was calculated. The results are showed in figure 4.14.

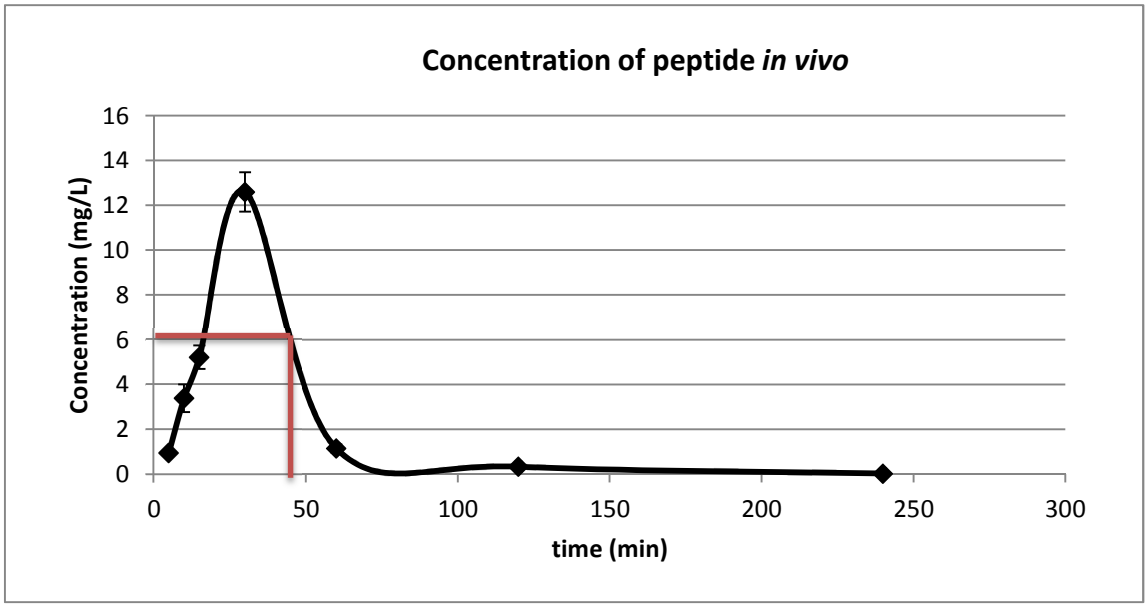


Figure 4.14.Concentration of MV5 *in vivo* at a different times using a dose of 10 mg/Kg.

The results obtained in the pharmacokinetic analysis are showed in the figure above indicate that the maximum concentration achieved by the peptide was around 12 mg/L at 30 minutes, and the half-life time of the MV5 was slightly less than 50 minutes as it is showed in the red line

In summary from the work reported in this chapter, we have indetified an hexacyclic peptide able to inhibit the virulence of several A .baumannii strains both in vitro and in vivo trough a novel mode of actionbased on blocking the binding of bacteria OmpAto host-cell fibronectin.

Discussion

Nowadays daptomycin and colistin are, together with the glycopeptides, the peptidic antibiotics used in the clinical setting. The resistance to daptomycin and colistin in the different microorganisms is steadily increasing, therefore our work in the first part of this doctoral thesis focused in the study of the mechanisms of resistance of *A. nosocomialis* and *S. mitis* to colistin and daptomycin, respectively.

The mechanism of action of daptomycin is associated with the depolarization of the membrane of the Gram-positive bacteria. Among the potential mechanisms of resistance to daptomycin an increase of the thickness of cell wall is considered. In daptomycin-resistant *S. aureus,* the thickness of the cell increases or decreases (166) independently of the resistant grade. In our study regarding the resistance to daptomycin in *S. mitis*, an slight increase in the thickness of the cell wall was observed in the daptomycin-resistant strain compared to its daptomycin-susceptible counterpart, however this difference was not significant enough to be considered as a mechanism of resistance to daptomycin, therefore, there was not an strong evidence that resistance to daptomycin implicates an increase of the cell wall thickness in *S. mitis.*

After not being successful with the first hypothesis of the implication of the increase in cell wall thickness, the idea of using a proteomic approach to compare the protein expression between daptomycin-susceptible and –resistant strains was choosen. Several proteins appeared to be overexpressed or repressed in the daptomycin-resistant strain compared to the original daptomycin-susceptible strain, however and due to the effect of daptomycin on the membrane we just focused in a first phase in the proteins related to the membrane. Two proteins have been identified to be overexpressed in the daptomycin-resistant strain. The first protein is streptococcal surface immunogenic protein which is up-regulated 2.26 fold. It is worthy of mention that this protein contains a lysine motif (LysM), the function of this motif is not well known however it is thought that it may have a general peptidoglycan binding function (189). This domain is typically found in the enzymes involved in the cell wall degradation (190). The second protein found has an unkown function, however it has a similar domain to a cell wall-active antibiotics response protein, in which YvqF/VraSR are included described in *S. aureus* (191) and *Bacillus subtilis* (192). In the article published by Kato *et al.* (193), it was found that in *S. aureus* strains resistant to teicoplanin (glycopeptide) some amino acidic changes were observed in the proteins YvqF and in the two component system VraSR. VraS is a two component histidine kinase sensor, and it has his analog LiaFSR in *Bacillus subtilis*, this protein is the one involved in the resistance to

bacitracin (194) and YvqF is a conserved hypothetical protein. Although the function of YvqF is not known there are strong evidences that it could be involved in the cell wall metabolism because is located upstream of vraS and the transcription of this protein is regulated by VraSR system.

These are the two candidates of being the proteins involved in the daptomycin resistance in *S. mitis*. If we compare this hypothetical proteins with the already known proteins involved in daptomycin-resistant to *S. aureus* and *Enterococcus*. VraSR was also found to be up-regulated in daptomycin resistant for *S. aureus* (195), and its homologue in *Enterococcus faecalis* (LiaFSR) was also reported as being involved in daptomycin resistant (7) by mutations in the gene when the genome of both daptomycin-susceptible and daptmycin-resistant strains were sequenced. As it has been reported in the resistance to daptomycin, a two-component system seems to be involved in the mechanism of resistance. Another two-component system is also involved in the resistance of another peptide which is colistin. The two-component system implicated in colistin resistance in *A. baumannii* is PmrAB. It induces the expression of the *pmr*C, a gene encoding a Lipid A phosphoethanolamine transferase, which adds phosphoethanolamine moieties into the phosphate of the Lipid A, which reduces the negative charge of the outer membrane and thereby decreases binding of positively charged colistin (89, 90). Some mutations in the *pmr*A and *pmr*B genes are the responsible of colistin-resistance (88). In addition, Moffatt et al. (91) indicated that the basis for polymyxin resistance in *A. baumannii* was mutations in the first 3 genes of the lipid A biosynthesis pathway, called, *lpx*ACD, and that this led to complete loss of LPS production and supersusceptibility to other antibiotics.

A. nosocomialis together with *A. pittii* and *A. baumannii* constitute the *A. baumannii* group. In the last years the nosocomial importance of *A. nosocomialis* and *A. pittii* has increased, even in some reports the prevalence of these microorganisms as a causative agent of nosocomial infections is higher that the reported to *A. baumannii* (196). Although overall *A. nosocomialis* clinical isolates are more susceptible to antibacterial agents than *A. baumannii*, colisitin-resistant *A. nosocomialis* clinical isolates have been reported (197), therefore an investigation of the molecular bases of colistin resistance in *A. nosocomialis* was carried out. In order to perform this study a high level colistin-resistant *A. nosocomialis* mutant was obtained by a multi-step method. During the first steps of selection, the mutants obtained tolerate colistin up to a concentration of 8 mg/L,

however the MIC of colistin of these mutants was below 1 mg/L, a sudden increase in the MIC from < 1 mg/L to 128 mg/L was observed. This tolerance may be related to the induction of specific genes by colistin. The high-colistin resistant mutant strain showed a different phenotype compared to the colistin-susceptible wild type strain, characterized by the lost of the ability to growth on MacConkey agar and to show increased susceptibility to different antibacterial agents. On comparing the genome sequence of the susceptible and highly-resistant colistin *A. nosocomialis* strains, no mutations were found in any of the three genes that conformed the *pmr*ABC operon, however although no mutations were neither found in the *lpx*A and *lpx*C genes, several mutations and a stop codon were found the *lpxD* gene. All the strains before the MIC increase had no mutations in any of the genes commented previously, however, after the break point several mutations, and a codon stop appeared.

In the study by Moffatt and colleagues several mutations in the *lpx*A and *lpx*C were found and only a point mutation in the *lpx*D gene consisting in a single base deletion at the nucleotide 952 generating a frameshift after amino acid K317. Therefore the stop codon that we found was located in the nucleotide 810, before the above described mutation. To corroborate that this truncated protein was detrimental for the synthesis of the LPS, the amount of LPS was quantified by a kinetic chromogenic LAL assay showing a huge difference in the production of the LPS of the colistin-susceptible strain compared with the colistin-resistant strain. This result corroborates that the complete lost of LPS production is associated with colistin resistance in *A. baumannii* related to Lpx deficiency (91).

A proteomics study of a colistin-resistant *A. baumannii* ATCC 19606 strain identified 35 proteins, including several outer membrane components, whose expression was downregulated relative to the colistin-susceptible phenotype (198).

Proteomic studies comparing both colistin-susceptible and colistin-resistant *A. nosocomialis* strains were performed, and the results obtained were really surprising observing different results compared with the proteomic comparison between colistin-susceptible and colistin-resistant of *A. baumannii* (198). Some of the proteins found in *A. nosocomialis* were the same as found in *A. baumannii*, however in *A. nosocomialis* were up-regulated, and in *A. baumannii* were down-regulated. Observing that these proteins are up-regulated or down-regulated does not mean that this is an effect of colistin-resistance, because observing the proteome comparison between a multi-drug resistant and very susceptible *A. baumannii* strain some of the proteins observed are the

same as in the others examples (199). It is also important to mention that the strain obtained has a very susceptible profile with very low MICs against most of the antibiotics. OmpA was overexpressed in *A. nosocomialis* 2-fold however in *A. baumannii* it was down-regulated around 3-fold, the same effect is observed in CarO downregulated 2.35 fold in *A. baumannii* and overexpressed 1.7 times in *A. nosocomialis*. This fact have a racional justification, in *A. nosocomialis* it is possible to observe a decrease in the MIC of ertapenem and imipenem due probably to the effect of CarO, however in *A. baumannii* despite of the fact that CarO was repressed the same MIC is observed for imipenem in both strains. CarO was reported to enhance the entrance of imipenem, and when it is deficient is a mechanism of resistance (200). An hypothesis of the overexpression of ompA in the resistant strain could be explained only with the theory that colistin could have a parallel intracellular mechanism. Colistin-resistant *A. nosocomialis* is less virulent than the colistin-susceptible even though OmpA is overexpressed, this information showed us that there are other virulence factors apart from the OmpA. In the case of OmpW the same contrary effect is observed compared with *A. baumannii*, were decreased levels of this proteins are observed when resistance to colistin exists (201). The hypotethical protein (2.1-fold) that belong to the NodT family have a role in the inner membrane in a efflux system, that export small molecules. The only proteins that are down-regulated in the resistance strain are Porin (OprB) Carbohydrate-selective porin (-1.9), this protein together with OmpA have recently been found as a immunogenic protein candidate for a potential vaccine (202) and signal peptide protein (-1.2). The first protein is involved in the transit of the carbohydrate, which probably is directly unrelated with colistin-resistantce, and the second protein, which is a peptide, has a role in the degradation MetA-pathway of the phenol. The remaining proteins seems to be irrelevant. It is important to highlight that the colistin-resistant *A. nosocomialis* strain showed a reduced virulence compared with its susceptible counterpart. This result is in agreement with what has been shown in *A. baumannii* in which it was found that a more striking reduction of the virulence was associated with LPS loss than with phosphoethanolamine addition (203; 48). However, it has also been described that compensatory mutations can restore the virulence of the colisitin-resistant *A. baumannii* strains (204)

We have studied the mechanism of resistance to peptidic antibiotics available in the pharmaceutical market such as daptomycin and colistin, although the knowledge of

these mechanisms of resistance is very important to adopt different approaches in the potential discovery of derivatives which circumvent the known mechanism of resistance, the search for new antibacterial agents active against multidrug resistant bacteria is desperate. In this sense, *A. baumannii* has currently described as an important problem since pandrug resistant clinical isolates have already been reported, therefore we have focused our efforts in this microorganism. In the last decade, due to the lack of develop of new antibacterial agents, the search of inhibitors of virulence factors have been improved with the aim of blocking the developing of the infection. This approach has several advantages such as, compounds able to inhibit a common virulence factor found in several bacteria or an inhibitor of a virulence factor of a specific bacteria can be developed, this later aspect is important since the host endogenous microbiome can be preserved, in addition, this inhibitor will likely exert a less selective pressure, therefore none selecting resistance mutants. Two different approaches were followed in order to discover new drugs active against colistin-resistant *A. baumannii* infections.

The first approach was based on the developing of antivirulence drugs. The main target selected was OmpA, a major porin found in *A. baumannii*, since it is one of the main proteins involved in the adherence of this microorganism to epithelial cells causing pneumonia, therefore our idea was to develop a compound capable to interact with this protein and hence to block the interaction between the bacteria and the cell. We though that designing cyclic peptides able to interact with the inside part of the channel of the porin we can achieve this goal. The initial approach was to design the cyclic peptides by using *in silico* tools, a virtual screening of an hexacyclic peptides library was performed against OmpA and afterwards the peptides were synthesized and tested *in vitro* to probe that indeed they were able to block the activity of the OmpA. Among the synthesized cyclic peptides, MV5 shows the best results and we proceed to the *in vivo* studies with this compound, showing an excellent result since a significant decrease of mortality after just one dose of the peptide was observed.

This is the first time that an inhibitor of the OmpA was developed. As mentioned above OmpA is one of the immunogenic proteins found in the outer membrane, therefore it has also been used as a vaccine to try to immunize mice and the results were good achieving high survival percentages (162), a similar approach was followed by Pachón's group, but in this case the vaccine was not only ompA, since they include other outer membrane proteins and it was called outer membrane complex (OMC) (205).

A similar approach searching for a compound able to inhibit LpxC, one of the proteins involved in the cascade of the synthesis of the LPS has been reported. This inhibitor did not kill the bacteria with a concentration lower than 512 mg/l, only inhibit the production of LPS. LPS is recognized by Toll-like receptor 4 (TLR4), therefore the aim of this strategy was to find a compound able to inhibit LpxC and the synthesis of LPS, whit the consequent no activation of the TLR4 and protection of the mice from lethal infection. (206)

The second approach used was the search for new antimicrobial agents. Three different type of compounds were used, different peptides from different sources, peptides isolated from frog skin secretions and a fully synthetic compounds like the ceragenins which were tested against three different species of colistin-resistant bacteria. Among the 15 peptides investigated, cecropin P1, colistin, melittin, indolicidin and mastoparan showed activity against colistin-susceptible *A. baumannii*, but only mastoparan and melittin showed good activity against both colistin-susceptible and colistin-resistant *A. baumannii*. It is worthy of mention that the HNP-1 showed good activity only against colistin-resistant *A. baumannii*. In previous studies performed by Saugar et al. (207) and Rodriguez-Hernandez et al. (132) several hybrid peptides composed of a mixture of cecropin A and melittin showed MICs of 2–8 mg/L against 13 clinical isolates of colistin-resistant (4–64 mg/L) *A. baumannii*. In our study, the MICs were 4 mg/L for mastoparan against colistin-susceptible *A. baumannii* and 1 mg/L for mastoparan against colistin-resistant *A. baumannii* with an MIC for colistin of 256 mg/L. On comparison of the MIC_{50} and MIC_{90} of cecropin A–melittin and mastoparan for colistin-resistant *A. baumannii*, they were found to be slightly lower, by one or two dilutions, for cecropin A–melittin than for mastoparan. Several studies have investigated mastoparan activity, but there has been no report on the activity of mastoparan against *A. baumannii* (208). In the study performed by Giacometti et al. (209), a similar set of peptides was used, but only against colistin-susceptible *A. baumannii*, with buforin II (MIC range 0.25–16 mg/L), magainin II (MIC range 0.50–16 mg/L) and cecropin A (MIC range 0.50–32 mg/L) being the most active AMPs against colistin-susceptible *A. baumannii*, in contrast to our study, in which only cecropin P1 showed good activity against colistin susceptible *A. baumannii*. Mastoparan is not the only peptide isolated from wasps. All of the peptides isolated from wasps have very similar sequences, with changes in only

some amino acids, but their activity can vary, changing from an MIC of 58 mg/L to one of 164 mg/L with just one amino acid substitution in the peptide (210). On comparison of the time-killing curves of mastoparan and cecropin A–melittin, similar behaviour was seen. Cecropin A–melittin was bactericidal at lower concentrations in some strains tested, but in others there was regrowth that was not observed in mastoparan time killing curves (132). In a study by Li et al. (211), a time-killing curve for colistin against ATCC 19606 colistin-susceptible *A. baumannii* was obtained. On comparison of this time-killing curve with that obtained in our study with mastoparan, it can be seen that the bactericidal effect of colistin was faster than that of mastoparan. Moreover, colistin was bactericidal at lower concentrations than mastoparan for colistin-susceptible *A. baumannii*, but, in contrast, regrowth was observed in the colistin time-killing curve that did not appear when mastoparan was used. The fact that mastoparan shows activity against both colistin- susceptible and colistin-resistant *A. baumannii* may suggest that the mechanisms of action used by mastoparan and colistin are different.The mechanism of action of colistin may be related to disruption of the bacterial membrane. Mastoparan could act as a cell-penetrating peptide, crossing the bacterial membrane (212). However, Li et al. (213) analysed the effect of mastoparan against *Escherichia coli* and *Staphylococcus aureus* by scanning electron microscopy, and suggested that mastoparan acts on the cell surface, resulting in different abnormalities depending on the microorganism. In conclusion, mastoparan showed a good *in vitro* bactericidal activity against both colistin-susceptible and colistin-resistant *A. baumannii*. Several analogs of mastoparan were synthesized in order to increase the stability in human serum and maintain the same activity as mastoparan. The only peptides synthesized that had the same activity as mastoparan were peptide 1, peptide 10 and peptide 13, however all of them showed higher cytotoxicity but an increase in the stability in human serum. Peptide 10 together with mastoparan were selected to be tested *in vivo* in a mouse model, however no activity was observed against infections caused by *A. baumannii*. We suggest that this loss of activity *in vivo* may be associated with the high binding to the proteins found in serum, specially albumin.

Previous studies have shown that the peptides [E4K]alyteserin-1c (183) and [D4K]B2RP (184) show high potency against colistin-susceptible *A. baumannii* clinical isolates. We have now extended these studies by demonstrating that these peptides show comparable, and in some cases even greater, potency against colistin-resistant *A. baumannii* and *A. nosocomialis* strains. In addition, three naturally occurring peptides

with low haemolytic activity (CPF-AM1, PLGa-AM1 and B2RP-ERa) and one non-haemolytic analogue of a naturally occurring peptide ([G4K]XT-7) have been identified that are active both against colistin-susceptible and colistin-resistant *Acinetobacter sp.* strains. Overall, [D4K]B2RP and [E4K]alyteserin-1c were the most effective against the colistin-resistant strains. [E4K]alyteserin-1c has the advantage of very low haemolytic activity (LC50 > 1000 μg/mL compared with 250 μg/mL for [D4K]B2RP) but is active against only Gram-negative bacteria (183). [D4K]B2RP, however, is active against Gram-positive bacteria and against *Candida* (184). In common with the majority of frog skin AMPs, the peptides used in this study do not show any secondary structural features in aqueous solution, but in a membrane-mimetic solvent (50% trifluoroethanol–water) they adopt a stable amphipathic α-helical conformation with the cationic lysine residues segregating on one face of the helix and the hydrophobic residues on the opposite face (214). There is no single mechanism by which the peptides produce cell death, but their mode of action usually involves binding to, and penetration into, the inner bacterial cell membrane with eventual destruction of membrane integrity (215). Conformation studies using proton nuclear magnetic resonance (1H NMR) indicate that the helix in [E4K]alyteserin-1c extends between residues Lys3 to Val21 and, in the presence of sodium dodecyl sulphate, dodecylphosphocholine and 1,2-dihexanoyl-sn-glycero-3-phosphatidylcholine micelles, the N-terminal domain (amino acid residues 1–19) in the peptide is inserted into the micelle (214).

