Studies of the effects of antimicrobial peptides on model membranes and relevant pathogens

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Dissertação de Mestrado em Bioquímica

Universidade do Porto

Faculdade de Ciências

Instituto Ciências Biomédicas Abel Salazar

2011

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Dissertação de Candidatura ao grau de Mestre em Bioquímica da Universidade do Porto

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2011

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

AIDS – Acquired Immune Deficiency Syndrome

AMPs – Antimicrobial Peptides

BMMØ – Bone Marrow derived Macrophages

CA(1-7)M(2-9) or CAM – hybrid of cecropin A (aa 1-7) and melittin (aa 2-9)

CFUs – Colony Forming Units

Di-LFampin – Hybrid of two LFampin265-284 coupled by a special lysine linkage

Di-LFcin– Hybrid of two LFcin17-30 coupled by a special lysine linkage

DMEM – Dulbecco's Modified Eagle's Medium

FBS – Fetal Bovine Serum

FITC – Fluorescein isothiocyanate

HBSS – Hank's Balanced Salt Solution

HIFCS – Heat Inactivated Fetal Calf Serum

H_{II} – Inverted hexagonal phase

HIV – Human Immunodeficiency Virus

hLFcin1-11 – lactoferricin peptide containing amino acids 1-11 from human lactoferrin

IC_{50} – Concentration that inhibits 50% of parasites growth at the end of 4h

IFN-γ – Interferon gamma

IL-10 – Interleukin 10

IL-12 – Interleukin 12

K6 – Lysine N^{ε}-trimethylated CA(1-7)M(2-9) at a lysine in position 6

K7 – Lysine N^{ε}-trimethylated CA(1-7)M(2-9) at a lysine in position 7

LCCM – L929 cell-conditioned medium

LD_{50} – Concentration that inhibits 50% of parasites proliferation at the end of 3 or 5 days for promastigotes and amastigotes, respectively

LF – Lactoferrin

LFampin265-284 – lactoferrampin peptide containing amino acids 265-284 from bovine lactoferrin

LFampin-LFcin – Hybrid of LFampin265-284 and LFcin17-30

LFChimera – Hybrid of LFcin17-30 and LFampin265-284 coupled by a special lysine linkage

LFChimera-R – Reverse peptide of LFChimera

LFcin17-30 – lactoferricin peptide containing amino acids 17-30 from bovine lactoferrin

LFcin-LFampin – Hybrid of LFcin17-30 and LFampin265-284

LPS – Lipopolysaccharide

L_α – Fluid lamellar phase

L_β – Gel lamellar phase

MAC – *Mycobacterium avium* complex

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

P:L – Peptide-to-Lipid molar ratio

PBS – Phosphate Buffered Saline

PC – Phosphatidylcholine

PE – Phosphatidylethanolamine

PG – Phosphatidylglicerol

POPE – 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine

SAXD – Small Angle X-ray Diffraction

TB – Tuberculosis

TGF-β – Transforming Growth Factor β

T_H – Lipid transition temperature from fluid lamellar to hexagonal phase

T_M – Lipid transition temperature from gel lamellar to fluid lamellar phase

TNF-α – Tumor Necrosis Factor α

WAXD – Wide Angle X-ray Diffraction

WHO – World Health Organization

ACKNOWLEDGMENTS

First of all I would like to thank my supervisors, Margarida Bastos and Salomé Gomes, for all the support, patience, for always being available for helping me and for giving me the opportunity to continue my work. In this year I had the chance not only to grow as a scientist but also as person, and much of that was due to both of them.

Also thank to all the people that have helped me during this year making possible the writing of this thesis, especially to:

- Guangyue Bai, that although is now very far away her teaching was essential for me.
- All the members of the Iron and Innate Immunity group and the Microbiology and Immunology of Infection group from IBMC, especially to Sílvia Costa, Sandro Gomes, António Barroso e Tânia Moniz, your help and friendship was fundamental.
- All the people that I have met in *Centro Investigaciones Biológicas* in Madrid, for making me feel at home even when I was so far away. Especially to the members of the eukaryotic antibiotic peptides group, Luis Rivas for his wisdom, you taught me a lot, to María Fernández-Reyes and María Ángeles Abengózar without whom my work would never be possible and Oscar Santos Calvo, for making me laugh and show me the amazing city that is Madrid.
- Daniela Uhríková (Faculty of Pharmacy, Comenius University, Bratislava, Slovak Republic) and Sergio Funari (Desy, Hasylab, Germany) for the introduction to the X-ray world (together with Margarida Bastos) that I completely ignored and now fascinats me.
- Jan Bolscher (ACTA, The Netherlands), David Andreu (Universitat Pompeu Fabra, Barcelona) and Paula Gomes (FCUP, Porto) for providing the peptides.