Cationic AMPs interact electrostatically with the outer membrane of Gram-negative bacteria and cross this barrier by a process termed ‘self-promoted uptake’ which is similar to that adopted by colistin. This involves the peptide competitively displacing divalent cations from surface LPS causing outer membrane perturbation and thereby promoting further peptide uptake (216). The fact that certain strains of *A. baumannii* are resistant to colistin but susceptible to the frog skin peptides implies that the initial binding site of the peptides on the outer membrane differs from that of colistin. It has been shown that a cecropin A–melittin hybrid peptide has a higher affinity than colistin towards LPS from colistin-resistant but not from colistin-susceptible strains (207). Despite the fact that the frog skin peptides used in this study are effective both against colistin-susceptible and colistin-resistant strains of *A. baumannii* and *A. nosocomialis* and show low cytotoxicity against human red blood cells, their therapeutic potential is limited, like all peptide-based drugs, by their short half-lives in the circulation. Attempts to prepare a long-acting derivative of [E4K]alyteserin-1c by coupling a palmitate group

to the α-amino group in the peptide were disappointing as the resulting analogue showed greatly increased haemolytic activity (183).

If we compared the results of the activity against *A. baumannii* of the frog skin secretions peptides with the mastoparan analogues the values of MICs were similar, however mastoparan and the best analogues have a high stability in human serum and probably the skin frog secretion peptides (formed by L-amino acids) will have a lower time life in human serum but they are less hemolytic than mastoparan compounds, therefore further optimization of these natural peptides should be performed in order to have a future candidate to treat invasive nosocomial infections. These peptides may be limited to topical applications, such as aerosol treatment of respiratory infections and treatment of *Acinetobacter* sp. infected burns and wounds in war trauma patients (217).

Ceragenins could avoid the problem of stability commented above due to the fact that they are not really peptides. Another advantage of this type of compounds is their ability of being effective against several type of bacteria, therefore showing a broad spectrum of activity. In our study, among the four ceragenins tests (CSA-13, -131, -44 and -138), CSA-131 showed the best activity with a MIC of 0.5, 2 and 8 mg/L for colistin-resistant *P. aeruginosa,* colistin-resistant *A. baumannii* and colistin-resistant *K. pneumoniae,* respectively. The MIC_{90} of CSA-131 for both colistin-resistant *P. aeruginosa* and colistin-resistant *A. baumannii* was of 2 mg/L. CSA-131 showed a rapid bactericidal activity at 2X, 4X and 8X the MIC against colistin-resistant *A. baumannii* strains. In the article reported by Pollard *et al.* (218) they compared the ability of acquiring resistance to colistin, ciprofloxacin (quinolone) and ceragenin in *P. aeruginosa* and *A. baumannii* and they found that ability of acquiring resistance to colistin and ciprofloxacin was much higher compared to ceragenin. CSA-13 was tested against carbapenem-resistant *A. baumannii* (219), and the results reported were similar as the observed in our study, this confirms the theory exposed that the MIC of ceragenins is independent of the level of resistance to several antibacterial agents of the strain tested. CSA-131 was the compound that gave us better results, however the similarity with CSA-13 is very high only differing in the length of the hydrophobic part. Further *in vivo* studies are needed to probe its use to treat systemic infections caused by multidrug-resistant bacteria.

Conclusions

1. Daptomycin resistant grade is not associated with thickness of the cell wall in *S. mitis.*
2. Two proteins identified as conserved hypothetical protein and group B streptococcal surface immunogenic protein are overexpressed 1.33 and 2.26 fold respectively. These proteins may play an important role in daptomycin resistance in *S. mitis* due to its high homology with proteins found in other bacteria to be involved in daptomycin resistance.
3. The MIC for colistin in *A. nosocomilais* increases drastically from <1 mg/L to 128 mg/L, when the microorganism is exposed to colistin, observing no growth on McConkey.
4. The MICs of several antibiotics for the colistin-resistant *A. nosocomialis* mutants generated decreased from 1 to 11-fold.
5. A decrease in the virulence of colistin-resistant *A. nosocomialis* mutant is showed compared to its colistin-susceptible counterpart.
6. Colistin resistance in *A. nosocomialis* was linked to the lost of LPS, associated with several mutations and a stop codon observed in the *lpxD* gene.
7. Several proteins were found to be up-regulated or down-regulated when comparing colistin-susceptible and colistin-resistant *A. nosocomialis* strains. Any of them seems to play an important role in the colistin resistance, however they may be important in the reduction of the MICs against several antibiotics.
8. Indolicidin, melittin and mastoparan show a good activity against both colistin-susceptible and colistin-resistant *A. baumannii* strains.
9. Mastoparan show a bactericidal behavior at 8-fold MIC in both colistin-susceptible and colistin-resistant *A. baumannii* strains, and 4-fold MIC in the colistin-resistant *A. baumannii*.
10. Mastoparan, a lineal peptide, shows an exceptional high time-life in human serum (6h), observing only the lost of the N-terminal isoleucine as resulting product of the action of proteases and peptidases.
11. Three peptides (peptide 1(Gu-INLKALAALAKKIL-NH_2), peptide 10 (inlkalaalakkil-NH_2) and peptide 13(INLKALAALAKKIL-$CH_2CH_2NH_2$) from all the analogs synthesized, show the same activity as mastoparan. The results observed in all the analogs highlight the importance of the N-terminal positive charge.

12. Mastoparan and peptide 10 show a very low hemolytic activity at MIC concentrations, modest cytotoxicity and higher toxicity *in vivo*, being 8 mg/Kg for peptide 10 and 32 mg/Kg for mastoparan.

13. No antibacterial effect was observed *in vivo*, in any of the conditions tried, for peptide 10 and mastoparan. The reason is likely associated with the strong bonding of both peptides with proteins present in the serum. Observing a higher MIC when MIC assays were performed in presence of serum. Albumin is the protein which mastoparan binds to when is incubated with serum.

14. Mechanism of antibacterial action of these above mentioned peptides may be related with membrane disruption as observed both by leakage assays and transmission electron microscopy.

15. [E4K]alyteserin-1c and [D4K]B2RP, peptides isolated from skin-frog secretions, show a good activity against both colistin-susceptible and colistin-resistant *Acinetobacter* species.

16. All the ceragenins tested, specially CSA 131, show a good activity against both colistin-susceptible and colistin-resistant *P. aeruginosa*, *A. baumannii* and *K. pneumonia*e.

17. A bactericidal effect of CSA 131 is observed against colistin-resistant *P. aeruginosa* and *A. baumannii* at all the concentrations tested, however a regrowth is observed at low concentrations.

18. All of the hexacyclic peptides tested as ompA inhibitors show inhibition of the OmpAmediated adherence to host cell.

19. MV5 (cyclo[pwrpwr]) decreases the binding between OmpA and fibronectin and increases the host cell viability in the presence of different *A. baumannii* strains tested. MV5 has also a very low *in vivo* toxicity, since all of the mices survive at 40 mg/Kg.

20. ED_{50} in mice was achieved in all the doses tested. At 10 mg/Kg a high decrease in the number of bacteria in lungs and spleen was observed, and reaches a concentration of around 12 mg/L and a half-life time of around 50 minutes.

Materials and methods

1. Microbiologycal methods.

1.1. Strains used in this thesis.

ATCC 1906 and ATCC 17978 are reference strains, ATCC 19606 col-R is the isogenic strain generated after incubation with colistin as explained in the thesis. Colistin-susceptible *A. baumannii* strains used in the assay of calculation of MIC_{50} and MIC_{90} of colistin were clinical isolates from REIPI (Spanish network research in infectious disease). Colistin-resistant *A. baumannii* strains used in the assay of calculation of MIC_{50} and MIC_{90} were clinical isolates from Hospital Virgen del Rocío (Sevilla), strains Ab113, Ab11, Ab22P and Ab1 used in the same chapter also belong to this group. CS01 is a colistin-susceptible *A. baumannii* strain, and CR17, CR86 are colistin-resistant *A. baumannii* strains, the three of them belong to the same clone, and were isolated in Hospital Virgen del Rocío (Sevilla) . Strain 77778 is a clinical isolate from Hospital General de Asturias, and strain G13 (*A. nosocomialis*) is also a clinical isolate from REIPI, colistin-resistant mutants were generated also with these two strains. Five multi-drug *A. baumannii* strains (NM8, NM35, NM75, NM109 and NM124) were isolated at four different hospitals in the Emirate of Abu Dhabi. Strain 113-16 is a clinical pan-resistant *A. baumannii* isolate from the Hospital Virgen del Rocío (Sevilla). Strain 77 is a multi-drug resistant *A. baumannii* strain isolated in the Hospital Clinic (Barcelona). Both colistin-susceptible and colistin-resistant *P. aeruginosa* strains were supplied by Dr. Oliver from Hospital Son Dureta (Palma de Mallorca). Both colistin-susceptible and colistin-resistant *K. pneumoniae* strains were obtained from the Magic Bullet project. *S. mitis* strains used were, D6-2, D6-7 and D6-14, which were isolated from the cardiac valve vegetations of rabbits infected with the *S. mitis* strain 351, a clinical isolate from Hospital Clinic (Barcelona) and treated with daptomycin.

1.2. Minimal inhibitory concentration

The MICs of all the peptides or peptidomimetics used in this thesis were determined against different *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* strains with the microdilution method, following the CLSI recommendations. The antibacterial activity of the peptides was measured in sterile 96-well plates. Samples of a final volume of 100μL were prepared as follows: aliquots (50 μL) of a suspension containing bacteria at

a concentration of 10^5 colony-forming units/mL in culture medium (MH, Muller Hinton Broth, Difco), adjusted at pH 7.4, were added to 50 µL of solution containing the peptide prepared from a stock solution of 1,024 mg/mL peptide in water in serial 2-fold dilutions in MH broth adjusted to pH 7.4. Inhibition of bacterial growth was determined observing no growth after an incubation of 24 h at 37 ºC (220). The concentrations of the peptides mastoparan, indolicidin, colistin, histatin 5, cecropin B, cecropin A, magainin II, buforin I, magainin I, histatin 8, bactenicin, melittin, peptide (1-14), CPF-AM1, PGLa-AM1, B2RP-Era, [E4K]alyteserin-1c, [D4K]B2RP, [G4K]XT-7, CSA-13, CSA-44, CSA-131, CSA-138, MV6, MV5, MV3B, MV8, MV9, MV10, NBA01011 and SXV4 ranged from 0.5 to 256 mg/L. The concentrations of HNP-1 and HNP-2 ranged from 0.09 to 50 mg/L, and those for cecropin P1 and β-defensin ranged from 0.05 to 25 mg/L. Mastoparan, indolicidin, colistin, histatin 5, cecropin B, cecropin A, magainin II, magainin I, histatin 8, melittin were purchased in Sigma. Buforin I and bactenicin were purchased in Anaspec and Genscript respectively. CPF-AM1, PGLa-AM1, B2RP-Era, [E4K]alyteserin-1c, [D4K]B2RP, [G4K]XT-7 were supplied by Prof. Conlon and , CSA-13, CSA-44, CSA-131, CSA-138 by Prof. Savage.

1.3. MIC using E-test.

With some strains the MICs of different antibiotics were carried out using E-test method. This method is performed preparing an initial inoculum of 10^8 cfu/ml (McFarland 0.5) and spread it homogenely into a Muller Hinton agar plate, after the spread, stick an E-test strip (Biomerieux) into the plate. (E-test strips are strips which contain increase concentrations of a certain antibiotic, this allow us to know the MIC of an antibiotic in a faster way compared to the normal microdilution method)

1.4. MIC with serum or albumin

In the assay to calculate the MIC of the peptides in presence of serum and albumin, the first two steps were the same as in a normal MIC determination experiment, however the volumes used are the double, using both 100 µl for Muller Hinton and peptide dilution. The third step changes slightly, an initial inoculum of 10^8 cfu/ml was adjusted, and a 1/1000 dilution was performed in 4X Muller Hinton, 3 ml of this pool was added

to a 12 ml falcon together with H_2O and Human serum (HS), the proportions are showed in the table below.

% HS supplement	**0**	**25**	**50**	**75**
HS (ml)	0	3	6	9
MH+CA (x4) (ml)	3	3	3	3
Sterile MilliQ water (ml)	9	6	3	0
Total (ml)	12	12	12	12

100 µl of each pool was added, and incubate during 24h at 37ºC. Next day read the 96 well plate.

1.5. Killing curves

Time-killing curves were obtained with mastoparan and CSA-131, the peptides were tested against both colistin-susceptible and colistin-resistant *A. baumannii* and the ceragenin was tested against colistin-resistant *A. baumannii* and *P. aeruginosa* strains. Initial inocula between 10^6 and $5 \cdot 10^6$ CFU/mL of bacteria in 10-mL aliquots of Mueller–Hinton broth were prepared for the time-killing curves. Concentrations of 1xMIC, 2xMIC, 4xMIC and 8xMIC were used for mastoparan and CSA-131. Samples were taken at 0, 4, 7 and 24 h for mastoparan and 0, 1, 4, 8 and 24 h for CSA-131 after the incubation. Drug carryover was addressed by dilution. An antibiotic was considered to be bactericidal when a reduction of 3 log10 CFU/mL as compared with the initial inoculum was achieved.

2. Synthesis of peptides

2.1. Solvent and Reagents

All the reagents employed for peptide synthesis have been purchased to the following suppliers: Anaspec, Bachem AG, Calbiochem-Novabiochem AG, Iris Biotech, KaliChemie, Luxemburg Industries, Neosystems and Sigma-Aldrich. All the solvents employed have been purchased to the following suppliers: Panreac, Scharlau, SDS and Sigma-Aldrich.

2.2. HPLC-UV

HPLC chromatograms were recorded on a Waters Alliance 2695 separation module coupled with a Waters 2998 photodiode array detector and Empower software (Waters) using a Sunfire C_{18} column (100 mmX 4.6 mm, 3.5 µm; Waters). Column flow was set at 1 ml/min, and solvents used were water (0.045 % TFA) and acetonitrile (0.036% TFA).

2.3. Semi-preparative HPLC

Peptides synthesized were purified by semi-preparative HPLC on a Waters 2700 sample manager equipped with a Waters 2487 dual λ absorbance detector, a Waters 600 controller, a Waters fraction collection II and Millenium chromatography manager software (Waters) using a symmetry C_{18} column (100 mm X 19 mm, 5 µm; Waters). Column flow was set at 15 ml/min, the solvents used were water (0.1% formic acid) and acetonitrile (0.07% formic acid).

2.4. MALDI-TOF

The MALDI-TOF used was a MALDI-TOF/TOF Applied Biosystems 4700 with a N_2 laser of 337 nm. The samples are prepared by mixing the peptide or fraction that want to be analysed (1µl) with the MALDI matrix (1µl) in the MALDI plate and let it dry in the air. The MALDI matrix was prepared by dissolving 10 mg of α-cyano-4-hydroxycinnamic acid (ACH) in H_2O and ACN ((1:1) containing 0.1% of TFA.

2.5. General synthesis

All the peptides synthesized during this thesis were carried out by SPPS using the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) strategy. All the synthesis were carried out manually in polypropylene syringes provided with a polyethylene porous disk. Washings between each step were DMF (5 x 30s) and DCM (5 x 30s), the volume used is the necessary to cover all the resin used.

2.6. Ninhydrin or Kaiser test

The Kaiser test, also known as ninhydrin test, is test used to detect primary amines. The resin is washed with DCM and few resin beads are transferred to a small glass tube in which the reaction takes place. Next step is to add 6 drops of reagent A and 2 drops of reagent B, the mixture is incubated during 3 minutes at 110 ºC. Blue colour on the resin beads showed free primary amines, meanwhile the none colour variation indicates a negative test.

Reagent A: 400 g of phenol are dissolved in 100 ml of EtOH and the mixture is heated until complete dissolution of the phenol, 20 ml of 10 mM KCN (65 mg in 100 ml H_2O) are added to 1 liter of freshly distilled pyridine over ninhydrin. Both solutions are stirred for 45 minutes with 40 g of Amberlite MB-3 ion exchange resin, filtered and mixed.

Reagent B: 2.5 g of ninhydrin are dissolved in 50 ml of absolute EtOH and the resulting solution is stored in a flask protected light.

2.7. Chloroanil test

The chloroanil test is used when secondary amines want to be detected, and is specially used when monitoring the couplings and the deprotection reactions involving secondary amines. The resin is washed with DCM and transferred to a small glass tube, 200 µl of acetone and 20 µl of saturated chloroanil solution is added (750 µg of 2,3,5,6-tetrachloro-1,4-benzoquinone in 25 ml of toluene). The mixture is thoroughly stirred for 5 minutes at room temperature. The blue colour indicated the presence of free secondary amines, meanwhile yellow is considered as negative test.

2.8. Resin conditioning

In the resin H-Rink amide ChemMatrix (PCAS Biomatrix) some conditioning was performed before the coupling of the first amino acid. For resins 1,2-Diamino-ethane trityl (Novabiochem) and 2-Chlorotrityl chloride (Irisbiotech) only 3x1 min washes with DCM are needed.

H-Rink amide ChemMatrix

Step	Treatment	Solvents/Reagents	Time
1	Wash	MeOH	5 x 1 min
2	Wash	DMF	5 x 1 min
3	Wash	DCM	5 x 1 min
4	Wash	DCM 1% TFA	5 x 1 min
5	Wash	DCM	5 x 1 min
6	Wash	DMF	5 x 1 min
7	Wash	DCM	5 x 1 min
8	Wash	DCM 5% DIEA	5 x 1 min
9	Wash	DCM	5 x 1 min
10	Wash	DMF	5 x 1 min

2.9. Fmoc group removal

The fmoc group was removed using basic conditions, for this reason the resin was treated with 20% piperidine in DMF (4 ml/g resin, 1 x 1 min and 2 x 10 min). Additionally when fmoc want to be removed from Fmoc-Pro-OH, an additional treatment with DBU, toluene, piperidine and DMF (5:5:20:70) (2 x 5 min) was performed.

2.10. Amino acid coupling and elongation

Three different protocols were used depending on the resin and the amino acids used, protocol A was used for H-Rink amide Chem Matrix and 1,2-Diamino-ethane trityl resin and Protocol B was used for 2-Chlorotrityl chloride, and Protocol C when used 2-Chlorotrityl chloride for coupling onto secondary amines.

Protocol A:

Step	Treatment	Solvents /reagents	Time
1	Wash	DMF	5 x 1 min
2	Coupling	Fmoc-Aa-OH (3 eq), TBTU (3 eq), DIEA (6 eq) in DMF	1 x 60 min
3	Wash	DMF	5 x 1 min
4	Fmoc group removal	20% piperidine in DMF	1 x 1 min, 2 x 10 min
5	Wash	DMF	5 x 1 min

Protocol B:

Step	Treatment	Solvents/reagents	Time
1	Wash	DCM	5 x 1 min
2	Coupling	Fmoc-Aa-OH (0.7 eq), DIEA (10 eq) in DCM // MeOH (0.8 ml/g resin)	1 x 60 min and 15 min after MeOH addition
3	Wash	DMF	5 x 1 min
4	Fmoc group removal	20% piperidine in DMF	1 x 1 min, 2 x 10 min
5	Wash	DMF	5 x 1 min
6	Coupling	Fmoc-Aa-OH (3 eq), PyBOP (4 eq), HOAt (3 eq) DIEA (6 eq) in DMF	5 x 1 min
7	Wash	DMF	1 x 60 min

Protocol C:

Step	Treatment	Solvents/reagents	Time
1	Wash	DMF	5 x 1 min
2	Coupling	Fmoc-Aa-OH (0.7 eq), DIEA (10 eq) in DCM // MeOH (0.8 ml/g resin)	1 x 60 min and 15 min after MeOH addition
3	Wash	DMF	5 x 1 min
4	Fmoc group removal	20% piperidine in DMF	1 x 1 min, 2 x 10 min
5	Wash	DMF	5 x 1 min
6	Coupling	Fmoc-Pro-OH (3 eq), PyBOP (4 eq), HOAt (3 eq) DIEA (6 eq) in DMF	2 x 60 min
7	Wash	DMF	5 x 1 min
8	Fmoc group removal	DBU, toluene, piperidine in DMF (5:5:20:70)	2 x 5 min
9	Fmoc group removal	20% piperidine in DMF	1 x 1 min, 2 x 10 min

2.11. N-terminal acetylation

After the peptide elongation, acetylation was performed in order to protect the N-terminal. Ac_2O (50 eq) and DIEA (50 eq) in were added to the resin together with DCM 30 minutes, before and after the treatment, washes of DCM (5 min) need were performed.

2.12. N-terminal guanidination

After the peptide elongation, acetylation was performed in order to protect the N-terminal, the reagents used were 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine (5 eq) and Et_3N (5 eq) in DCM during 18 hours, before and after the treatment, washes of DCM (5 min) need to be performed.

2.13. Cleavage of peptides from the resin

Peptides synthesized using Protocol A were cleaved using a cocktail that contains TFA, H_2O and TIS (95:2.5:2.5). The mixture of the resin and peptide with the cocktail was located in the shaker and allow it to react during 2 hours in a Falcon tube. After 2h the supernatant was evaporated using N_2, to dry the resin as much as possible. Once the resin is dried 30 ml of methyl *t*-butyl ether were added to allow the peptide released from the resin to precipitate, incubate during 10 minutes on ice and centrifuge during 10 minutes at 4ºC (3,500 rpm), discard the supernatant carefully, add again methyl *t*-butyl ether, and repeat again the procedure three times more. Dissolve the white precipitate using H_2O and ACN (1:1), and filtrate it to separate the resin from de peptide, after the separation the peptide was lyophilized.

For the peptides synthesized using Protocol B and C, which were the cyclic peptides, a different procedure was used. The cocktail used contained 2% of TFA in DCM, and 5 washes of 30 seconds to the resin were performed. This procedure only cleaves the peptide from the resin, and did not remove the protecting groups present in the side chains of the amino acids. The resultant from the washes was collected in a container with H_2O, mixed with ACN and lyophilized.

2.14. Cyclization and deprotection

For cyclization the solvent used was DCM/DMF (98:2), PyAOP (2 eq) was dissolved in DMF and afterwards the peptide (5 mM) was dissolved with DCM and once is mixed 6 equivalents of DIEA were added, the reaction was completed in 2 or 3 hours. The end of the reaction was checked by HPLC. The mixture was rotavapored until an oil (DMF) was obtained, the oil was treated with the same cocktail as used for peptides synthesized with protocol A (TFA/TIS/ H_2O.95:2.5:2.5) and the same procedure as explained previously was carried out, except that no separation from the resin was needed.

3. Characterization of peptides

3.1 Tickets of all the peptides synthesized

Amino acids in lower case have a D configuration

Gu-INLKALAALAKKIL-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1521 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 3.4 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.3 min
MALDI-TOF, $[M+H]^+$: 1521.1 Da

iNLKALAALAKKIL-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1479 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 5.6 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.2 min
MALDI-TOF, $[M+H]^+$: 1479 Da

InLKALAALAKKIL-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1479 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 5.9 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.2 min
MALDI-TOF, $[M+H]^+$: 1479 Da

NLKALAALAKKIL-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1365.8 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 6.0 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.2 min
MALDI-TOF, $[M+H]^+$: 1365.9 Da

Ac-INLKALAALAKKIL-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1520.9 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 5.1 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.7 min
MALDI-TOF, $[M+H]^+$: 1521 Da

INLKALAALAKKIL-NH_2 (Mastoparan)

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1479 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 6.5 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.3 min
MALDI-TOF, $[M+H]^+$: 1479 Da

inLKALAALAKKIL-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1479 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 4.1 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.2 min
MALDI-TOF, $[M+H]^+$: 1479 Da

LIKKALAALAKLNI-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1479 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 7.1 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.7 min
MALDI-TOF, $[M+H]^+$: 1479.1 Da

likkalaalaklni-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1479 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 6.8 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.6 min
MALDI-TOF, $[M+H]^+$: 1479.1 Da

inlkalaalakkil-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1479 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 8.1 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.3 min
MALDI-TOF, $[M+H]^+$: 1479.1 Da

INLKAKAALAKKIL-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1493.9 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 6.3 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.5 min
MALDI-TOF, $[M+H]^+$: 1494.2 Da

In(Orn)kalaalakkil-NH_2

Side-chain protecting groups: Lys (Boc), Asn (Trt), Orn (Boc)
Resin: H-Rink amide Chem Matrix
Initial loading: 0.49 mmol/g
MW: 1480 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 6.5 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.7 min
MALDI-TOF, $[M+H]^+$: 1479.9 Da

INLKALAALAKKIL-$CH_2CH_2NH_2$

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: 1,2-Diamino-ethane trityl resin
Initial loading: 1.18 mmol/g
MW: 1522 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 4.6 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 6.1 min
MALDI-TOF, $[M+H]^+$: 1522.2 Da

LIKKALAALAKLNI-$CH_2CH_2NH_2$

Side-chain protecting groups: Lys (Boc), Asn (Trt)
Resin: 1,2-Diamino-ethane trityl resin
Initial loading: 1.18 mmol/g
MW: 1522 Da
Cleavage cocktail: 95% TFA/ 2.5% H_2O/ 2.5% TIS
Yield: 6.1 %
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.6 min
MALDI-TOF, $[M+H]^+$: 1521.9 Da

Cyclo[RpWRpW]
Side-chain protecting groups: Arg (Pbf), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.65 mmol/g
MW: 879.04 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 25%
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.8 min
HPLC-MS: 879.36 Da

Cyclo[WpRWpR]
Side-chain protecting groups: Arg (Pbf), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.74 mmol/g
MW: 879.04 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 38%
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.7 min
HPLC-MS: 879.29 Da

Cyclo[rPWrPW]
Side-chain protecting groups: Arg (Pbf), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.62 mmol/g
MW: 879.04 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 23%
HPLC, t_R (min, G0-100% MeCN in 8 min): 5.0 min
HPLC-MS: 879.49 Da

Cyclo[RPwRPw]
Side-chain protecting groups: Arg (Pbf), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.63 mmol/g
MW: 879.04 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 32%
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.6 min
HPLC-MS: 879.52 Da

Cyclo[SpWSpW]
Side-chain protecting groups: Ser (tBu), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.69 mmol/g
MW: 741.33 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 16%
HPLC, t_R (min, G0-100% MeCN in 8 min): 6.0 min
HPLC-MS: 741.15 Da

Cyclo[EpWEpW]
Side-chain protecting groups: Glu (tBu), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.62 mmol/g
MW: 825.35 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 23%
HPLC, t_R (min, G0-100% MeCN in 8 min): 825.26 min
HPLC-MS: 825.26 Da

Cyclo[ASpASp]
Side-chain protecting groups: Glu (tBu), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.61 mmol/g
MW: 511.50 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 19%
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.0 min
HPLC-MS: 511.19 Da

Ac-WpRWpR-OH
Side-chain protecting groups: Arg (Pbf), Trp (Boc)
Resin: 2-Chlorotrityl chloride
Initial loading: 0.60 mmol/g
MW: 938.48 Da
Cleavage cocktail: 2% TFA/ 98% DCM
Yield: 42%
HPLC, t_R (min, G0-100% MeCN in 8 min): 4.5 min
MALDI-TOF, $[M+H]^+$: 939.51 Da

3.2. Stability assay

Peptides were incubated at 37ºC in the presence of 100% human serum (from human male AB plasma). At a range of times, 50 µL aliquots were extracted and serum proteins were precipitated by the addition of 150 µL of acetonitrile at 4ºC to stop degradation. After 30 min at 4ºC, the samples were centrifuged at 10,000 rpm (9,300 x g) for 10 min at 4ºC. The supernatant was analyzed by HPLC. Fractions were also analized by MALDI-TOF mass spectrometry.

3.3. MTT cytotoxicity assay

For these experiments HeLa cells were used for mastoparan and analogues and A549 pulmonar cells were used for the cyclic peptides. Their doubling time and the lineal absorbance at 570 nm were taken into account for seeding purposes. Cell viability in the presence of peptides was tested using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For each assay, $5x10^3$ HeLa cells or A549 pulonar cells were seeded on a 96-well plate (Nalge Nunc) and cultured for 24 h. Samples were added at concentrations ranging from 1µM to 500 µM depending on the peptide, and 0.25, 0.5 and 1 mg/ml for the peptides from the EXORIS library. Cells were incubated for 24 h at 37°C under a 5% CO_2 atmosphere. After 20 h, medium with compounds were removed, and MTT was added to a final concentration of 0.5 mg/ml. MTT was incubated for a further 4 h, and the medium was then discarded. DMSO was added to dissolve the formazan product, and absorbance was measured at 570 nm after 15 min. Cell viability percentages were calculated by dividing the absorbance value of cells treated with a given compound by the absorbance of untreated cells.

3.4. Hemolysis assay

In the hemolysis test, peptides concentrations ranged from 32 to 1024 mg/L for peptides isolated from frog skins and from 0.5-256 mg/L for mastoparan and peptide 10. Peptides were incubated with washed human erythrocytes from a healthy donor in 100 µL of Dulbecco's phosphate-buffered saline (pH 7.4) for 1 h at 37◦C. Following centrifugation (12,000 x g for 15 s), the absorbance of the supernatant at 450 nm was measured. A parallel incubation in the presence of 1% v/v Tween 20 was carried out to determine the absorbance associated with 100% hemolysis.

3.5. *In vivo* toxicity assay

The Reed and Munch method (221) was applied. Groups of six C57BL/6 mice were intraperitoneally (i.p.) inoculated with a single 250 μL dose of peptide, starting at 32 mg/kg for mastoparan and peptide 10 and starting at 10 mg/Kg for MV5, this initial doses were further increased or decreased until 100% mortality was reached. Maximum tolerated dose (LD0), lethal dose 50 (LD50), and lethal dose 100 (LD100), were defined as those causing 0%, 50%, and 100% mortality, respectively.

3.6. Calculation bacterial minimal lethal dose (BMLD)

Three groups of four C57BL/6 mice were i.p inoculated with a single 250 μL dose of 10^5, 10^6 and 10^7 cfu/ml of a pan-resistant *A. baumannii* strain. The survival of the mice was monitored during one week.

3.7. Calculation of the Effective dose 50 (ED_{50})

Groups of different amount of mice were used in all the ED_{50} experiments, but the procedure was the same. C57BL/6 mice were inoculated i.p with the BMLD, and after certain time (1h, 2h and 4h was tested) an i.p dose of the peptide was administered. The evolution of the mice was monitored during one week. ED_{50} is achieved when the 50% of the mice survive to the infection.

3.8. Monitoring the infection

Four groups of three C57BL/6 mice were used for this experiment, all the mice were infected i.p with the BMLD, after 1, 2, 3 and 4h, each group was sacrificed at a different time point and lungs and spleen were aseptically removed and weighted, followed by an homogenization (2 min) in 2 mL of sterile saline solution (Tekmar Co.). After ten-fold dilution of the homogenate, 100 μL aliquots were plated on Columbia sheep blood agar and CFUs determined and expressed as log10 CFU/g of tissue. Number of bacteria in each organ at all the time points was calculated.

3.9. Murine pneumonia model

Seven groups of three C57BL/6 mice were used for this experiment. The animals were anaesthetized ip with 5% (w/v) sodium thiopental (Braun Medical, Spain) and intratracheally inoculated with the BMLD and different doses of the peptides were administered i.p. The first group was sacrificed and the amount of bacteria present in lungs was calculated with the same procedure explained above but just in lungs, the other six groups were the control and two peptides at 2h and 24h, at each time point the amount of bacteria in lungs was calculated.

3.10. Murine peritoneal sepsis model

Three groups of ten C57BL/6 mice were used for this experiment, all the mice were infected i.p with the BMLD, and 1h after the infection different doses of peptides were administered, after another hour the mice were sacrificed. The CFUs in peritoneal fluid were determined by i.p. injection of 2 ml sterile saline serum and, after mild abdominal massage, 1 mL of peritoneal fluid was aspirated with a sterile pipette tip. One hundred μL aliquots of serial ten-fold dilutions were plated on Columbia sheep blood agar and incubated at 37°C. CFUs were counted after 24 h and expressed as of log10 CFU/mL.

3.11. Leakage assay

Aliquots containing the appropriate amount of lipid in chloroform/methanol (1:1, v/v) were placed in a test tube, the solvents were removed by evaporation under a stream of O2-free nitrogen, and finally traces of solvents were eliminated under vacuum in the dark for more than 3 h. After, 1 ml of buffer containing 10 mM HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4 buffer and carboxyfluorescein at a concentration of 40 mM was added, and multilamellar vesicles were obtained. Large unilamellar vesicles (LUVs) with a mean diameter of 200 nm were prepared from the multilamellar vesicles by the LiposoFast device from Avestin, Inc., using polycarbonate filters with a pore size of 0.2 μm (Nuclepore Corp). Breakdown of the vesicle membrane leads to content leakage, i.e., CF fluorescence. Non-encapsulated CF was separated from the vesicle suspension through a Sephadex G-25 filtration column eluted with buffer containing 10 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA, pH 7.4. Leakage of intraliposomal CF was assayed by treating the probe-loaded liposomes (final lipid concentration, 0.125mM)

with the appropriate amount of peptide in Costar 3797 round-bottom 96-well plates, each well containing a final volume of 100 µl. The microtiter plate was incubated at RT for 1 h to induce dye leakage. Leakage was measured at various peptide concentrations. Changes in fluorescence intensity were recorded using the FL600 fluorescence microplate reader with excitation and emission wavelengths set at 492 and 517 nm, respectively. One hundred percent release was achieved by adding Triton X-100 to a final concentration of 1% v/v to the microtiter plates. Fluorescence measurements were made initially with probeloaded liposomes, afterwards by adding peptide and, finally by adding Triton X-100 to obtain 100% leakage. The results were expressed as percentage of CF released relative to the positive control (Triton X-100).

3.13. Transmission electron microscopy

Bacteria were grown in LB media and in the mid-log exponential phase of growth were incubated with mastoparan and peptide 10 for 1h at 37ºC, using the MIC concentration. A control without peptide was also performed. After the incubation, centrifugation at 3,500 rpm 4ºC was performed. The pellets were then fixed for 1h with 2% of gluteraldehyde, three times washed with water and fixed again with 1% of OsO_4. The post-fixation positive stain was carried out with 3% of uranyl acetate acqueous solution during 1.5 h, afterwards graded ethanol series (30, 50, 70, 90 and 100%) every 15 min were carried out in order to be dehydrated. The samples were embedded in a epoxy resin. Tecnai Spirit microscope (EM) (FEI) equipped with a LaB6 cathode. Images were acquired at 120 kV and room temperature with a 1376 x 1024 pixel CCD camera (FEI).

4. Proteomic approaches

4.1. iTRAQ

4.1.1. Protein extraction

An overnight culture of bacteria was grown in BHI (Becton and Dickinson), Fresh BHI was inoculated with a 1:50 dilution of the overnight culture and grown to a OD 0.6 aprox. at 37ºC with shaking. Bacteria were harvested by centrifugation at 3,500 rpm (Thermo Scientific) for 30 minutes and washed twice with 10 ml of 0.9% NaCl,

supernatant was discarded and the pellet was resuspended in Tris 10mM pH 8.0/NaCl 1% (Merck). Bacteria were incubated with lysozime and lysostaphine in order to weaken the cell membrane of the bacteria. Samples were sonicated at maximum Hz (Sonics) on ice during 10 minutes with intervals of 1 min. After the sonication, the extract was centrifuged 3,500 rpm and the pellet was discarded.

4.1.2. Digestion

Protein extraction samples from *S. mitis* were quantified using Micro BCA™ Protein Assay Kit (Thermo Scientific), and 300 μg of each were digested with trypsin using the filter aided sample preparation (FASP) digestion approach. Briefly, samples were diluted up to 500 μL with 500 mM triethyl ammonium bicarbonate (TEAB; pH 8.0-8.5), reduced with tris (2- carboxyethyl) phosphine (5mM; 60 min at 60 oC) and alkylated with iodoacetamide (30 mM; 20 minutes at 21 ºC in the dark). Afterwards, Samples were loaded onto an Amicon Ultra filter (10KDa, 0.5mL capacity; Millipore) to clean-up and denature sample proteins by two washes with 300 μL of 2M Urea/500 mM TEAB exchange buffer (12000g, 30 min, RT). Finally the filtered sample protein were resuspended on the filter with a 300 μL of a solution containing 1M Urea/ 500 mM TEAB, and then digested with 4μg trypsin/sample of porcine trypsin (Sequence grade modified Trypsin, Promega) at 37ºC for 16h. After tryptic digestion, the resulting peptide mixture was recovered by centrifugation (12,000g, 15 min, RT), and washing the filter twice with 200 μL of 500 mM TEAB solution as well. The peptide mixture were dried-down on a SpeedVac system and stored at -20ºC.

4.1.3. Peptide labeling

Samples were resuspended in 100 μL 1% formic acid (FA) solution and digests were desalted and concentrated by a C18 microcolunm (toptip, PolyLC) and eluted from the tip with 200 μL (2X 100 μL) of 70% acetonitrile (ACN)/0.1% FA and dried in SpeedVac. The peptide labeling was done with iTRAQ (iTRAQ™ 4plex Multiplex kit) according to the product specifications. Briefly, samples were resuspended in 60 μL 500 mM TEAB and divided by two to be labeled with two different markers. 70 μl of ethanol were added to each vial of iTRAQ labeling reagents, vortexed for 1 minute and spinned. The contents of label vials were transferred to the each sample tube, mixed

well and incubated at room temperature for 1 h. Samples were labeled as following: sample 351 was labeled with 114, and D6 G14 with 115 and 117. A small portion of each reaction was analyzed to ensure complete labeling. Reaction mixtures were diluted with 100 µl of water to quench the reaction and labeled samples were combined.