Finally, but not least, to my parents and my boyfriend, for their infinite patience and love you are my rocks and the ones that keep my feet on the ground.

ABSTRACT

Once thought to be a solved problem, the treatment of bacterial infections is currently a major human health concern. The increasing level of bacterial resistance to the existing antibiotics, together with the lack of any new effective antibacterial compounds in several decades, poses a great challenge to the development of new therapies.

Antimicrobial peptides (AMPs) are one potential alternative to fight infectious diseases. These peptides are present in almost all living organisms, as part of their immune system, varying in length, sequence and structure. The mode of action of AMPs is still under debate, but in most cases this is thought to involve membrane disruption (by a variety of mechanisms) and in some cases they can also have internal targets. Beyond their capacity of directly killing bacteria and other microorganisms, the possibility of their use together with conventional antibiotics leading to a synergistic effect has also been described.

In the present work the effect of AMPs from two families, cecropin A-melittin derivatives and lactoferrin-derived peptides, were tested for their effects on model membranes, and on two relevant pathogens: *Leishmania* and *Mycobacterium avium*. These are both intracellular pathogens of macrophages, which frequently act as opportunistic pathogens, in immunocompromised hosts.

Lactoferrin-derived peptides were found to be leishmanicidal against *L. donovani* promastigotes and *L. pifanoi* amastigotes. In order to investigate the leishmanicidal mechanism of AMPs the permeabilization of *Leishmania* plasma membrane was evaluated by several assays allowing us to conclude that the mechanism of action of these peptides is based on the permeabilization of the plasma membrane.

Cecropin A-melittin peptides directly inhibited the growth of *M. avium* in axenic cultures at high concentrations and they were toxic against macrophages. A Lactoferrin-derived peptide, lactoferricin, was active against *M. avium* in axenic cultures and when combined with ethambutol we observed a synergistic effect against *M. avium* growing inside macrophages. At odds, another peptide of the same family, LFChimera, significantly potentiated the growth of *M. avium* inside macrophages.

In an attempt to contribute to the understanding of the mechanism of action of antimicrobial peptides, the interaction of AMPs with model membranes of phosphatidylethanolamine (PE), the major phospholipid in the bacterial cell membrane, was evaluated by Small Angle X-ray Diffraction (SAXD). Our results lead us to conclude that AMPs induce cubic phases in these model systems, which can be seen as a possible mechanism of their bactericidal action.

RESUMO

O tratamento de infeções bacterianas constitui atualmente um dos maiores problemas de saúde humana. Os níveis crescentes de resistência de bactérias patogénicas aos antibióticos disponíveis, juntamente com a falta de novos fármacos eficazes em várias décadas, representam um grande desafio no desenvolvimento de novas terapias.

Os péptidos antimicrobianos (PAMs) são uma potencial alternativa no combate às doenças infeciosas. Estes péptidos estão naturalmente presentes em quase todos os seres vivos, como parte do seu sistema imunológico, variando no tamanho, sequência e estrutura. Em geral, é aceite que o seu mecanismo de ação consiste em disrupção membranar por variados processos, podendo também atuar em alvos intracelulares. Além da sua capacidade para matar diretamente os microrganismos, os PAMs têm também a capacidade de atuar em conjunto com antibióticos resultando num efeito sinergistico.

Neste trabalho foi avaliado o efeito de péptidos de duas famílias de PAMs, nomeadamente derivados da cecropina A-melitina e derivados da lactoferrina. O seu efeito foi testado em patogénios relevantes, *Leishmania* e *Mycobacterium avium*, ambos patogénios intracelulares de macrófagos, frequentemente responsáveis por infeções oportunistas em doentes imunocomprometidos, assim como em membranas modelo.