The cleanup of the samples was done in two steps: C18 clean-up (reverse phase, toptip C18) and then SCX cleanup (strong cationic exchange, P200 toptip, PolySULFOETHYL A). Firstly, samples were resuspended in 100 µL 1% FA solution, loaded to a C18 toptip, washed twice (50 µL 0.1% FA), eluted from the tip with 200 µL (2X 100 µL) of 70% ACN/0.1% FA and dried in SpeedVac. Then, samples were resuspended in 100 µL in 20% ACN/0.1% FA (pH 2.7-3), loaded to a SCX P200 toptip, washed (3X 50 µL 20% ACN/0.1% FA) and finally were eluted from the tip with 200 µL (4X 50 µL) 5% ammonium hydroxide/30 %methanol. 100 µL of water (HPLC grade) were added to the samples and then were dried down in SpeedVac.

4.1.4. LC-MS/MS analysis

The dried-down peptide mixture was analyzed in a nanoAcquity liquid chromatographer (Waters) coupled to a LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer. Tryptic labeled peptides were resuspended in 5% ACN/1% FA solution and an aliquot (500 ng) was injected for their chromatographic separation. Peptides were trapped on a Symmetry C18TM trap column (5µm 180µm x 20mm; Waters), and were separated using a C18 reverse phase capillary column (75 µm Øi, 25 cm, nano Acquity, 1.7µm BEH column; Waters). The gradient used for the elution of the peptides was 2 to 35 % B in 155 minutes, followed by gradient from 35% to 45% in 20 min (A: 0.1% FA; B: 100% ACN, 0.1%FA), with a 250 nL/min flow rate.

Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTipTM, New Objective) with an applied voltage of 2000V. Peptide masses (*m/z* 350-1700) were analyzed in data dependent mode where a full Scan MS in the Orbitrap with a resolution of 30,000 FWHM at 400m/z was obtained. Up to the 10 most abundant peptides (minimum intensity of 2000 counts) were selected from each MS scan and then fragmented using HCD (Higher Energy Collision Dissociation) in C-trap using nitrogen as collision gas, with 50% normalized collision energy and analyzed in the Orbitrap with a resolution of 7,500 FWHM at 400m/z. The scan time settings were:

Full MS: 250ms (1 microscan) and MSn: 300ms (2 microscans). Generated raw data were collected with *Thermo Xcalibur (v.2.1.0.1140).*

4.1.5. Database search

Thermo Proteome Discover *(v.1.3.0.339)* was used to generate a text file (mgf) to perform a decoy database search by Mascot search engine against NCBI database. Both target and decoy database were searched to obtain a false discovery rate (FDR), and thus estimate the number of incorrect peptide-spectrum matches that exceed a given threshold. From database search, Mascot generated a DAT file which was used to relative quantify the ratios based on reporter ions intensities.

4.2. Outer membrane protein (OMP) extraction

The protocol for the isolation of outer membrane proteins is the same as commented for the extraction of proteins for the iTRAQ, however extra steps to extract the outer membrane proteins from the rest of the proteins were needed. After the sonication the supernatant was ultracentrifuged (Thermo) during 45 minutes 70,000 x g at 4ºC, prior to be introduced in the ultracentrifuge it is important to check that all the tubes introduced have the same weight. After centrifugation, the supernatant was discarded and the pellet was resuspended with 100 µl of N-lauryl-sarcosyl 0.3% and let it react during 30 minutes at room temperature, the action of the N-lauryl-sarcosyl it to solubilize the inner membrane protein from the cell envelope proteins. The pellet, outer membrane proteins, was solubilized using the suitable buffer. Proteins were quantified using Bradford method.

4.3. One-dimensional SDS-PAGE.

Gel is prepared following the proportions showed in the table below, the first lower part of the SDS-PAGE gel is prepared the day before running the gel, and let it polymerize overnight. The upper part or stacking part is prepared the same day.

12.5%	Lower	Upper
H_2O	13.02mL	6.49 mL
40% Acrylamide/Bisacryilamide 37,5:1 (N,N-methylene-bis-acrylamide)	9.41 mL	1.002 mL
Upper (Tris 1.5 M pH 6.8 + SDS)	-	2.505 mL
Lower (Tris 1.5 M pH 8.8 + SDS)	7.53 mL	-
PSA 10%	0.3 mL	0.1 mL
TEMED	0.012 mL	0.01 mL

5 µg of outer membrane proteins were mixed with Laemmli buffer 5X, and boiled during 5 minutes. The samples were loaded into the gel and run at 25 mA until the blue front has overtaken the stacking gel, at that time the voltage can be increased to 30 mA. The electrophoresis was run until the blue tracking front arrived to 1 cm of the end of the gel.

After the electrophoresis was run, gel was removed from the cassette and silver stained. Briefly, the gel was fixed with ethanol (40%) and acetic acid (10%) during at least 30 minutes. This step can be extended to over-weekend. After the fixation step the gel was sensitized with Ethanol 30%, 2g sodium thiosulphate and 68g sodium acetate in 1 liter during another 30 minutes, three washes with water of 5 minutes each were performed previous to the incubation with the silver solution (2.5 g silver nitrate in 1 liter) during 20 minutes. Two fast washes of 1 minute were carried out previously to the development solution [50 gr sodium carbonate, 800 ul formaldehide 37% (Add just before use) in 1 liter] the time of incubation is depending on the abundance of proteins in the gel but normally is around 10 minutes or until the desired contrast is reached. At that time the gel is incubated with the stopping solution (14.6 gr EDTA-Na_2 in 1liter) during 20 minutes to finish the reaction. For silver staining high purity reagents were used.

4.4. 2D gel electrophoresis

The 2-DE was performed using GE Healthcare reagents and equipment. First-dimension isoelectric focusing was performed on immobilized pH gradient strips (24 cm, pH 3–10) using an Ettan IPGphor System (GE Healthcare). Samples were applied near the basic end of the strips by cup-loading, after being incubated o/n in 450 µl of rehydration

buffer (7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 2% ASB-14 w/v, 0.5% pharmalytes, pH 3–10, 100 mM DeStreak reagent). After focusing at 70 kVh, strips were equilibrated, first for 15 min in 10 ml of reducing solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 5 mg/ml dithiothreitol [DTT]) and then in 10 ml of alkylating solution (6 M urea, 100 mM Tris-HCl, pH 8, 30% v/v glycerol, 2% w/v SDS, 22.5 mg/ml iodoacetamide) for 15 min on a rocking platform. Second dimension SDS-PAGE was performed by laying the strips on 12.5% isocratic Laemmli gels (24×20 cm), cast in low fluorescence glass plates, on an Ettan DALT Six system. Gels were run at 20°C at a constant power of 2.5 W per gel for 60 min followed by 17 W per gel until the bromophenol blue tracking front had run off the end of the gel. Four replicas were run for each sample using independent biological replicates. Fluorescence images of the gels were obtained on a Typhoon 9400 scanner (GE Healthcare). Cy2, Cy3 and Cy5 images were scanned at excitation/emission wavelengths of 488/520 nm, 532/580 nm and 633/670 nm, respectively, at a resolution of 100 µm. Both image analysis and statistical quantification of relative protein levels were performed using Progenesis SameSpots V.4 (Nonlinear Dynamics). The data were analyzed as pairwise comparisons.

4.5. DIGE sample preparation

OMPs samples were resuspended in lysis solution (8 M urea, 2 M thiourea, 2.5% 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS), 2% ASB-14, 40 mM Tris-HCl, pH 8.8). ASB-14 was used to increase the presence of membrane proteins in the 2DE. Then samples were centrifuged in order to discard any insoluble debris. In order to remove salts and other contaminants, samples were cleaned with 2D Clean-Up Kit (GE Healthcare). Resulting pellets were resuspended in the above-mentioned lysis solution. Protein concentration was determined with 2D-Quant Kit (GE Healthcare) and adjusted to 2 mg/ml by the addition of a DIGE labeling buffer (7 M urea, 2 M thiourea, 2.5% w/v CHAPS, 40 mM Tris, pH 8.8). A pool consisting of equal amounts of each of the two samples analyzed in the experiment was prepared as an internal standard for quantitative comparisons. The colistin-resistant strain was labeled with Cy3 in two replicas and the colistin-susceptible was labeled with Cy5, while colistin-resistant strain was labeled with Cy5 in the other two replicas and the colistin-susceptible was labeled with Cy3. This experimental design avoids possible bias as

result of differences in the labeling reaction. A third fluorescent dye, Cy2, was used to label the internal standard sample. Labeling was carried out by the addition of 400 pmol of the required Cydye in 1 µl of anhydrous N,N-dimethylformamide per 50 µg of protein. After 30 min of incubation on ice in the dark, the reaction was quenched with 10 mM lysine and the samples incubated for a further 10 min. Samples were combined according to the experimental design, using 50 µg of protein per Cy dye per gel, and diluted two-fold with isoelectric focusing (IEF) sample buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% dithiothreitol [DTT], 2% pharmalytes, pH 3–10). One colistin-resistant and colistin-susceptible strain sample, together with an aliquot of the internal standard pool, were then separated by two-dimensional electrophoresis (2-DE) in each of the gels. This experimental design allows the accurate quantification and statistical assessment of the differences in protein abundances observed between the two sample groups.

For spot picking, gels were silver stained as described previously.

4.6. In gel triptic digestion

Silver-stained spots were destained with 200 µL of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate (1:1) for 20 min in the dark, and then spots were washed with Milli-Q water until they were completely clear. Before tryptic digestion, reduction and alkylation with DTT/IAA was performed by incubating samples with 200 µL of 10 mM DTT in 50 mM ammonium bicarbonate for 1 h at 56 °C, followed by alkylation with 200 µL of 55 mM IAA in 50 mM ammonium bicarbonate for 30 min, protected from light. Gel pieces were digested overnight with 6 ng/µL trypsin at 37 °C. The peptide extraction was carried out with three consecutive washes with 0.2% TFA for MALDI-TOF identifications. The eluted peptides were dried in a SpeedVac and stored at −20 °C until they were analyzed by mass spectrometry.

4.7. Protein identification by MALDI-TOF

For MALDI analysis, 1 µL of sample was mixed with the same volume of a solution of α-cyano-4-hydroxy-trans-cinnamic acid matrix (0.5 mg/mL in ethanol/acetone 6:3) and spotted onto a MALDI target plate (Bruker). The drop was air-dried at room temperature. MALDI-mass spectra were recorded in the positive ion mode on an

Ultraflex Extreme time-of-flight instrument. Ion acceleration was set to 25 kV. All mass spectra were externally calibrated using a standard peptide mixture containing angiotensin II (1046.54), angiotensin I (1296.68), substance P (1347.74), bombesin (1619.82), rennin substrate (1758.93), adrenocorticotropic hormone 1-17 (2093.09), adrenocorticotropic hormone 18-39 (2465.20), and somatostatin 28 (3147.47). Calibration was considered good when a value below 1 ppm was obtained. For PMF analysis, the MASCOT search engine (Matrix Science) was used with the following parameters: one missed cleavage permission, 50 ppm measurement tolerance, and at least four matching peptide masses. Cysteine carbamidomethylation was set as fixed modification when appropriate, with methionine oxidation as the variable modification. Common contaminants were removed using the contaminants database available in the Mascot search engine. Searches were carried out using the NCBInr July 2014 database. Positive identifications were accepted with a Mascot score higher than that corresponding to a P value of 0.05.

5. Molecular biology tools

5.1. Polymerase chain reaction (PCR)

PCR of the *lpx*ACD and *pmr*ABC genes of three colistin-susceptible and three colistin-resistant *A. nosocomialis* strains were performed.

These are the primers used for the PCRs which were designed specifically for this study.

Primers	**Forward**	Annealing Temperature (ºC)	**Reverse**	Annealing Temperature(ºC)
*pmr*A	ATGACAAAAATCTTGATGATTGAAGAT	59.2	TTATGATTGCCCCAAACGGTAG	60.3
*pmr*B	GACTTCATGAACGTGCAT	51.6	CAAGAGCTTAGTTAAGCTC	53.0
*pmr*C	CAGCAGTTTCTGTGCCATGT	58.4	TTTTGCATAGCCAAGTTGACC	57.4
*lpx*A	CCTTATTGTATTATTGGTCC	52.3	CGTACAATTCCACGCTC	52.4
*lpx*C	GCGAGTGGAATAGGTCTT	53.8	CGTATGGAATTGGACAGTC	55.2
*lpx*D	GCCTATGACGCTAAGTATGA	55.3	TCTGCATTGGTAATTCAGGG	55.3

Three or four colonies were added to 50 µl of water, the mixture was incubated during 10 minutes at 96ºC in order to destroy all the bacteria, centrifugation at 12,000 rpm for 10 min. was performed and the pellet was discarded.

Reagent	Volume
DNA	5 µl
Primer forward (5 µM)	5 µl
Primer reverse (5 µM)	5 µl
dNTPs (2 mM)	5 µl
Mg_2Cl (15 mM)	5 µl
Buffer	10 µl
Taq polymerase	0.4 µl
H_2O	14.6 µl

A mixture of the reagents indicated above was performed before the PCR.

The PCR conditions were as follow:

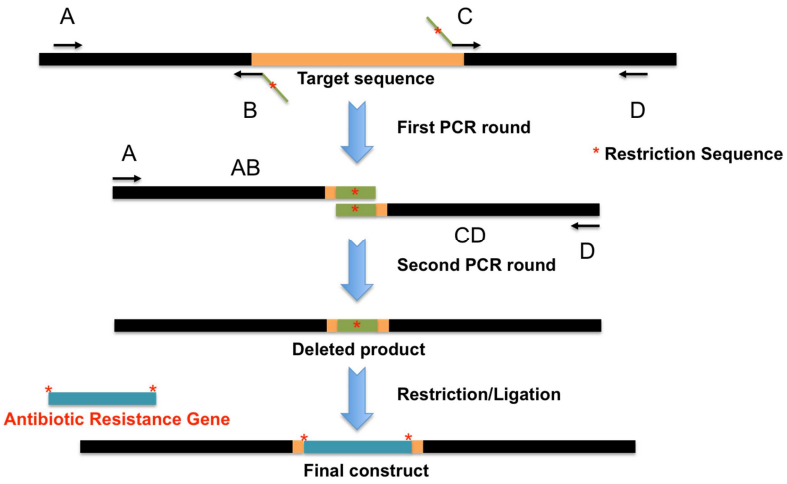
Temperature	Time	
95ºC	3 min	
95ºC	30 seg	X 30 cycles
Annealing temperature	2 min	
72ºC	1.5 min	
72ºC	7 min	
12ºC	∞	

The PCR products were loaded into a 1% agarose (Conda) gel, and runned the electrophoresis until the end of the agarose gel. Due to the present of Sybr Safe (Invitrogen) into the gel it was possible to observe the DNA fragment amplified, these PCR products were removed from the agarose gel and purified using the kit (Omega). Once the PCR product was clean, it was send to (Beckman Coulter Genomics) for elucidation of the sequence amplified.

5.2. Preparation of the knockout genes

The nucleotide sequences of the target genes were used to design flanking primers pairs to perform a two-step nested PCR mutagenesis reaction. The general procedure for the two-step nested PCR mutagenesis is depicted in **Fig. 2** and it involves designing two flanking primer pairs on either side of the target sequence to create an internal deletion and introduce a restriction site to allow the insertion of a selection marker cassette.

Primers **B** and **C** in showed in the figure below contain an extra sequence tail complementary to each other and incorporate a restriction site (BamHI). The complementary sequences allow the overlap of the PCR products after the first round of amplification (using primer pairs **A/B** and **C/D**). The resulting PCR products from the first round of amplification were then mixed together in the second PCR round using only primers **A** and **D**. The overlapping ends allow both fragments to hybridise creating a final PCR product with a deleted region within the target sequence. The restriction site introduced during the PCR reaction allowed the subsequent insertion of a selection marker by BamHI enzymatic digestion/DNA ligation. In this study we introduced an *armA* cassette encoding a 16S rRNA methylase that confers resistance to all aminoglycosides.



Name	Primer forward	Annealing T (ºC)	Primer Reverse	Annealing T (ºC)
Immunoprot Upstream	GAACCTTATT GGACTCTTAC G	50.4	CAGCTAGACAG TCGATGGATCC TGGATTTGCTC AACGCT	66.7
Immunoprot Downstream	GGATCCATCG ACTGTCTAGC TGCAACTACA TCATCTGGAA GC	66.4	AAGTGCTCCAA GCCTATCTC	54.3
Membraneprot Upstream	GGAGATGTGA TGAAGTTACG	50.4	CAGCTAGACAG TCGATGGATCC GCTGATGTTAG ATGACGAC	66.2
Membraneprot Downstream	GGATCCATCG ACTGTCTAGC TGTTGAAACC TCCTTTGGTG C	67.1	TCGGCGGATAT TTACTCT	49.9

Construction of knock-out strain in the gens that encoded the up-regulated poteins found in the iTRAQ assay was performed following the procedures explained before. The PCR products after the first round of amplification were loaded into an agarose gel (1 %) and two bands corresponding with the expected size were recovered and gel-purified.

Next step was to perform the second PCR round by adding 1 µl of each PCR product recovered in the previous step. Upon loading an agarose gel, a PCR product of the expected size (containing the target flanking sequences as well as an internal deletion) was observed and purified from the gel. This truncated gene was inserted into a cloning vector (pGEM-T) by A/T cloning, since the terminal transferase activity of the Taq DNA polymerase adds a single 3´-A overhang to each end of the PCR product.

For the insertion of the PCR product of the truncated gene into the plasmid (1µl of the PCR product of the truncated gene, 1µl pGEM-T, 1µl T4 DNA ligase, 2µl H_2O, 5µl buffer 2X) overnight at 16ºC.

The plasmid with the inserted truncated gene was transformed using competent cells of *E. coli* DH5α strain. Transformants were selected into LB agar plates containing 50 µg/ml ampicillin (pGEM-T plasmid contain an ampicillin resistant gene) and a cell-cracking procedure was used to confirm carriage of the plasmid. Cracking method consist in adding several colonies to 25µl of H_2O, afterwards 25µl of cracking lysis buffer (860µl H_2O, 40µl NaOH (5M), 20µl SDS 10%) was added and incubated at room temperature for 5 minutes. The mixture was then loaded into a 0.7% agarose gel, and an intense band belonging to the plasmid should be observed. As soon as the presence of the plasmid was confirmed, an overnight bacterial culture in the presence of ampicillin was set up. An extraction of the plasmid from the overnight culture was carried out using a commercial plasmid extraction kit (Promega). The plasmid was digested with BamHI (1µl buffer 10X, 6µl H_2O, 2 µl plasmid and 1µl enzyme) and a BamHI-digested *armA* cassette (encoding a 16S rRNA methylase that confers resistance to all the aminoglycosides) was ligated with the previous construct (2µl T4 DNA ligase buffer 10X, 2µl *armA*, 2µl plasmid, 1µl T4 DNA ligase, 13µl H_2O). The ligation reaction was performed at 16ºC for 4 h after which it was introduced into *E. coli* DH5α competent cells as explained above. Transformants were selectec on LB-agar plates containing 50 50 µg/ml kanamycin. The presence of the plasmid was checked as before and plasmids extracted from the *E. coli* cells were used to naturally transform an overnight culture of *S. mitis* (351). *S. mitis* is a natural transformant and no competent cells are needed, only

the action of a stimulating peptide (Genscript) to enhance the efficiency of transformation (222, 223). Mutants arisen from a double crossover event were further selected on LB agar plates supplemented with gentamicin and evaluated by PCR after DNA extraction using a standard kit (Promega).

5.3. Preparation of competent cells

An overnight culture of *E. coli* DH5α was diluted 1/1000 in LB and was incubated at 37ºC until OD of 0.6 aprox. was achieved. Bacteria were incubated on ice during 15 minutes, and centrifuge at 7,000 rpm during 10 minutes at 4ºC. Supernatant was discarded and the cells were resuspended in 1/10 of the previous volume of 0.05M $CaCl_2$ -glycerol 25% (cold). Store in 200 µl aliquots at -80ºC.

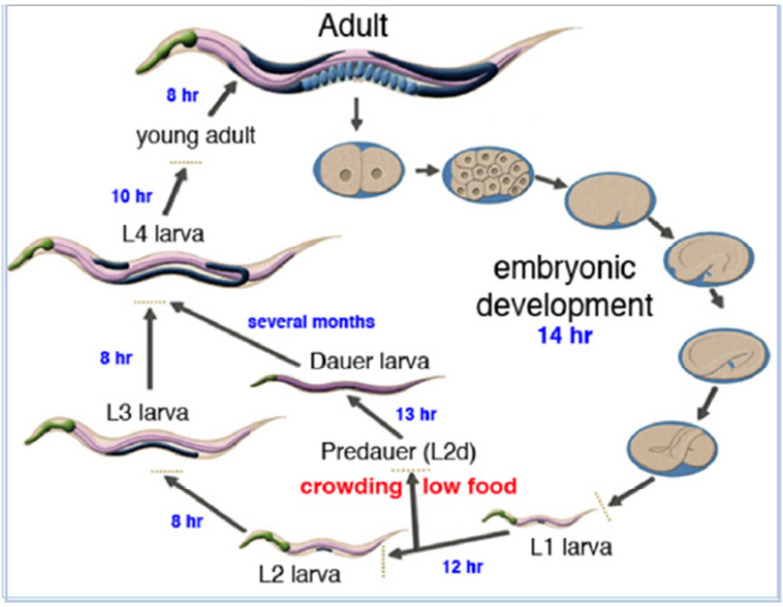
5.4. Genome sequence

DNA from the strains sequenced was extracted using the DNA extraction kit (Promega). Genomic DNA from *A. nosocomialis* strains was fully sequenced by pyrosequencing using the genome sequencer Titanium (454 Life Sciences). A library of paired-end fragments was created following the manufacturer's instructions (454 Life Sciences). This library was sequenced using the GS Titanium sequencer (454 Life Sciences). Reads originated from each strain were assembled into contigs using Newbler 2.53 (454 Life Sciences). The assembly was verified using the CLC genomics software (CLC Bio). Predicted protein proteins were compared against a nonredundant Genbank database using BLASTP for functional annotation.

6. Other methodologies.

6.1. Caenorhabditis elegans model

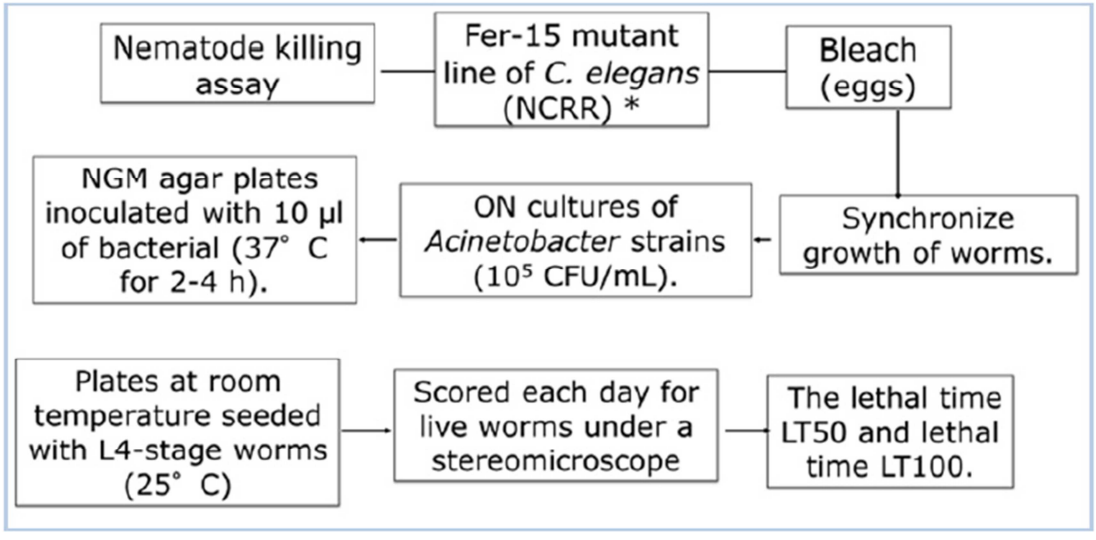
The aim of using the *in vivo* model of *C. elegans* was to have an idea of the virulence of the generated mutants compared to the original strain. The *C. elegans* killing assay was performed using *E. coli* OP50 as a control, and the two *A. nosocomialis* strains (colisitin-susceptible and colistin-resistant).



C. elegans life cycle at 22ºC. (artwork by Altun and Hall, Wormatlas)

C. elegans embryos develop rapidly and hatch after 14 hours. The first larval stage is completed after another 12 hours and the animals proceed through four molt cycles before becoming adults. Under crowded conditions or in the absence of food larvae can choose an alternative development pathway leading to the dauer larva, which does not feed but can survive adverse conditions for several months. When life gets better normal development is resumed, the animals exit the dauer larval stage and develop into the normal fourth larval stage before becoming adult. Adult animals are hermaphrodites and produce both sperm and eggs. Over the course of 3-4 days some 300 eggs are laid. The overall life span of *C. elegans* is 2-3 weeks.

The method used in the killing assay is showed in the figure below.



6.2. Quantitation of the LPS

The procedure to determine the amount of LPS present in a certain bacteria was the commercial Kit QCL-1000 Limulus amebocyte assay from Lonza, following the instructions.

6.3. Docking and molecular modeling

Molecular docking is a computational method which predicts the preferred orientation of one molecule (ligand) to a second (protein) when bound to each other to form a stable complex. This knowledge is used to predict the strength of association (binding affinity) between the two molecules using scoring functions.

We used Glide XP scoring function and molecular docking software from SCHRODINGER package (www.schrodinger.com). Protein structure was obtained with the help of homology modeling technique. Homology modeling constructs a model of the "*target*" protein from its amino acid sequence and an experimental three-dimensional structure (X-Ray crystal or NMR) of a related homologous protein (the "*template*").

I-TASSER online server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used for homology modeling. The amino acid sequence is taken from Genbank, and template structures used in the modeling are taken from the ompA structures present in the PDB. Best ranked model was prepared (all preparation was done with SCHRODINGER including short MD simulations) for docking calculations. EXORIS library was used for docking.

6.4. Bacterial adherence

An overnight bacterial culture was grown in LB (Luria Broth), at the same time 10^5 cells (A549, pulmonary cells) were seeded in a 24 wells microplate, DMEM together with bacteria and different concentration of peptides were incubated with cells at 37ºC during 2h, after two hours the supernatant was separated and cells were treated with EDTA and trypsin, this mixture was also treated with 0.5% of Triton X-100 to disrupt all the cells, microdilutions to the extract were performed prior to be seed into blood agar plates in order to count the amount of bacteria present in each well, 100% of bacterial adherence is considered when the peptide was not added

6.5. Adherence to fibronectin

An overnight incubation of 1.25 μg of fibronectin in 1X of PBS in a 96 well plate was performed to allow fibronectin to bind to the internal wall of the well. Next day BSA (Bovine serum albumin) was added for 1h in order to block the free positions present in the 96 wells plate, after 1h, bacteria together with different concentrations of the peptides were added during 2 hours, after that supernantant was removed and 0.5% of triton X-100 was added to 96 well plate, microdilutions were performed and seeded into blood agar plates, which were incubated during 24h at 37ºC. 100 % adherence was considered when no peptide inhibitor was incubated.

6.6. Cell viability analysis

The procedure of determining the cell viability is similar to the MTT assay, however bacteria are incubated together with the peptide, the rest of the procedure is the same.

6.7. Pharmacokinetic assay

A dose of 10 mg/Kg was injected into 30 mice, and 3 of them were sacrificed at a different time points (5, 10, 15 and 30 minutes, and 1, 2, 4, 6, 12 and 24 hours). The maximum blood of the mice was extracted and incubated with EDTA, afterwards the serum was obtained from the blood. Once the serum was obtained , the rest of the protocol was the same as the calculation of the stability of the peptide. However, once the peptide was in solution, a certain amount of pure peptide (in a knowledge concentration) was added, therefore in the HPLC the peak will increase and we will have the contribution of the same peptide from two different sources, this fact will allow us to calculate the concentration of peptide we will have in serum at a certain time point.

Resum

Introducció.

Avui en dia estem davant d'un problema molt greu d'abast mundial, aquest problema té dos factors importants que són: i. el augment d'infeccions resistents a la majoria dels antibiòtics disponibles. ii. la poca inversió per part de la indústria farmacèutica en el desenvolupament de nous antibiòtics. A l'últim informe de la OMS es va alertar sobre aquesta situació. Tenint en conte la situació mencionada anteriorment es va anomenar ESKAPE (***E****nterococcus faecium*, ***S****taphylococcus. aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, ***E****ntereobacter* species) al grup de bacteris més resistents. El grup ESKAPE està composat per bacteris tant Gram-positius com Gram-negatius, i realment son dianes molts atractives per al disseny de nous fàrmacs.

Una altre factor important a tindre en conte es l'escala de resistència d'un bacteri en front a certs tipus d'antibiòtics. S'ha definit com a multi resistència quan el bacteri es no sensible a ≥ 1 agent en ≥ 3 categories d'agents antimicrobians, el següent grau de resistència es el de resistència extensa, el qual es defineix com a no sensible a ≥ 1 agent de com a màxim dues categories, i per últim pan resistència, que es defineix com la resistència a tots els tipus i famílies d'antibiòtics. Fins fa uns anys no hi havia controvèrsia en el tema de definir el grau de resistència del antibiòtics degut a que no s'havien aïllat soques resistents a tots els antibiòtics, però en els últims anys el nombre de soques pan resistents han augmentat considerablement sobretot *A. baumannii*, fent necessari que hi hagi una definició del grau de resistència d'una bacteri.

Es pot apreciar l'evolució d'*A. baumannii* en els estudis realitzats, on es pot veure el augment de la resistència a imipenem del 3 al 70% entre el període de 1993 i 2007, el mateix efecte s'observa en quinolones amb un increment del 30 al 97% durant el mateix període de temps.

Com hem comentat anteriorment hi ha una necessitat urgent de nous antibiòtics, i especialment per antibiòtics actius en front a bacteris Gam-negatius degut al fet que els últims antibiòtics llençats al mercat (daptomicina i linezolid) només son efectius en front bacteris Gram-positius. Tot i esser els últims antibiòtics llençats al mercat ja s'han aïllat soques que presenten resistència a aquests dos antibiòtics.

Com em comentat anteriorment ens centrarem en *Acinetobacter*, que són principalment patògens nosocomials i oportunistes, això vol dir que normalment afecten a pacients amb un sistema immunològic deteriorat. Entre el 2 i el 10 % de les infeccions produïdes a les UCI son causades per aquest patogen. Solen ser una de les causes més freqüents en pneumònia però també estan implicats en bacterièmia, infeccions del tracte urinari o meningitis.

El gènere *Acinetobacter* està format per diverses especies, les quals tenen en comú que son coc-bacils Gram-negatius, no fermentadors, incapaços de reduir nitrats, catalasa positius, oxidasa negatius, i sense motilitat, d'aquí es d'on prové el nom *Acinetobacter*, que es deriva del terme grec (*a-kineto*) que significa no mòbil, per això indica que aquest gènere no te motilitat en superfícies. Actualment, al 2014, 34 especies diferents del gènere *Acinetobacter* han estat definides.

Les colònies formades per les especies pertanyents al gènere *Acinetobacter* son d'un color groc però poden arribar tindre un color blanc grisós i entre 1 i 2 mm de diàmetre. El test d'oxidasa es el emprat per diferenciar el gènere *Acinetobacter* d'altres bacteris Gam-negatius no fermentadors. Per a un aïllament ràpid de soques clíniques s'utilitza un medi sòlid selectiu que no permeti el creixement d'altres microorganismes, tipus Herellea agar o medi Leeds *Acinetobacter*.

Les dues característiques mes importants que fan que *Acinetobacter* sigui un gènere tan resistent es l'habilitat per desenvolupar patrons de multi resistència i l'habilitat per sobreviure en diferents condicions. Les especies d'*Acinetobacter* poden ser trobades al sòl, aigua, animals o humans, creixent normalment a totes les mostres aïllades del sòl o l'aigua. Els llocs on han sigut aïllades aquests bacteris son tan diversos com aigua fresca natural, mostres d'aire d'hospitals o fins i tot a escoles o escoles bressol. Aquesta alta resistència a qualsevol condició ambiental prové de la habilitat d'emprar diferents fonts de carboni, això es pot veure clarament en la comparació entre les soques tipus (ATCC) i les soques dels aïllats clínics.

Si comparem la capacitat de supervivència amb altres bacteris la diferencia es notable essent al voltant de 24 hores per *E. coli* i menys de 24 hores per *P. aeruginosa*, quan a les mateixes condicions qualsevol bacteri pertanyent al gènere *Acinetobacter* sol viure varis dies inclús arribant a 25 com en el cas d'*A. baumannii*.

Els factors de virulència són necessaris per la patogènia d'infeccions causades per *A. baumannii*, aquest factors son claus alhora d'infectar i colonitzar el hoste. Hi ha molts tipus de factors de virulència, com per exemple el biofilm, el qual es defineix com una matriu polimèrica formada per carbohidrats, proteïnes, àcids nucleics i d'altres macromolècules que manté units els bacteris un cop adherits a la cèl·lula del hoste i els ajuda a sobreviure. Un altre factor de virulència es el de la motilitat i adherència, com hem comentat anteriorment el gènere *Acinetobacter* manca de motilitat degut a l'absència de flagels, però això no implica que no tingui mobilitat tipus twiching, degut a la presencia de pilis.

El lipopolisacàrid (LPS) present a la membrana del bacteri també es un factor important de virulència, com es pot apreciar en les soques d'*Acinetobacter* tant *baumannii* com *nosocomialis*, la pèrdua del LPS implica també una perduda de la virulència de la soca. Un altre component de les membranes dels bacteris Gram-negatius, com son les outer membrane proteins (OMPs), son també importants factors de virulència, on cal destacar OmpA, que s'ha descrit com la proteïna involucrada a l'adherència dels bacteris a les cèl·lules del pacient infectat.

Hi han altres factors de virulència que no pertanyen a la estructura de la bacteri sinó que son elements mòbils com son les outer membrane vesicles, que son nano vesícules esfèriques compostes per DNA, RNA, lípids, LPS o inclús OMP, la seva funció principal es la de transportar factors de virulència. Un altre factors important es l'acció dels sideròfors, compostos que tenen l'habilitat de quelar el ferro, que com es conegut es un factor determinant en el creixement bacterià i per últim, el quòrum sensing, que es un sistema de comunicació entre bacteris depenent de la densitat cel·lular o la fase de creixement, aquestes molècules poden controlar diversos factors de virulència comentats anteriorment tals com el biofilm o la motilitat.

Com s'ha comentat anteriorment, *Acinetobacter* te una facilitat innata per adquirir elements de resistència a tot tipus d'antibiòtics.

La resistència a β-lactamics, pot esser de dos tipus, el mediat per un enzim (β-lactamases), el qual actua obrint l'anell β-lactamic de la molècula hidrolitzant-lo i així desactivar l'efecte antibacterià del compost, o per altre banda, mecanismes no enzimàtics.

Hi ha diferents tipus de β-lactamases depenent del compost sobre el que actuen, hi ha per exemple les AmpC que actuen inhibint penicil·lines i cefalosporines d'espectre estès excepte cefepime, o les oxacilinases capaces d'inhibir l'efecte de l'oxacilin i el cloxacilin i per últim les metallo-β-lactamases, esteses mundialment i caracteritzades pel seu ampli espectre d'acció degut a que son capaces d'inhibir tots els antibiòtics β-lactamics excepte aztreonam.

Els mecanismes no enzimàtics estan relacionats amb alteracions a la membrana com poden ser la sobre expressió de bombes de flux o alteracions a les OMPs, o fins i tot modificacions a les PBPs (Penicilin binding proteins).

La resistència a aminoglicòsids també està dividida en dos tipus de mecanismes diferents com son les acetiltransferases, nucleotidiltransferases o fosfotransferases, enzims que modifiquen el grup amino o hidroxil dels aminoglicòsids, i per altre banda, les bombes de flux en especial la bomba AdeABC que confereix resistència a aminoglicòsids apart d'altres tipus d'antibiòtics.

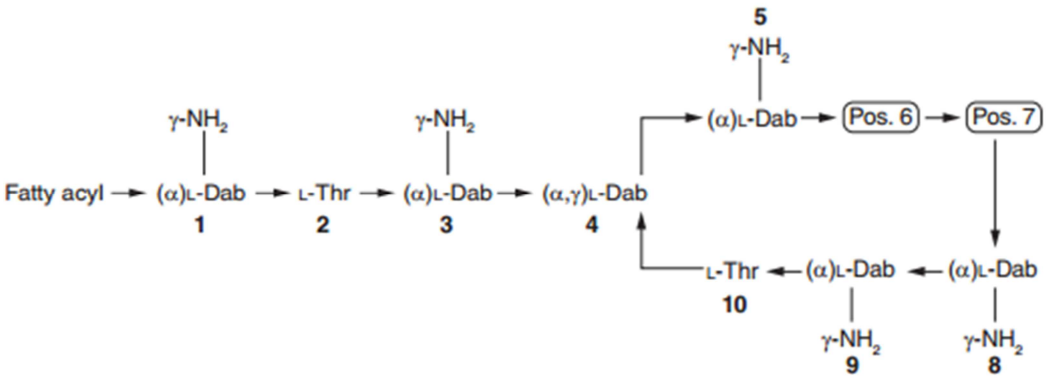
La resistència a quinolones ve donada per mutacions, com succeix a totes les especies, a la zona QRDR (Quinolone-resistance determinig region) de la subunitat A de la DNA girasa i de la DNA topoisomerasa IV. Paral·lelament a aquestes mutacions també s'han descrit diverses bombes d'expulsió involucrades en la resistència a quinolones.

La resistència a polimixines estan basats principalment en la modificació de gens que estan involucrats en algun procés de la síntesi d'algun component del LPS del bacteri, com per exemple mutacions al sistema regulador de doble component *pmrABC*, que esta relacionat amb la modificació del lípid A del LPS o mutacions als gens *lpxACD* que estan directament relacionats amb la biosíntesi del lípid A.

Actualment dos tipus d'antibiòtics peptídics, colistina i daptomicina, son emprats per a tractar certes infeccions produïdes per bacteris Gram-negatius i Gram-positius respectivament.

Colistina es un antibiòtics peptídic aïllat als anys seixanta però reemplaçat pels aminoglicòsids als setanta degut a la seva nefrotoxicitat. Però en els últims anys degut al increment de la resistència dels antibiòtics emprats s'ha hagut de tornar a reutilitzar aquest tipus d'antibiòtic, però tot i així ja s'han aïllat diverses soques resistents a colistina. Arrel de la reutilització de la colistina com a antibiòtic, els estudis de la seva

toxicitat s'han intensificat, trobant que la toxicitat d'aquest compost està al nivell d'altres antibiòtics utilitzats com la tobramicina, o que realment lo important es la dosi acumulada més que la dosi diari administrada, per tant ara es coneix com aquesta toxicitat es totalment reversible amb el temps.