Péptidos derivados da lactoferrina foram activos contra promastigotas de *L. donovani* e amastigotas de *L. pifanoi.* Para tentar esclarecer o mecanismo de ação destes péptidos, a permeabilização da membrana plasmática da *Leishmania* foi avaliada por diferentes técnicas permitindo-nos concluir que o mecanismo de acção destes péptidos contra *Leishmania* consiste na permeabilização da membrana plasmática.

Péptidos derivados da cecropina A-melitina inibiram directamente o crescimento de *M. avium* mas apenas em concentrações muito altas, sendo também tóxicos para macrófagos. No caso dos péptidos derivados da lactoferrina, lactoferricina foi activa contra *M. avium* em culturas axénicas e quando em combinação com ethambutol existiu um efeito sinergistico contra *M. avium* a crescer dentro de macrófagos. LFChimera por outro lado potenciou o crescimento deste patogénio no interior dos macrófagos.

Na tentativa de clarificar o mecanismo de ação destes péptidos, a interação de PAMs com membranas modelo de fosfatidiletanolamina (PE), o principal fosfolípido das membranas bacterianas, foi avaliada por difração de raios-X (Small Angle X-ray Diffraction – SAXD). Os nossos resultados mostram que os PAMs induzem a formação de fases cúbicas nestas membranas modelo, podendo a sua formação ser considerada como um possível mecanismo de ação bactericida.

THESIS ORGANIZATION

This thesis is organized into four parts.

- **Part I** comprises the introduction to the thesis theme, where the first section gives a global overview of the area and subsequent sections develop the more relevant topics.
- **Part II** describes the objectives of this master thesis
- **Part III** encompasses the experimental procedures and the results. This part is divided into three sections, corresponding to different projects performed during the course of the thesis. Each section includes its own Materials, Methods, and Results.
- **Part IV** contains the overall Discussion and Conclusions of the work and Bibliography.

PART I

INTRODUCTION

1. GENERAL INTRODUCTION

The introduction of the first antibiotics in the clinical practice in the 1940s (Aminov, R.I. 2009) started one of the greatest successes in the history of medicine. These pharmaceutical compounds saved millions of human lives throughout the last decades and made people believe that all bacterial infections could be treated and they would rapidly become a problem of the past. However this belief was proven to be wrong. After a few years antibiotic-resistant pathogens started to emerge and to disseminate. In response to that, new antibiotics had to be brought to the clinic and, during several decades, the number of deaths caused by infections continued to decline. Nowadays we face a re-emergence of infectious diseases and the appearance of multidrug-resistant "super-bugs", the treatment of which is increasingly costly and prone to failure. Examples of these multidrug-resistant bacteria are: the methicillin-resistant *Staphylococcus aureus* (MRSA) that is responsible for a high percentage of hospital-acquired infections and that is spreading outside the hospital zones (Lohner, K. 2009), multidrug-resistant *Mycobacterium tuberculosis* with approximately 440 000 new cases emerging annually, causing at least 150 000 deaths (WHO 2011a), among many others. This alarming situation was originated from a combination of factors, such as, the excessive and inappropriate use of antibiotics in human and animal health (Lohner, K. & Blondelle, S.E. 2005) and the almost complete absence of discovery of new antibiotics in recent years (only two new classes of antimicrobial compounds were introduced in the market in the last three decades) (Lohner, K. 2009).

Figure 1 – Publicity poster from the World Health Organization (WHO) for the World Health Day 2011, where the aim was to reach attention for the antimicrobial resistance problem (WHO 2011c).

Considering all this there is an urgent need to develop new and effective antimicrobial therapies. In this context antimicrobial peptides (AMPs) are one potential alternative to fight infectious diseases because their mode of action makes them less prone to resistance induction than currently used antibiotics and permits high activity against a vast range of microorganisms. AMPs are present in almost all living organisms, having broad functions in innate immunity, including immunomodulation, and providing a first line of defense against a wide variety of pathogens (Hancock, R.E. & Sahl, H.G. 2006; Lohner, K. 2009). Their mechanism of action is not fully understood, but it is widely accepted that they act by disrupting the cellular membrane of pathogens, with the possibility of also acting on intracellular targets. Most of these peptides have multiple modes of action, and they work in a multiple-hit strategy which increases their efficiency and capacity to evade potential resistance mechanisms (Nguyen, L.T. *et al.* 2011). These characteristics may make AMPs useful in the clinic for a longer time, as compared to conventional antibiotics (which become obsolete, due to microbial resistance in 1 to 2 decades on average) (Hancock, R.E. & Sahl, H.G. 2006).