Polymyxin	Fatty acyl group	Pos. 6	Pos. 7
B_1	(*S*)-6-methyloctanoyl	D-Phe	Leu
B_1-Ile	(*S*)-6-methyloctanoyl	D-Phe	Ile
B_2	6-methylheptanoyl	D-Phe	Leu
B_3	Octanoyl	D-Phe	Leu
B_4	Heptanoyl	D-Phe	Leu
B_5	Nonanoyl	D-Phe	Leu
B_6	3-hydroxy-6-methyloctanoyl	D-Phe	Leu
E_1	(*S*)-6-methyloctanoyl	D-Leu	Leu
E_2	6-methylheptanoyl	D-Leu	Leu
E_3	Octanoyl	D-Leu	Leu
E_4	Heptanoyl	D-Leu	Leu
E_7	7-methyloctanoyl	D-Leu	Leu
E_1-Ile	(*S*)-6-methyloctanoyl	D-Leu	Ile
E_1-Val	(*S*)-6-methyloctanoyl	D-Leu	Val
E_1-Nva	(*S*)-6-methyloctanoyl	D-Leu	Nva
E_2-Ile	6-methylheptanoyl	D-Leu	Ile
E_2-Val	6-methylheptanoyl	D-Leu	Val
E_8-Ile	7-methylnonanoyl	D-Leu	Ile

Pos.: Amino acid position.

Figura. R.1. Estructura químicade la colistina i els anàlegs aïllats

L'estructura química de la colistina està formada per un decapèptid cíclic i un àcid gras, la unió per formar el cicle està entre el àcid diaminobutíric de la posició 4 i la treonina de la posició 10. De totes les polimixines aïllades la única variació present, son els 2 aminoàcids de la posició 6 i 7 i l'àcid gras.

La colistina o polimixina E, té una D-Leu a la posició 6 i un Leu a la posició 7. S'ha de tindre en conte que la colistina inclou diferents especies però una gran majoria (més del 80%) pertanyen a la mescla entre polimixina E_1 i polimixina E_2.

Com s'ha comentat anteriorment el mecanisme de resistència a colistina esta lligat d'alguna manera al LPS i per això el mecanisme d'acció de les polimixines està relacionat amb la membrana dels bacteris Gram-negatius tot i que no està del tot clar quin es el mecanisme exacte.

Un altre pèptid emprat com a antibiòtic es la daptomicina, aquest antibiòtic va ser aprovat per la FDA al 2003, té una estructura molt semblant a la de les polimixines, amb un pèptid cíclic i un àcid gras. La part peptídica esta composada per 13 aminoàcids, 10 d'ells estan formant un cicle, la ciclació del pèptid es produeix entre els aminoàcids L-Kyrunenine (residu 13) i L-treonina (residu 4).

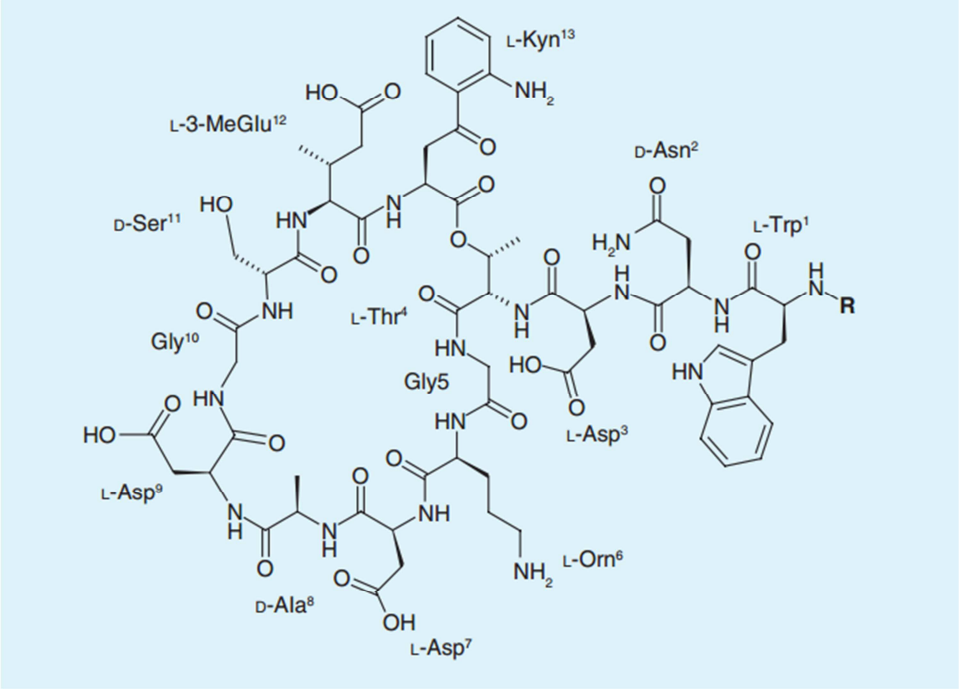
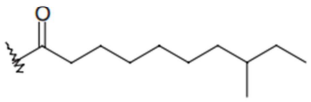
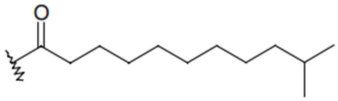
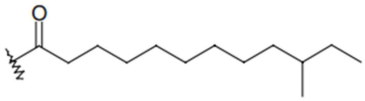
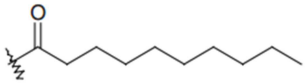


Figura. R.2. Estructura química de la daptomicina

Compound	R	MIC (μg/ml) *Staphylococcus aureus*
1 A21978C$_1$		1
2 A21978C$_2$		0.5
3 A21978C$_3$		0.13
4 Daptomycin		0.125–0.5

Taula.R.1 Diferents cadenes d''acid gras de la daptmicina relacionat amb la seva activitat en front *S. aureus*.

Diferents compostos varen ser aïllats al mateix temps que la daptomicina, la única diferencia entre ells es l'estructura de l'àcid gras, però de tots ells la daptomicina era el que presentava un millor balanç entre toxicitat i activitat.

El mecanisme d'acció de la daptomicina està relacionat amb la membrana dels bacteris Gram-positius però tal com passa en el cas de les polimixines, el mecanisme complert no ha estat elucidat, de totes maneres es coneix que l'acció de la daptomicina esta intrínsecament relacionat amb la concentració de Ca^{2+} present al sèrum.

To i que la daptomicina es un antibiòtic relativament nou, varis casos de soques resistents s'han aïllat en *S. aureus* i en *Enterococcus*. Com en el cas de la colistina, tots els bacteris aïllats resistents a daptomicina presenten algun tipus de modificació relacionat amb la membrana.

Fins ara només em comentat sobre els problemes que hi ha en el tractament d'infeccions però no em proposat alternatives als antibiòtics ja emprats, aquí es descriuen diferents opcions pel tractament d'*A. baumannii*.

Antimicrobial peptides.

Hi ha diferents maneres per obtindré un pèptid actiu, alguna d'elles son utilitzant extractes naturals i fent un "screening" en front el bacteri desitjat, o d'altres maneres com pot esser la dissenyar el compost d'acord amb una diana concreta. El ús de pèptids com a agents antibacterians té certs avantatges com són el ampli espectre d'acció, o la inespecificitat de la diana que fa que sigui més difícil d'adquirir resistència, també te certs desavantatges com son la baixa estabilitat en sèrum humà i la seva ràpida excreció.

Algunes soques comercials han estat testades en front de soques d'*A. baumannii*, amb diferents perfil de resistència de les soques emprades, els millors resultats s'observen en el cas de buforin II i magainin II, amb una MIC_{50} i MIC_{90} de 2 i 16 mg/l respectivament, la mateixa MIC_{90} es observada en el cas de cecropin P1 i melittin però la MIC_{50} presenta una dilució més (4 mg/l). Cecropin A, indolicidin i ranalexin presenten uns valors de MIC més elevats. Dos dels pèptids comentats anteriorment, cecropin A (CA) i melittin (M), van ser barrejats per obtindré una millor activitat en front a soques d'*A. baumannii* resistents a colistina. CA (1-7)M(2-9) va ser testat en front de soques multi resistents i en soques colistina resistents d'*A. baumannii*. El millor resultat es el obtingut en front de soques colistina resistents amb uns valors de MIC_{50} i MIC_{90} de 2 i 4 mg/L respectivament, comparat amb la soca multi-resistent (MIC_{50} 4 mg/l, MIC_{90} 12 mg/l).

La font d'on els pèptids antimicrobians poden ser trobats es molt diversa des de secrecions de pell de granota a verí d'escorpí australià.

Fins ara només em parlat de pèptids naturals, tot i així pèptids totalment sintètics han estat també testats en front soques d'*A. baumannii*, com es el cas d'un pèptid format només per lisines i leucines (LKLLKKLLKKLLKLL), el mateix pèptid però amb un 33% de D-aminoàcids va ser sintetitzat i també té una millor activitat 5.6 mg/l. Altres pèptids que contenen majoritàriament lisines i leucines van ser sintetitzats però en aquest cas una o dues cisteïnes van ser funcionalitzades. $C(LLKK)_2C$ i $(LLKK)_2C$ van ser sintetitzats i testats amb valors de MIC molt elevats però tot i així deguts a la seva poca toxicitat *in vivo*, presenten una alta efectivitat quan son provats en ratolins.

Polimixines.

Com s'ha comentat anteriorment, un dels problemes de la utilització de les polimixines es la seva nefrotoxicitat, per aquest motiu una de les opcions es intentar sintetitzar

anàlegs de les polimixines intentant reduir la seva nefrotoxicitat i augmentar la seva activitat en front soques tant sensibles com resistents a colistina d'*A. baumannii*. Una de les opcions per augmentar l'efectivitat del compost es introduir nous grups hidrofòbics a les posicions 6 i 7 i a la cua d'àcid gras, es important mencionar que l'activitat d'aquests nous compostos es independent de la MIC a colistina per part de la soca. Un altre grup va escollir una altre estratègia que va ser canviant el Dab de la posició 3 per un Dap (àcid 2,3-diaminopropionic) i sobre aquest esquelet canvien la cua d'àcid gras, es pot veure com l'anàleg amb la cua com a 6-oxo-1-fenil-1,6-deshidropiridina-3-carbonil, té millor resultats *in vitro* però *in vivo* falla en comparació amb la polimixina B.

A part dels típics compostos antimicrobians, hi ha altres maneres de tractar les infeccions causades per *A. baumannii*.

Una de les opcions es la utilització de compostos capaços de quelar el ferro, que com hem comentat anteriorment es realment important pel creixement bacterià. Una opció alternativa a quelar el ferro es la de substituir al catió Fe^{3+} pel Ga^{3+}, on es produeix una competició degut a que tenen la mateixa valencia i semblant radi atòmic. La diferencia es que el Ga^{3+} no pot fer les funcions que fa el ferro i per això el bacteri mor a causa de la deficiència de ferro.

Una altre tipus de teràpia alternativa es inhibint la generació de biofilm, on s'ha trobat un compost marí amb l'habilitat per inhibir el biofilm i posteriorment ha estat optimitzat obtenint un compost amb una activitat elevada.

Es conegut que els fags poden tindre la capacitat de llisar les bactèries, per tant es un altre alternativa d'ús en front de soques d'*A. baumannii*, on després d'èsser optimitzat s'han assolit bons resultats *in vivo*.

L'ús de vacunes com a prevenció per l'adquisició d'infeccions causades per *A. baumannii* es una altre bona estratègia, tenint en conte que l'adquisició d'aquest tipus d'infeccions be donada en llocs i en pacients molt puntuals. Fins ara diferents tipus de vacunes en front *A. baumannii* han sigut testades assolint resultats *in vivo* força bons.

L'última teràpia alternativa i potser la més innovadora es la teràpia fotodinamica, on l'idea es generar oxigen reactiu que interactuarà amb el DNA o proteïnes de membrana i així destruint el bacteri.

Mecanisme de resistència a daptomicina en *S. mitis*.

Com hem comentat breument, el mecanisme d'acció de la daptomicina es a nivell de membrana dels bacteris Gram-positius, per això el mecanisme de resistència del bacteri ha d'estar lligat d'alguna manera a la membrana, així que la nostra primera hipòtesi va ser la de veure si es produïa un augment del gruix de la paret cel·lular del bacteri quan s'adquiria resistència a daptomicina. Es van analitzar el gruix de la paret cel·lular de 3 soques resistents a daptomicina i una soca sensible a daptomicina i els resultats obtinguts son els següents.

a) 351 b) D6-2

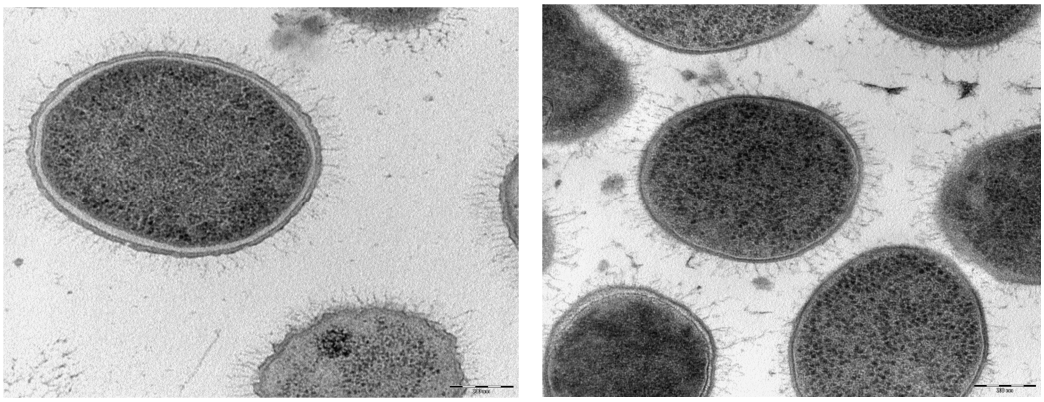


Figura.R.3. a) TEM de la soca *S. mitis* susceptible a daptomicina. b) TEM de la soca *S. mitis* resistent a daptomicina

Soques	Gruix de la membrana (nm)	MIC a daptomicina (mg/L)
351 (daptomycin S)	20.03±1.47	0.38
D6-2 (daptomycin R)	25.21±2.05	>256
D6-7 (daptomycin R)	22.35±2.84	>256
D6-14 (daptomycin R)	24.44±2.56	>256

Taula.R.2. Gruix de la membrana del bacteri relacionat amb la seva activitat en front daptomicina

Es pot apreciar com hi ha un lleuger augment en el gruix però sense arribar a ser significativament diferent. En altres bacteris resistents a daptomicina tals com *S. aureus*, no sempre es produeix un augment de la paret cel·lular quan la soca adquireix resistència a daptomicina per tant es un factor secundari.

Com a segona hipòtesi vàrem decidir utilitzar tècniques de proteòmica comparativa per veure si hi havia alguna proteïna interesant que varies el seu nivell d'expressió significativament, per això es va emprar el mètode d'ITRAQ, i els resultats obtinguts es poden veure a les taula inferior.

Diferencia Dapto R/Dapto S	Proteïna identificada
0,24	NADP-specific glutamate dehydrogenase [Streptococcus agalactiae COH1]
0,30	PF08006 family protein [Streptococcus oralis SK100]
0,36	30S ribosomal protein S3 [Streptococcus pneumoniae TIGR4]
0,55	PTS system transporter subunit IIB [Streptococcus pneumoniae TIGR4]
0,62	glutamate--ammonia ligase [Streptococcus oralis ATCC 35037]
0,64	ABC transporter, ATP-binding protein, partial [Streptococcus oralis SK255]
0,65	peptidyl-prolyl cis-trans isomerase [Streptococcus sp. oral taxon 071 str. 73H25AP]
0,65	peptidyl-prolyl cis-trans isomerase [Streptococcus oralis ATCC 35037]
0,69	ABC superfamily ATP binding cassette transporter, binding protein [Streptococcus sanguinis ATCC 49296]
0,72	hypothetical protein HMPREF0851_00776 [Streptococcus sp. M334]
0,73	PTS family maltose and glucose porter, IIABC component [Streptococcus oralis ATCC 35037]
0,74	peptidyl-prolyl cis-trans isomerase [Streptococcus sp. GMD6S]
1,19	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase [Streptococcus oralis ATCC 35037]
1,22	serine protease HtrA [Streptococcus oralis ATCC 35037]
1,27	phosphotransacetylase [Streptococcus mitis B6]
1,29	putative PTS system, IIC component [Streptococcus sp. M143]
1,33	**conserved hypothetical protein [Streptococcus oralis ATCC 35037]**
1,36	L-lactate oxidase [Streptococcus pneumoniae G54]
1,36	**group B streptococcal surface immunogenic protein [Streptococcus sanguinis ATCC 49296]**
1,48	glycogen biosynthesis protein,glucose-1-phosphate adenylyltransferase [Streptococcus oralis Uo5]
1,50	pyruvate oxidase [Streptococcus oralis SK100]
1,59	glucose-1-phosphate adenylyltransferase [Streptococcus oralis ATCC 35037]
2,26	**group B streptococcal surface immunogenic protein [Streptococcus mitis ATCC 6249]**

Taula. R.3.Proteines identificades en *S. mitis* amb un grau diferent d'expressió entre soques susceptible i resistent a daptomicina

Es pot apreciar com hi ha moltes proteines que varien el seu nivell d'expressió però ens centrarem només en aquelles que estiguin relacionades amb la membrana i puguin tindre algun homòleg en altres bacteris que estigui implicat en la resistència a daptomicina.

Aquestes proteïnes son la conserved hypothetical protein que esta sobre expressada 1,33 vegades, i la group B streptococcal surface immunogenic protein que esta sobre expressada 2,26 vegades, aquesta última proteïna conté un domini amb homologia a la proteïnaYvqF/VraSR involucrada a la resistència a daptomicina en certs bacteris, i la hypothetical protein te un grau d'homologia amb un domini de lisina motif que es pot trobar en enzims involucrats en la degradació de la paret cel·lular

Un cop em decidit que aquestes podrien ser les proteïnes involucrades en la resistència a daptomicina un knockout dels gens que les codifiquen s'han dut a terme per comprovar que realment esta involucrades, les construccions plasmídiques s'ha dut a terme perfectament però la transformació en *S. mitis* no funcionà però futurs experiments es duran a terme per verificar la funció d'aquestes proteïnes.

Mecanisme de resistència a colistina en *A. nosocomialis*.

Soques resistents a colistina d'*A. nosocomialis* va ser generades *in vitro* degut al fet que mai ha estat descrit resistència a colistina per part d'aquest tipus d'espècie d'*Acinetobacter*. La curiositat d'aquests mutants generats va ser la seva alta tolerància a colistina sense generar resistència, a 8mg/L de colistina on hi ha un punt d'inflexió en que la MIC passa de ser menor a 1 mg/l a 128 mg/l.

Aquests mutants generats presenten una disminució considerable de la MIC a gairebé tots els antibiòtics testats a part d'una disminució important de la virulència en el model de *C. elegans*.

Tenint en conte que mai abans s'ha descrit el mecanisme de resistència a colistina en soques d'*A. nosocomialis* , la única referencia es la de resistència a colistina en soques d'*A .baumannii*, els operons *pmrABC* i *lpxACD*, son els descrits que presenten mutacions que confereixen resistència a colistina. En el operó *pmrABC* no es pot apreciar cap tipus de mutació entre la soca colistina sensible i la soca colistina resistent.

En canvi al operó *lpxACD*, es poden veure mutacions presents al gen *lpxD*, a més a més d'un codó stop a la proteïna lpxD, mai descrit anteriorment. El operó *lpxACD* està directament involucrat en la síntesi del LPS de la membrana del bacteri Gram-negatiu, aquestes mutacions i el codó stop porten a una modificació del procés de síntesis d'una part del LPS que porta a la inexistència de LPS en les soques colistina resistents, com s'ha comprovat utilitzant un kit comercial.

També s'ha comprovat que les mutacions presents al gen *lpxD*, estan presents a totes les soques resistents a colistina i desapareixen quan les soques son sensibles a colistina.

Per una altre part també s'han dut a terme estudis de proteomica comparatius entre la soca sensible i la soca resistent. Primer es van comparar mitjançant gels bidimensionals però posteriorment es va realitzar pel mètode comparatiu de DIGE , aquí es pot observar el gel resultant i les proteïnes amb variació en l'expressió.

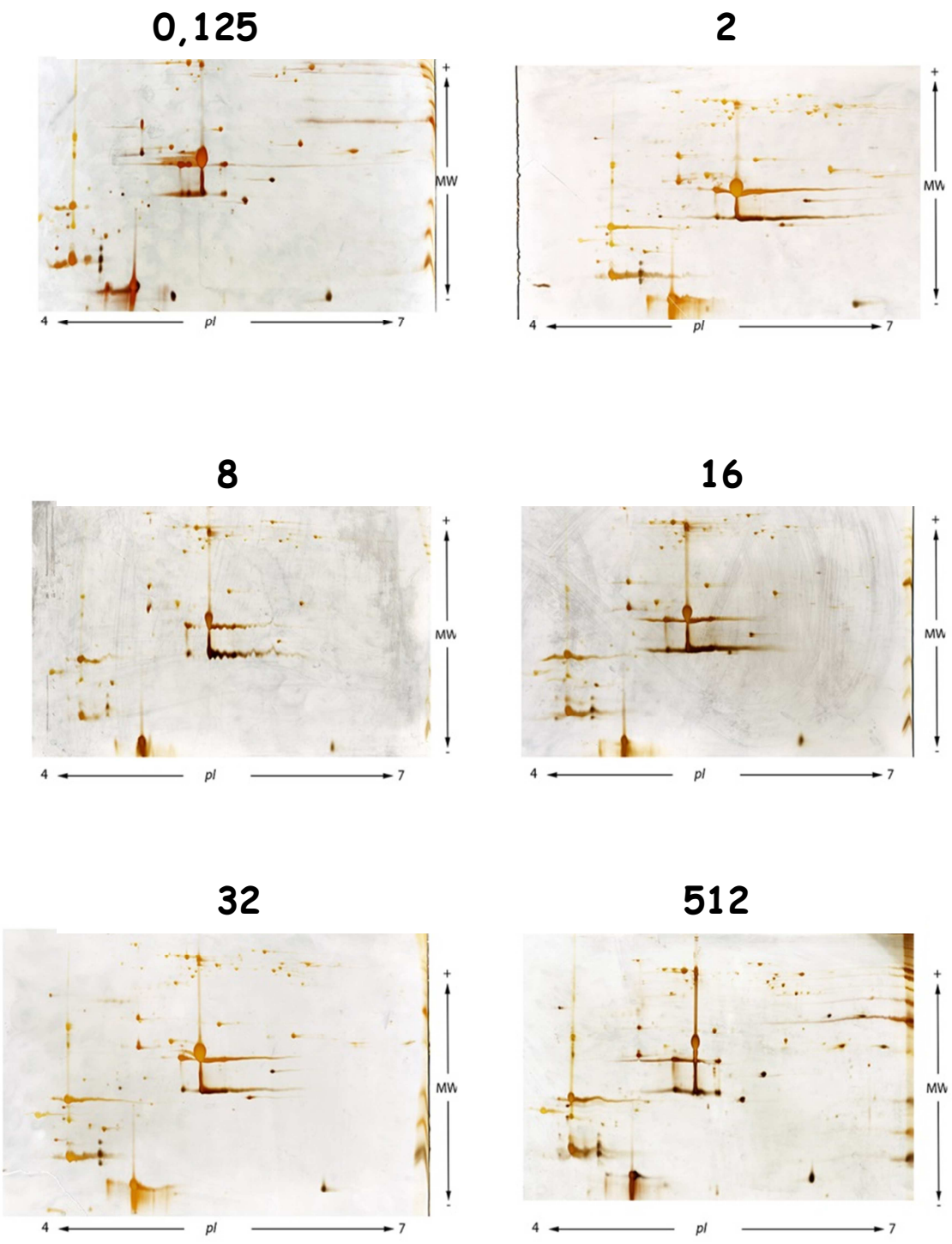


Figura.R.4. Gel bidimensional de proteïnes de soques d'*A. nosocomialis* colistina sensibles (0.125/2/8) i colistina resistents (16/32/512).

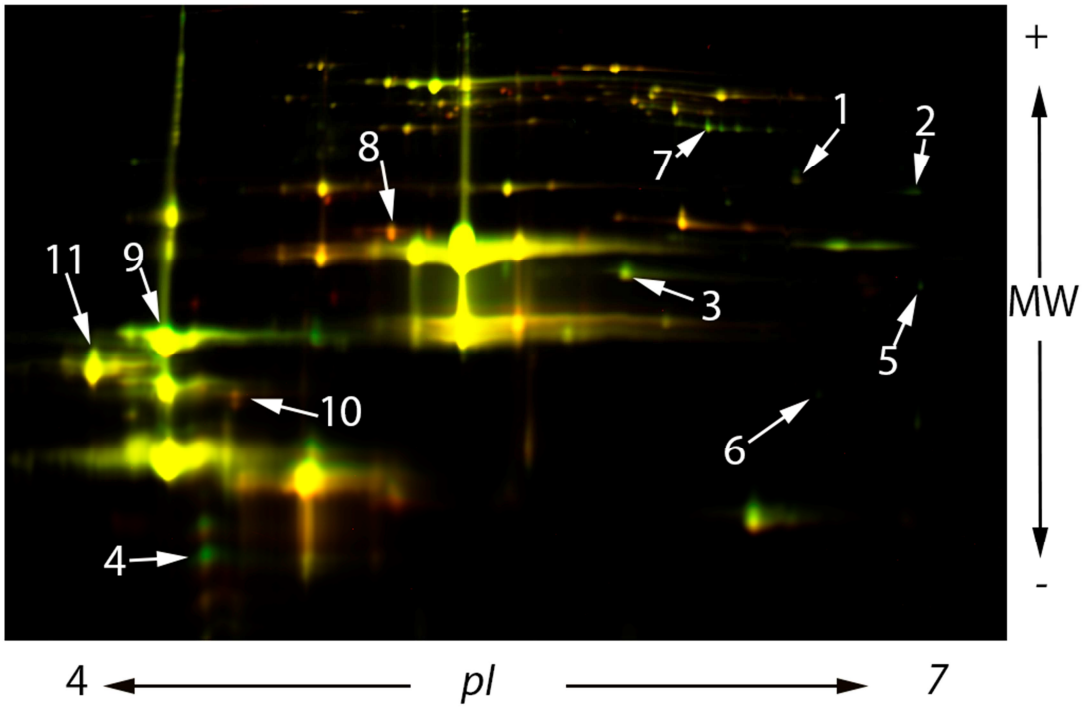


Figura.R.5. Gel comparatiu de DIGE.

Número spot	Proteïna id	Proteïna	Canvi a l'expressió*	Anova P-valors
1	gi|407441282	OmpA_C-like	1.3	0.0239
2	gi|490848590	hypothetical protein F984_02367 (NodT family RND efflux system)	2.1	0.00911
3	gi|497190250	hypothetical protein (tetratricopeptide repeat family protein)	1.5	0.0167
4	gi|593656836	putative carbapenem-associated resistance protein (CarO)	1.7	0.00914
5	gi|446899273	OmpW-like protein	1.4	0.0274
6	gi|354459714	OmpA (Isoform)	2	0.0233
7	gi|487978330	succinate dehydrogenase flavoprotein subunit	1.7	0.000768
8	gi|487978520	Porin (OprB) Carbohydrate-selective porin	-1.9	0.00533
9	gi|493628869	membrane protein (outer membrane beta-barrel domain protein)	1.4	0.0774
10	gi|487981035	signal peptide protein	-1.2	0.0237
11	gi|587819016	putative porin	1.1	0.0613

Taula.R.4. Proteines identificades en *A. nosocomialis* amb un grau diferent d'expressió entre soques susceptible i resistent a colistina

Aquí es pot veure el grau d'expressió comparativa de les proteïnes de membrana entre les soques de colistina sensible i colistina resistent d'*A. nosocomialis*. Gairebé totes les proteïnes estan sobre expressades en la soca resistent a colistina, que contradiu totalment els resultats publicats per colistina resistent en *A .baumannii*, però aquesta diferent expressió de proteïnes ja es pot apreciar quan es comparen una soca sensible amb una multi-resistent, així que aquestes variacions en l'expressió de les proteïnes siguin degudes al canvi en les MICs dels altres antibiòtics.

Fins ara només hem estat comentant els mecanismes de resistència de dos dels antibiòtics peptídics emprats habitualment pel tractament d'infeccions, però per altre part també es important proposar alternatives per a poder solucionar el problema de la resistència.

En aquesta tesi em utilitzat dos tipus d'aproximació diferents,. La primera aproximació, es la típica aproximació provant diferents compostos fins a trobar-ne un d'actiu en front la soca desitjada i posterior optimització, i per altre banda la segona aproximació es més innovadora inhibint la virulència creada per la proteïna OmpA.

Pèptids i peptidomimetics actius en front de soques d'*Acinetobacter* resistents a colistina.

Hi ha pocs compostos testats *in vitro* actius en front de soques *d'Acinetobacter* resistents a colistina, per tant diferents pèptids comercials van ser provats en front de dues soques d'*A. baumannii*, una sensible a colistina i l'altre resistent. Els millors resultats pertanyen als pèptids mastoparan, melittin i indolicidin. Aquest últim amb MICs de 8 i 16 mg/l en soques colistina sensible i colistina resistent respectivament. Melittin té una MIC de 4 mg/l quan es testat en front d'una soca sensible a colistina i disminueix fins a 2 mg/l quan la soca es resistent, i per últim mastoparan, amb la mateixa MIC en soques sensibles a colistina i una MIC d'1 mg/l quan la soca es resistent a colistina. Dos dels tres pèptids amb millor activitat, melittin va ser descartat degut a la seva toxicitat, va ser testats en front de més soques d'*A. baumannii* tant sensibles com resistents a colistina. Els resultats obtinguts son molts semblants als que es pot apreciar al screening inicia, essent més elevats en el cas d'indolicidin comparat amb mastoparan, per això ens centrarem en mastoparan.

Corbes de letalitat de diverses concentracions de mastoparan en front de soques d' *A. baumannii* colistina sensible i colistina resistent va ser dutes a terme, on es pot apreciar el comportament bacteriostàtic a 8 vegades la MIC en soques d'*A. baumannii* colistina

sensible i colistina resistent i a 4 vegades la MIC en soques colistina resistents sense observar cap recreixement.

Hem escollit mastoparan com el pèptid candidat a ser optimitzat, i com hem comentat anteriorment la baixa estabilitat en sèrum es un dels inconvenients d'utilitzar els pèptids com a fàrmacs. L'estabilitat del mastoparan en sèrum va ser calculada i els punts més susceptibles a proteases. L'estabilitat obtinguda va ser sorprenent tenint en conte que es un pèptid lineal (6h) però tot i així la seva estabilitat va ser optimitzada tenint en conte que l'únic lloc observable susceptible a proteases era la leucina present al extrem N-terminal. Sis pèptids anàlegs juntament amb el mastoparan van ser sintetitzats.

		Concentració mínima inhbitoria mg/l (μM)					
	Antimicrobial peptide	**CR17**	**CR86**	**Ab11**	**Ab113**	**Estabilitat en sèrum humà**	**Toxicitat MTT IC_{50} (HeLa)**
1	Gu-INLKALAALAKKIL	4 (2,6)	4 (2,6)	4 (2,6)	4 (2,6)	> 24h	13 μM
2	iNLKALAALAKKIL	32 (21,6)	32 (21,6)	64 (43,3)	64 (43,3)	> 24h	41 μM
3	InLKALAALAKKIL	32 (21,6)	64 (43,3)	128 (86,5)	256 (173)	> 24h	73 μM
4	NLKALAALAKKIL	32 (19,2)	64 (38,4)	128 (76,8)	128 (76,8)	> 24h	109 μM
5	Ac-INLKALAALAKKIL	16 (10,5)	32 (21)	64 (42)	64 (42)	> 24h	29 μM
6	**Mastoparan**	**4 (2,7)**	**4 (2,7)**	**4 (2,7)**	**4 (2,7)**	**6h**	**32 μM**
7	inLKALAALAKKIL	128 (86,5)	128 (86,5)	128 (86,5)	256 (173)	> 24h	21 μM

Taula.R.5. Activitat en front soques amb alta resistència d'A. baumannii, estabilitat en sèrum humà i citotoxicitat en HeLa de diferents anàlegs del mastoparan i el mastoparan.

El pèptid 1, que porta un grup guanidini al extrem N-terminal es el únic compost que presenta una activitat igual que el mastoparan i a més a més te una estabilitat major a les 24h, però té una toxicitat més elevada comparat amb el mastoparan.

Tenint en conté els resultats obtinguts als primers anàlegs i informació obtinguda a la literatura sobre toxicitat d'anàlegs del mastoparan, set nous anàlegs van ser sintetitzats.

		Concentració mínima inhbitoria mg/l (μM)					
	Antimicrobial peptide	**CR17**	**CR86**	**Ab11**	**Ab113**	**Estabilitat en sèrum humà**	**Toxicitat MTT IC_{50} (HeLa)**
8	LIKKALAALAKLNI	256 (173)	256 (173)	256 (173)	256 (173)	2h	192 μM
9	likkalaalaklni	256 (173)	256 (173)	256 (173)	256 (173)	> 24h	200 μM
10	inlkalaalakkil	4 (2,7)	4 (2,7)	4 (2,7)	4 (2,7)	> 24h	10 μM
11	INLKAKAALAKKIL	256 (171,5)	256 (171,5)	256 (171,5)	256 (171,5)	6h	290 μM
12	in(or)kalaalakkil	128 (86,5)	64 (43,2)	64 (43,2)	128 (86,5)	>24h	200 μM
13	INLKALAALAKKIL-$CH_2CH_2NH_2$	4 (2,6)	4 (2,6)	2 (1,3)	4 (2,6)	6h	5 μM
14	LIKKALAALAKLNI-$CH_2CH_2NH_2$	64 (42)	128 (84,1)	128 (84,1)	64 (42)	2h	150 μM

Taula.R.6. Activitat en front soques amb alta resistència d'A. baumannii, estabilitat en sèrum humà i citotoxicitat en HeLa de diferents anàlegs del mastoparan i el mastoparan

En aquest cas 2 anàlegs conserven l'activitat del mastoparan, el seu enantiòmer (pèptid 10) i el mastoparan original amb una càrrega positiva extra al extrem C-terminal (pèptid 13), aquests dos pèptids, com passa amb el pèptid 10, tenen una toxicitat superior al mastoparan i el pèptid 10 una estabilitat superior (>24h) però el pèptid 13 igual que el mastoparan.

Dels quatre pèptids obtinguts amb activitat s'havia d'escollir quins son els que s'ha de provar *in vivo*, ens vàrem decantar pel mastoparan degut a que es el menys tòxic i pel pèptid 10 que al estar format per D-amino àcids es molt més estable tot i ser més tòxic.

Aquests dos pèptids van ser provats *in vivo* en tot tipus d'experiments sense veure cap tipus d'activitat antibacteriana, la qual cosa ens va fer sospitar sobre alguns tipus d'unió a alguna proteïna, per aquest motiu vàrem fer assajos d'inhibició en presencia de sèrum i vàrem veure com la MIC dels dos pèptids pujava progressivament amb la concentració de sèrum a l'assaig.

MIC mg /l(µM)	CR17				Ab14			
% of Sèrum	**0%**	**25%**	**50%**	**75%**	**0%**	**25%**	**50%**	**75%**
Mastoparan	4(2.7)	16(10.8)	64(43.3)	128(86.5)	4(2.7)	32(21.6)	128(86.5)	128(86.5)
Pèptid 10	4(2.7)	16(10.8)	64(43.3)	128(86.5)	2(1.35)	32(21.6)	64(43.3)	128(86.5)

Taula.R.7. Activitat de mastoparan i pèptid 10 en front soques pan-resistents d'*A. baumannii* en presencia de sèrum.

Com hi ha moltes proteïnes presents al sèrum vàrem testar en front albúmina que es una de les més abundants.

MIC mg/l (µM)	CR17				Ab14			
% of albúmina	**0%**	**25%**	**50%**	**75%**	**0%**	**25%**	**50%**	**75%**
Mastoparan	4(2.7)	16(10.8)	32(21.6)	64(43.3)	4(2.7)	16(10.8)	32(21.6)	64(43.3)
Peptide 10	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)	4(2.7)

Taula.R.8. Activitat de mastoparan i pèptid 10 en front soques pan-resistents d'*A. baumannii* en presencia de d'albumina.

Es pot observar com la MIC de mastoparan augmenta quan augmenta la concentració de sèrum però en canvi amb el pèptid 10 la concentració roman constant, això ens indica que mastoparan té una unió amb l'albúmina però el pèptid 10 amb alguna altre proteïna present al sèrum més minoritària.

També s'han estudiat els mecanismes d'acció dels anàlegs a mastoparan sintetitzats utilitzant el mètode de leakage i els pèptids utilitzats *in vivo*, per microscòpia electrònica de transmissió.

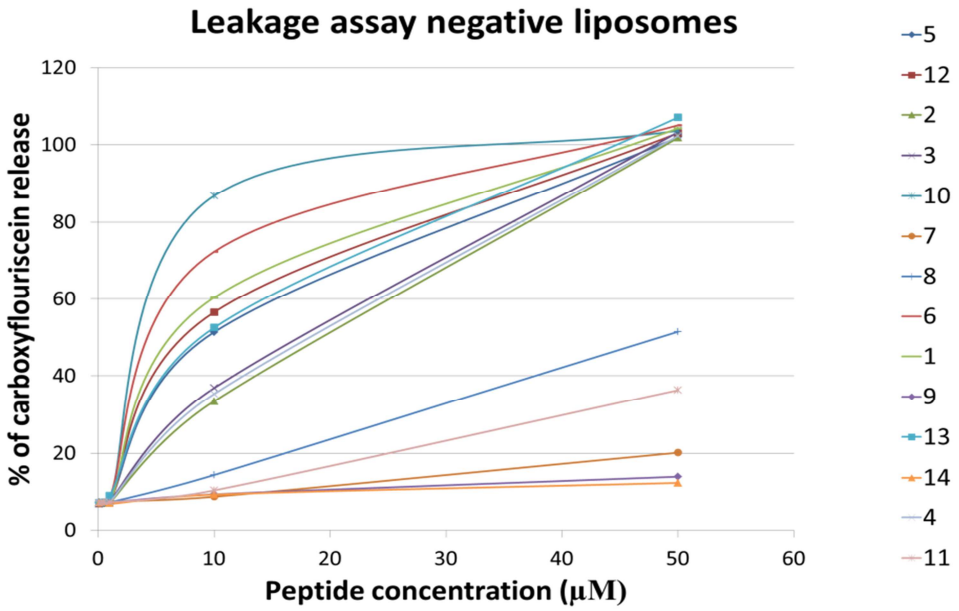


Figura R.6. Percentatge d'alliberació de carboxifluorisceina en liposomes negatius de tots els pèptids sintetitzats a diferents concentracions.