2. PATHOGENS

In this work the antimicrobial activity of AMPs was assessed on different pathogens, namely *Leishmania* and *Mycobacterium avium*. In the next sections a brief introduction will be made to these microorganisms focusing on the epidemiology, structural characteristics, pathology, on the current treatments available and on the status of AMPs as potential agents to fight the diseases inflicted by these pathogens.

2.1. *LEISHMANIA*

Protozoan parasitic diseases, especially malaria, leishmaniasis, and trypanosomiasis, are considered neglected diseases and remain an unsolved public health problem in certain areas of the world, with extremely high death rates. The high mortality in developing countries is often due to the poor sanitary conditions and lack of efficient prophylactic measures, whereas in developed countries, some of these diseases that were eradicated are re-appearing especially as opportunistic diseases in immunocompromised hosts (Piscopo, T.V. & Mallia Azzopardi, C. 2007; Zucca, M. & Savoia, D. 2011).

Leishmania is a genus of protozoan parasites that are transmitted to the mammalian host (*e.g.* canines, rodents and humans) by the bite of phlebotomine sand flies, and are the causative agents of a set of clinical diseases collectively known as leishmaniasis. The World Health Organization estimates that 350 million people are at risk of contracting leishmaniasis and approximately 2 million new cases occur per year. Visceral infections, caused by *L. donovani* and *L. infantum*, are highly fatal if not treated, and are responsible for 50 000 deaths annually, a rate surpassed among parasitic diseases only by malaria (WHO 2010).

Leishmaniasis is endemic in more than 60 countries worldwide, mostly found in tropical and subtropical regions of the world. This disease is also endemic to all European Mediterranean countries including Portugal, mostly as canine leishmaniasis, in humans the opportunistic co-infection with HIV is of special relevance (WHO 2010).

2.1.1. LEISHMANIASIS

Clinical manifestations of leishmaniasis differ widely depending on the *Leishmania* species (there are at least 20 species) as well as on host factors such as immunity status (Goto, H. & Lindoso, J.L. 2010). The interplay between *Leishmania* and the mammalian host response is manifest not only in terms of clinical outcome but also in the rate of

spontaneous healing and recurrent disease. Three main types of disease patterns occur: cutaneous, mucocutaneous and visceral infections (figure 2) (Piscopo, T.V. & Mallia Azzopardi, C. 2007; Zucca, M. & Savoia, D. 2011).

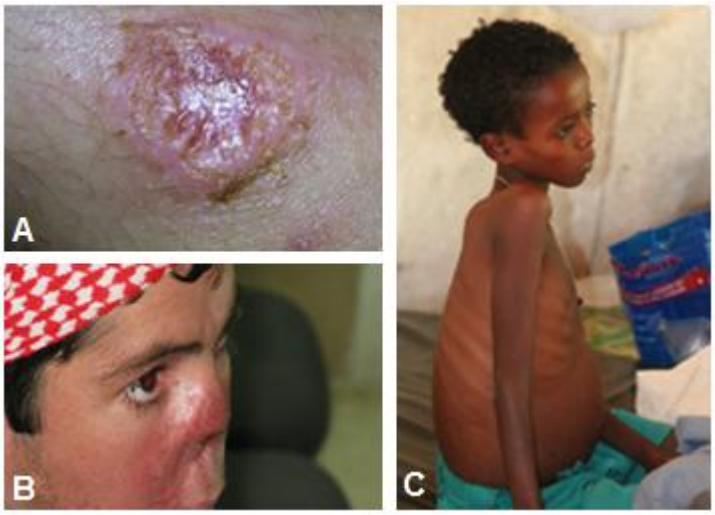

Figure 2 – Clinical forms of leishmaniasis. A) Cutaneous, **B)** mucocutaneous and **C)** visceral leishmaniasis (WHO 2011b).

Cutaneous leishmaniasis can potentially be associated with all *Leishmania* species, being the most common form of the disease characterized by the appearance of ulcers at the site of the bite on exposed parts of the body, such as the face, arms and legs. Normally these cutaneous forms of the disease are benign and the lesions spontaneously heal leaving scars. However there are some species of *Leishmania* that cause more complicated outcomes, for instance **mucocutaneous leishmaniasis** (WHO 2010). This form of the disease is caused by metastasis to the mucosal tissues of the mouth and upper respiratory tract by lymphatic and haematogenous dissemination potentially evolving to disfiguring lesions. Some *Leishmania* species that normally do not cause this type of leishmaniasis, have been reported to induce similar lesions in immunosuppressed patients (Goto, H. & Lindoso, J.L. 2010; WHO 2010). **Visceral leishmaniasis** is the most serious and complicated form of the disease and fatal if not treated. The infection is due to the invasion by the parasites of mononuclear phagocytic cells of the liver, spleen, bone marrow and intestinal mucosa. Aspects such as malnutrition, genetic factors and co-infections that lead to immunosuppression are risk factors to develop the disease (Piscopo, T.V. & Mallia Azzopardi, C. 2007; WHO 2010).

2.1.2. LIFE CYCLE

The protozoan parasite *Leishmania* has two major forms: the promastigote, the flagellated form that is transmitted to the vertebrate host by sand fly bites, and the intracellular amastigote, which lives inside mononuclear phagocytic cells of a wide variety of mammals (figure 3).

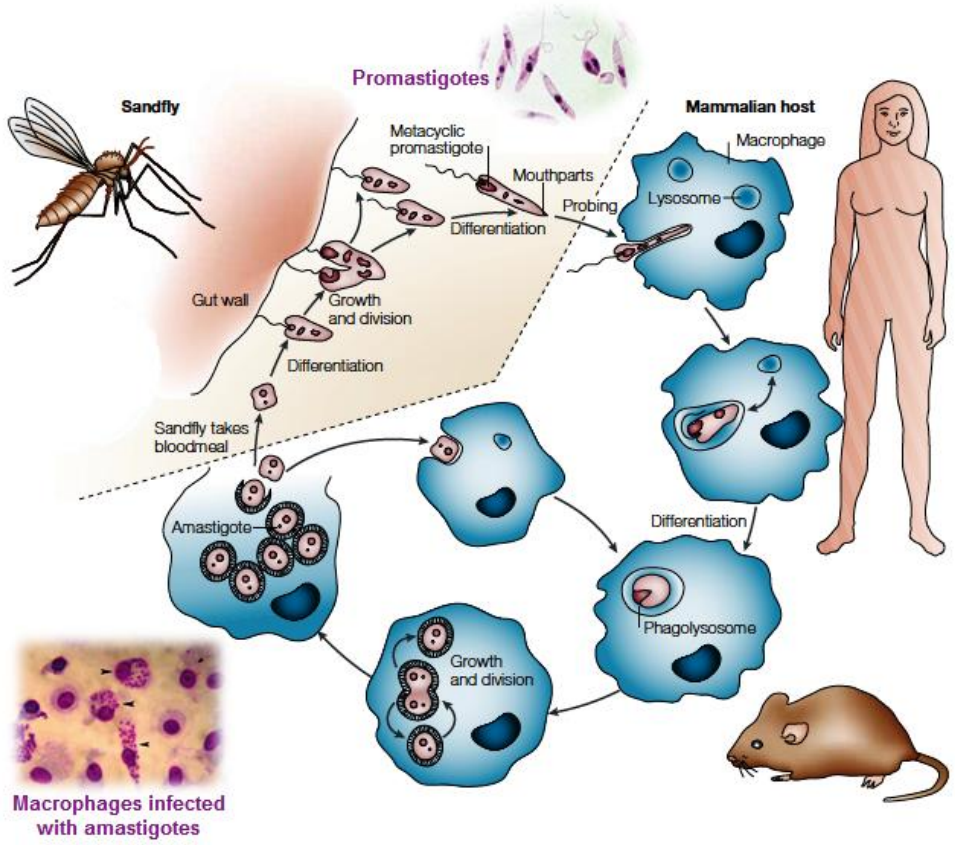

Figure 3 – Life cycle of *Leishmania*. Schematic representation of the different stages of development of the parasite in the invertebrate host, sand fly, and in the vertebrate host, mammals (Adapted from: Banuls, A.L. *et al.* 2007; Sacks, D. & Noben-Trauth, N. 2002).