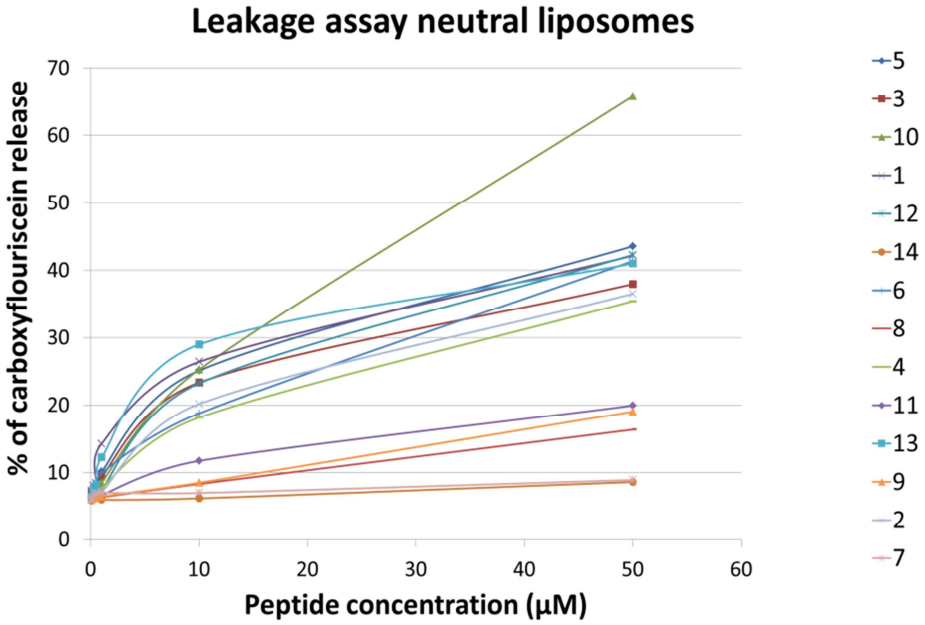


Figura.R.7. Percentatge d'alliberació de carboxifluorisceina en liposomes neutres de tots els pèptids sintetitzats a diferents concentracions.

En els assajos de leakage es pot apreciar com els pèptids que tenen millor activitat *in vitro*, també son els que alliberen més fluorofor del liposoma. Els assajos de leakage

s'ha dut a terme utilitzant dos tipus de lípids diferents, un conjunt de lípids amb carrega global negativa, mimetitzant la membrana d'*A. baumannii*, on es pot veure una activitat força elevada dels millors pèptids, en canvi quan la carrega global del liposoma es neutre el efecte dels pèptids es molt menor inclús nul per algun pèptid.

Utilitzant microscòpia electrònica de transmissió es pot apreciar comparant el control sense pèptid amb els bacteris incubats tant amb mastoparan com amb pèptid 10 uns forats a la membrana del bacteri.

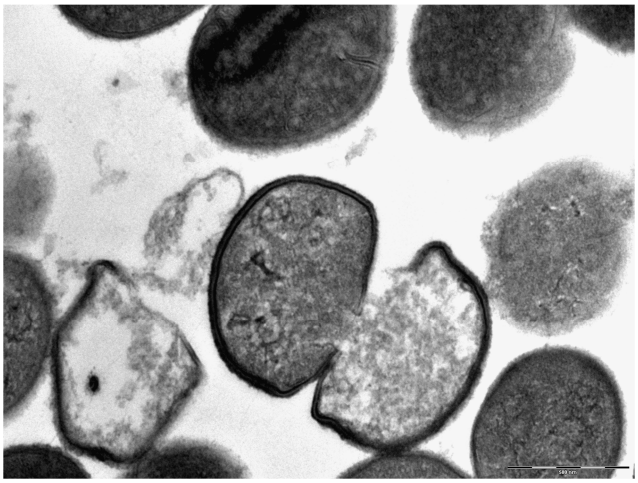


Figura.R.8. Imatge utilitzant TEM del efecte del mastoparan sobre una soca d'*A. baumannii*

En aquesta part de la tesi em trobat un compost actiu *in vitro* i la posterior optimització, tot i no tindre èxit *in vivo*, hem trobat els motius pels quals aquests compostos no són actius i d'aquesta manera es poden trobar diferents alternatives o modificacions per a poder fer aquests pèptids actius *in vivo*.

Hem utilitzat una altre tipus d'estratègia que es provant pèptids naturals extrets directament del cru natural, en aquest cas vàrem fer una col·laboració amb el Prof. Conlon de la universitat del Emirats àrabs. Diferents pèptids aïllats de secrecions de pell de granota vàrem ser provats en front de soques d'*Acinetobacter* especies tant resistents com sensibles a colistina.

Pèptid	***Acinetobacter* sp.Strains**			**colistina**	**sensible**			
mg/L (μM)	**19606**	**77778**	**G13**	**NM8**	**NM35**	**NM75**	**NM109**	**NM124**
[G4K]XT-7	32(17.3)	32(17.3)	4(2.2)	32(17.3)	32(17.3)	32(17.3)	16(8.7)	16(8.7)
[E4K]alyteserin-1c	8(3.5)	8(3.5)	8(3.5)	8(3.5)	16(7)	8(3.5)	4(1.8)	4(1.8)
PGLa-AM1	64(30.9)	64(30.9)	128(61.9)	16(7.7)	16(7.7)	16(7.7)	16(7.7)	16(7.7)
B2RP-Era	32(17)	32(17)	8(4.2)	32(17)	32(17)	16(8.5)	32(17)	32(17)
CPF-AM1	64(39.5)	64(39.5)	128(78.9)	16(9.9)	16(9.9)	16(9.9)	16(9.9)	16(9.9)
[D4K]B2RP	16(6.9)	8(3.4)	8(3.4)	8(3.4)	8(3.4)	8(3.4)	4(1.7)	4(1.7)
Colistin	0.5(0.43)	2(1.7)	<0.5(<0.43)	1(0.86)	1(0.86)	1(0.86)	<0.5(<0.43)	<0.5(<0.43)

Taula.R.9. Assaig d'activitat de diferents pèptids aïllats de les secretions de les granotes en front soques d'*Acinetobacter* susceptible a colistina

Pèptid	***Acinetobacter* sp.Strain**		**colistina**	**resistent**		
(mg/L)	**19606 mutant**	**77778 mutant**	**G13 mutant**	**Ab22P**	**Ab1**	**Ab113**
[G4K]XT-7	4(2.2.)	32(17.3)	4(2.2)	8(4.3)	32(17.3)	64(34.7)
[E4K]alyteserin-1c	4(1.8)	8(3.5)	8(3.5)	4(1.8)	8(3.5)	16(7)
PGLa-AM1	16(7.7)	64(30.9)	16(7.7)	16(7.7)	64(30.9)	128(61.9)
B2RP-ERa	8(4.2)	64(34)	8(4.2)	8(4.2)	32(17)	64(34)
CPF-AM1	4(2.5)	64(39.5)	8(4.9)	8(4.9)	64(39.5)	128(78.9)
[D4K]B2RP	4(1.7)	16(6.9)	16(6.9)	8(3.4)	16(6.9)	16(6.9)
Colistin	256 (221.6)	>256 (>221.6)	>256 (>221.6)	64(55.4)	>512 (>443.3)	256 (221.6)

Taula.R.10. Assaig d'activitat de diferents pèptids aïllats de les secretions de les granotes en front soques d'*Acinetobacter* resistent a colistina

De tots els pèptids testats [E4K]alyteserin-1c i [D4K]B2RP són els que presenten una millor activitat tant en soques resistents com en soques sensibles a colistina.

Per últim, els tipus de compostos que vàrem testar en front de soques colistina resistents i colistina sensibles van ser uns compostos anomenats ceragenines. Les ceragenines són compostos provinents del àcid còlic, algunes modificacions del àcid còlic són

introduïdes dins l'estructura de tal manera que queda una part catiònica i una part hidrofòbica. Aquest tipus d'estructura, molt semblant als pèptids antimicrobians, poden anomenar-se peptidomimetics. Aquests compostos van ser testats en front de soques colistina sensible i colistina resistents en *A. baumannii*, *K. Pneumoniae* i *P. aeruginosa*.

MIC (mg/l) [μM]	***A. baumannii***		***K. pneumoniae***		***P .aeruginosa***	
ceragenines	**Col-S**	**col-R**	**col-S**	**col-R**	**col-S**	**col-R**
138	2 [2.7]	4[5.4]	16[21.4]	16[21.4]	1[1.3]	1[1.3]
13	4[5.9]	4[5.9]	8[11.8]	16[23.6]	<0,5[<0.7]	<0,5[<0.7]
131	2[2.7]	2[2.7]	8[10.9]	8[10.9]	<0,5[<0.7]	<0,5[<0.7]
44	8[10.9]	4[5.4]	4[5.4]	16[21.8]	1[1.4]	1[1.4]

Taula.R.11. Assaig d'activitat de diferents pèptids ceragenines en front soques tant resistents com sensibles a colistina de *A. baumannii*, *K. pneumoniae* i *P. aeruginosa*.

Tots els compostos presenten bones activitats en front de totes les soques, de totes maneres caldria destacar el compost 131 per sobre dels altres i especialment en front de soques de *P. aeruginosa*. També es pot apreciar com l'activitat dels diferents peptidomimetics es independent del grau de resistència de les soques emprades.

Fins ara tots els tipus d'aproximació utilitzats son el clàssic antimicrobià, però la idea que vàrem utilitzar parteix de la idea d'inhibir la adherència dels bacteris a les cèl·lules mitjançant la inhibició de la proteïna ompA.

Aprofitant una llibreria d'hexapèptids cíclics van ser provats *in silico* com a inhibidor de la proteïna ompA. Els resultats obtinguts *in silico* demostren que els pèptids que contenen Pro/ Trp /Arg tenen major afinitat per la proteïna.

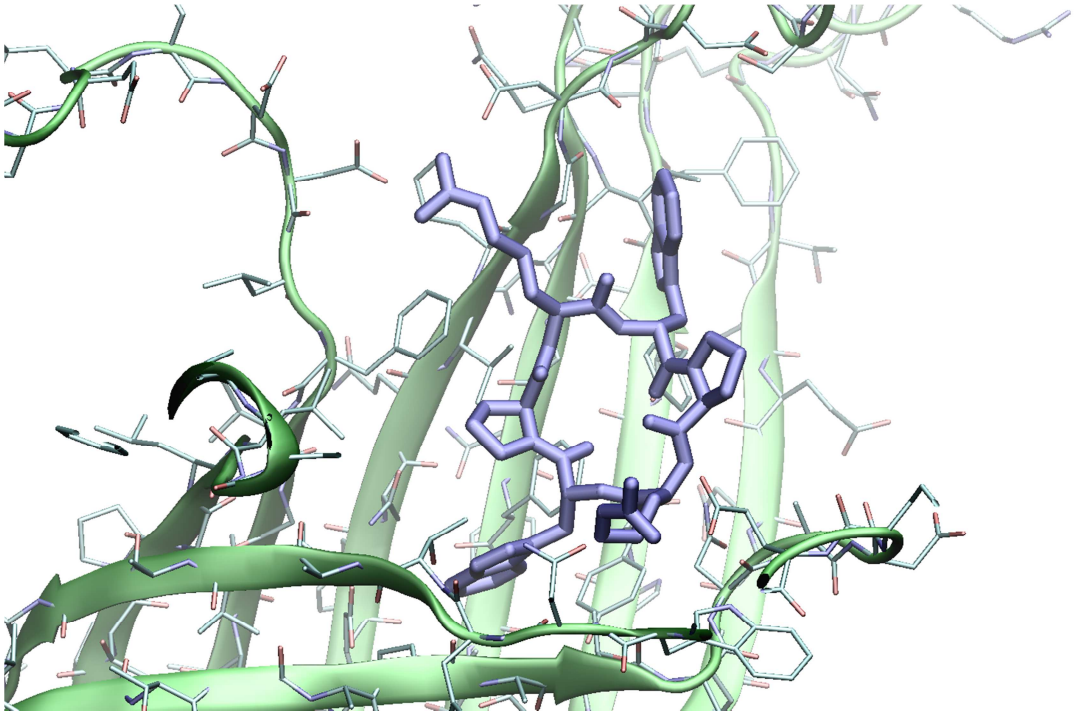


Figura.R.9. Imatge de la interacció entre un compost de la llibreria EXORIS i la proteïna OmpA.

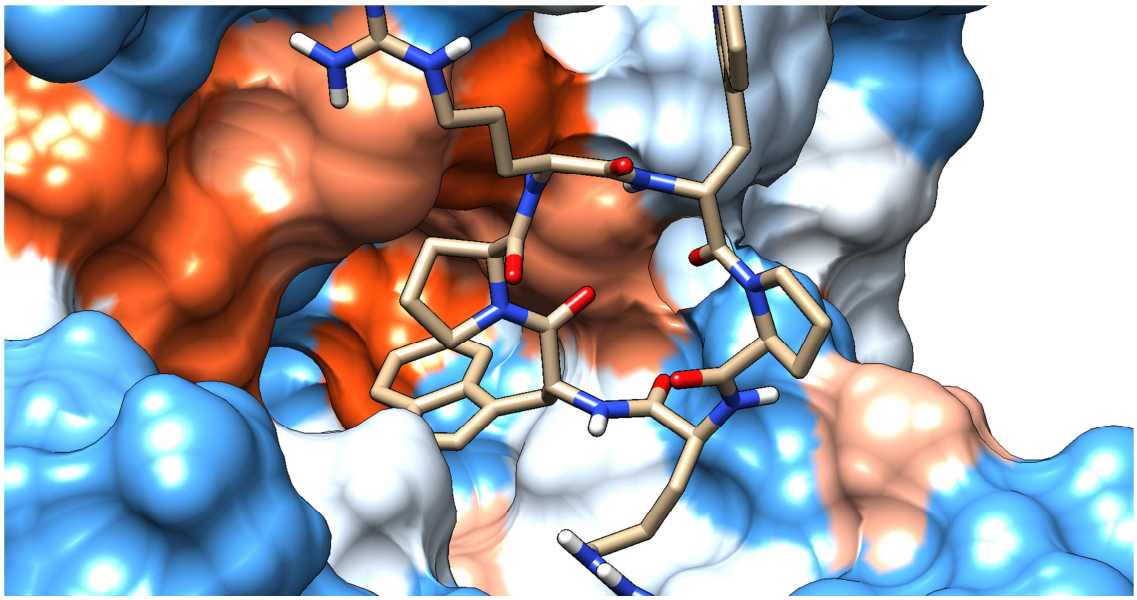


Figura.R.10. Imatge de la interacció entre un compost de la llibreria EXORIS i la proteïna OmpA.

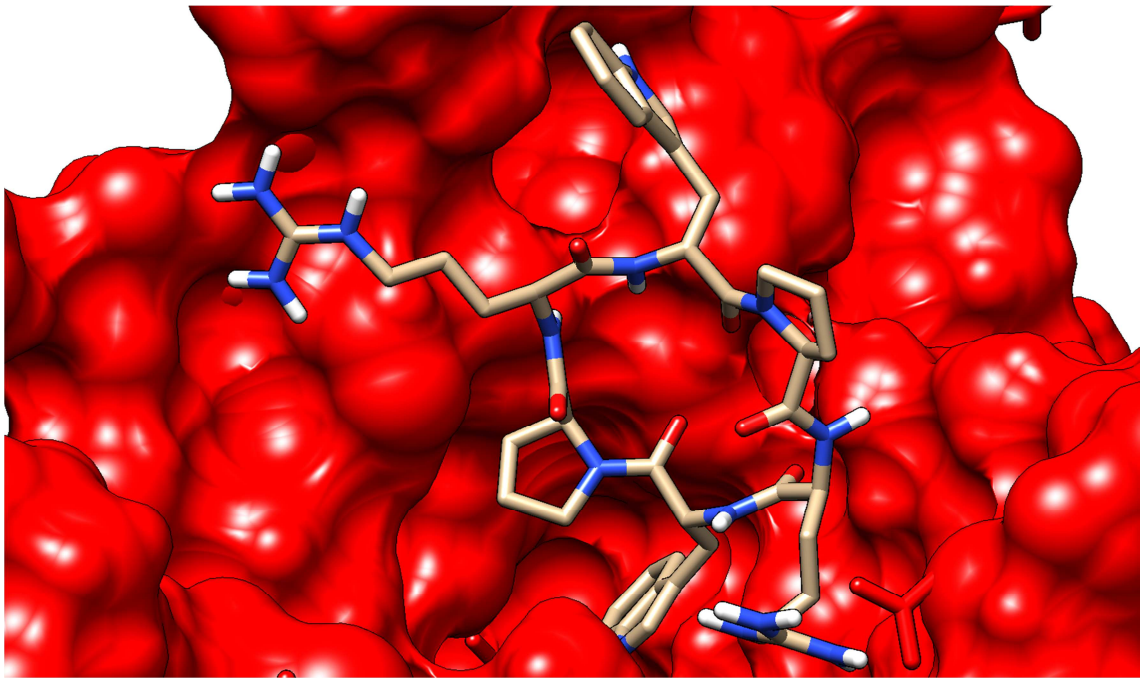
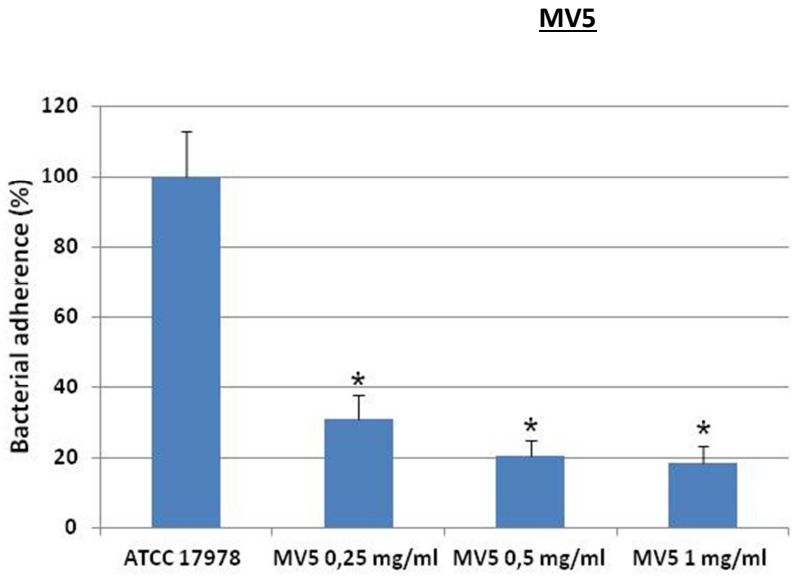


Figura.R.11. Imatge de la interacció entre un compost de la llibreria EXORIS i la proteïna OmpA.

Diferents pèptids vàrem ser provats com a inhibidors de la adherència de bacteris en cèl·lules.



De tots els pèptids provats, el que te millor activitat es el compost MV5, a més a més de presentar una bona activitat inhibidora, també es poc tòxic i no es antibacterià.

MV5 ciclo [Trp-D-Pro-Arg-Trp-D-Pro-Arg]

L'activitat d'aquest pèptid va ser testada utilitzant soques més resistents i es va veure com seguia mantenint la seva efectivitat.

Després de veure que era un pèptid efectiu, es va provar en ratolins. Primer de tot només el pèptid per veure la toxicitat *in vivo,* on es va veure que a una dosi de 40 mg/kg tots els ratolins sobrevivien. Posteriorment es va duu a terme la dosi efectiva 50 a diferents concentracions.

	MV5 (mg/kg)		
	10	**20**	**40**
Survival (%)	66,67	83,33	50

A les 3 concentracions utilitzades es pot veure com hi ha dosi efectiva 50 perquè es veu una supervivència superior al 50% de la població, fent èmfasi especialment en 20 mg/kg on sobreviuen 5 ratolins dels 6 tractats.

Annex VI