In the invertebrate host the promastigote has an elongated shape and a long flagellum. This form infects the mammalian host when a parasitized female sand fly feeds from the blood of the vertebrate. The promastigotes that enter into the mammals organism are opsonized by serum components and then phagocytosed, primarily by macrophages and dendritic cells (Kima, P.E. 2007; Sacks, D. & Noben-Trauth, N. 2002). Inside the phagocytic cells, within the phagolysosome, the promastigotes undergo several structural and morphological changes to give rise to the intracellular amastigotes, which are ovoid and nonflagelated. Amastigotes proliferate within the phagolysosomes (or parasitophorous vacuoles) where they reproduce by binary fission until the number of

parasites increases enough for the cell to burst and free the amastigotes to infect other cells and continue their life cycles. The parasites could then go back to the invertebrate host when a sand fly ingests blood that contains mononuclear phagocytic cells infected with amastigotes (Banuls, A.L. *et al.* 2007; Kamhawi, S. 2006; Olivier, M. *et al.* 2005).

One of the most remarkable accomplishments of *Leishmania* is that they successfully live inside mammalian cells that are responsible for killing pathogens, the macrophages. For that *Leishmania* had to evolve a range of sophisticated mechanisms to subvert host surveillance by altering the macrophage signal transduction machinery, thereby modulating the macrophage environment in its favor. Some examples of these mechanism are the production of lipophosphoglycan at the surface of the parasites that confers resistance to the lytic action of the complement system, allowing the infection of macrophages and creating appropriate conditions for the promastigote-to-amastigote differentiation (Lodge, R. & Descoteaux, A. 2005). Other examples include preventing the activation of macrophages defense mechanisms such as the production of oxygen and nitrogen reactive species and also inhibition of an adequate and protective T cell response by inhibiting the production of pro-inflammatory signals such as IL-12 and promoting the release of anti-inflammatory cytokines, like IL-10 and TGF-β. (Kima, P.E. 2007; Olivier, M. *et al.* 2005).

2.1.3. TREATMENTS

Drug treatment for leishmaniasis exists since the 20th Century, but only a limited range of drugs are available. The efficacy of these drugs is affected by a combination of factors, such as the differences in the sensitivity of *Leishmania* species to the drugs and their pharmacokinetic properties, and the immune status of the patient (Goto, H. & Lindoso, J.L. 2010).

The current treatment of leishmaniasis consists in the administration of pentavalent antimonials as a first option. As second line drugs there are some alternatives such as amphotericin B, which is effective in antimonials-resistant cases, miltefosine which was the first orally active drug made available against leishmaniasis, pentamidine that can also be used in the treatment of resistant cases of visceral leishmaniasis, as well as paromomycin (Goto, H. & Lindoso, J.L. 2010; Piscopo, T.V. & Mallia Azzopardi, C. 2007; WHO 2010).

Parasite resistance to these conventional treatments, lack of efficacy, toxicity and high costs associated with them lead to a clear need to improve the existing drugs and to

identify new targets and in that way develop new therapies (Piscopo, T.V. & Mallia Azzopardi, C. 2007). However, the highest prevalence of parasitic diseases such as leishmaniasis occurs in the poorest areas of the world, and from the total investment in health research in the world, the work related with malaria, leishmaniasis and trypanosomiasis accounts for only about 0.1% whereas the contribution of these diseases to total burden is 5%. Nevertheless in the last years the search for new antiparasitic treatments has received new impulse thanks to new technical and political developments, and the appearance of new therapies, such as protease and topoisomerase inhibitors, RNA interference- based approaches, nano-drug delivery and proteomics, as well as antimicrobial peptides (Zucca, M. & Savoia, D. 2011).

2.1.4. *LEISHMANIA* AS A TARGET OF AMPS

In what concerns AMPs, protozoans have received much less attention as a target than pathogens such as bacteria or fungi, although a variety of *in vivo* and *in vitro* antiparasitic assays suggest that these compounds could represent a powerful tool for the development of new therapies as well as to complement current ones (Rivas, L. *et al.* 2009).

Following the discovery of AMPs, magainins and cecropins were the first antimicrobial peptides to display antiparasitic activities along with hybrids of cecropin A-melittin (Andreu, D. *et al.* 1992; Boman, H.G. *et al.* 1989; Mor, A. 2009). Histatin 5, a human salivary AMP, is also active against *Leishmania*, not by acting in the plasma membrane, as usual for AMPs, but by decreasing the mitochondrial ATP synthesis leading to cell death (Luque-Ortega, J.R. *et al.* 2008).

Although *Leishmania* are eukaryotic pathogens, their cytoplasmic membranes have higher levels of negative phospholipids, such as phosphatidylserine and phosphatidylinositol than higher eukaryotes, conferring them more susceptibility to the action of AMPs than mammal cells (Wassef, M.K. *et al.* 1985). *Leishmania* is an interesting model as a target for AMPs primarily due to the absence of barriers external to the plasma membrane contrary to what happens in bacteria. The promastigote form of the parasite is endowed with an anionic glycocalix, essentially composed of lipophosphoglycan (LPG), an anionic oligosaccharide, anchored to the membrane through glycosylinositolphospholipids (GIPLs) (figure 4). Abundant proteolytic activity also exists at the surface, due to the presence and activity of GPI-proteins and proteophosphoglycans (PPGs) (figure 4) (Rivas, L. *et al.* 2009). These structures present at the surface of the membrane have some duality in what concerns resistance and/ or susceptibility to AMPs. The presence of a negatively

charged glycocalix can favor peptide interaction however they can also delay the interaction of the peptides with the cytoplasmic membrane diminishing their efficacy; the proteolytic activity at the surface of the membrane, can also confer resistance to AMPs due to increased possibility of peptide degradation. A second important characteristic of *Leishmania* cells is the fact that endo- and exocytosis are confined to a special area accounting for about 2% of the plasma membrane known as the flagellar pocket (deep invagination of the plasma membrane that is located at the base of the flagellum devoid of attached microtubules) (Morgan, G.W. *et al.* 2002; Overath, P. *et al.* 1997). Theoretically, this may somehow limit the repairing capacity of the membrane upon exposure to AMPs (Luque-Ortega, J.R. & Rivas, L. 2010).

Although amastigotes can appear more vulnerable to AMPs due to the much less developed glycocalix (figure 4), their location inside phagocytic cells and the substantial changes in the membrane composition adopted by these parasites along their life cycle, can be barriers to the action of AMPs (Luque-Ortega, J.R. & Rivas, L. 2010).

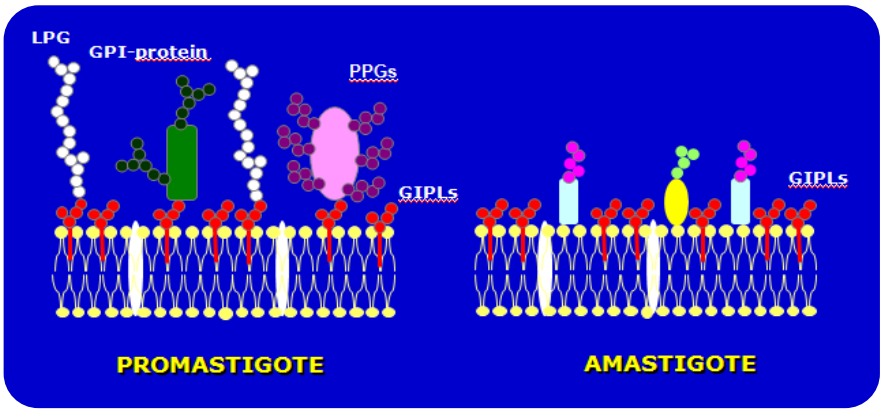

Figure 4 – Cytoplasmic membrane of *Leishmania* parasites. Comparison between the composition of the cellular membrane of promastigotes and amastigotes. LPG: lipophosphoglycan; PPGs: Proteophosphoglycans; GIPLs: glycosylinositolphospholipids (diagram kindly provided by Doctor Luís Rivas).

2.2. *MYCOBACTERIUM*

Tuberculosis (TB) remains today one of the most deadly human diseases. WHO estimates that one-third of the world's population is currently infected with the TB bacillus with 5-10% of people developing the disease. In 2009 approximately 2 million people died of TB. The incidence of tuberculosis in the United States had been declining since the turn of the 20th century, however since 1985 it has been rising mainly due to the AIDS epidemic, the lack of efficacy of the anti-tuberculosis vaccine and the emergence of multidrug-resistant strains (Clemens, D.L. & Horwitz, M.A. 1995) The genus *Mycobacterium* includes not only *Mycobacterium tuberculosis,* the causative agent for TB discovered in 1882 by Robert Koch (Koul, A. *et al.* 2011), but other pathogenic species such as *M. leprae* and *M. ulcerans*, and non-pathogenic species, usually referred to as nontuberculous mycobacteria (NTM). More than 130 species of NTM are known and most of them are typically environmental organisms present in water, soil, dust and plants. Contact with these contaminated environments may be responsible for infection in humans and animals with the possibility of transmission from human to human being almost null (Pieters, J. 2001; Tortoli, E. 2006; Tortoli, E. 2009). The large majority of NTM are nonpathogenic for healthy individuals, but they can act as opportunists in immunocompromised patients, especially in AIDS patients. The consequences of mycobacterial infection depend on the virulence of the infecting *Mycobacterium* and the resistance of the host. In humans the outcome can range from relatively mild and transient symptoms, mostly caused by pulmonary infections, to a widely disseminated disease (Pieters, J. 2001; Tortoli, E. 2009).

2.2.1. MYCOBACTERIA CELL WALL

Mycobacteria cells are irregular rods, aerobic, and unable to form spores (Prescott, L.M. *et al.* 2002). *Mycobacterium* species are characterized by an extremely complex and highly impermeable cell wall, composed of mycolic acids, glycolipids, lipoglycans, polysaccharides and pore forming proteins (figure 5). This unique cell wall contributes to the capacity of the pathogen to survive inside the host and to resist the chemotherapy (Nigou, J. *et al.* 2003).

The cell wall skeleton determines the size and shape of mycobacteria, and contains three different covalently linked layers, peptidoglycan, arabinogalactan and mycolic acids (Guenin-Mace, L. *et al.* 2009). The covalent linkage of the mycolic acids results in a hydrophobic layer of extremely low fluidity and high impermeability. Also bound to the mycolic acids by hydrophobic links, in the external side, are various free lipids such as

phenolic glycolipids, sulpholipids, among others. The outer layer of the cell wall that some authors refer to as capsule is mainly composed of polysaccharides such as glucan and arabinomannan (Abdallah, A.M. *et al.* 2007).

The presence of mycolic acids and other lipids outside the peptidoglycan layer makes the mycobacteria acid-fast. This means these bacteria are not stained by the Gram stain, and have to be stained with a harsher treatment, the Ziehl-Neelsen method (Prescott, L.M. *et al.* 2002).

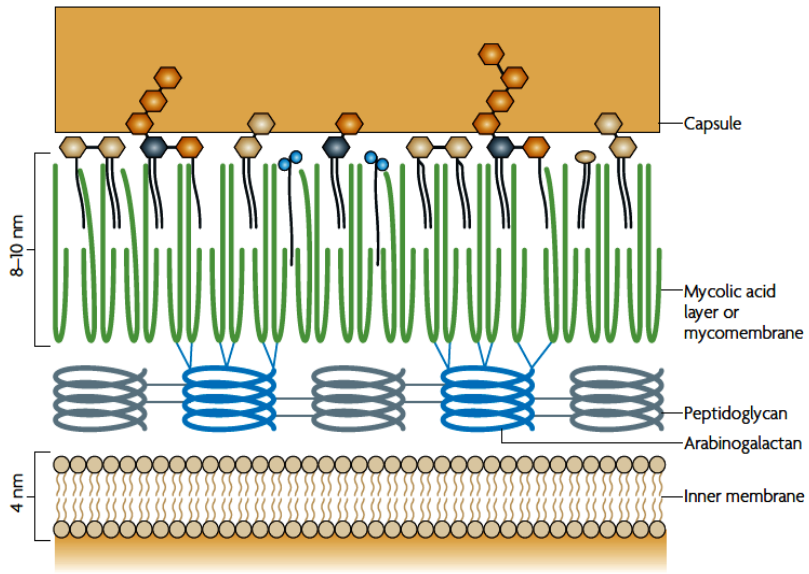

Figure 5 – Mycobacterial cell wall. Schematic representation of one of the current views of the mycobacterial cell wall (Abdallah, A.M. *et al.* 2007).

Mycobacterium plasma membrane, which is protected by the cell wall, is an asymmetric bilayer mostly composed of lipids and proteins, as any biological membranes. The phospholipid composition is very similar to all the other bacterial membranes being essentially composed of phosphatidylethanolamine, phosphatidylglicerol, diphosphatidylglicerol, and phosphatidylinositol mannosides, these last three conferring the membranes an overall negative charge. (Guenin-Mace, L. *et al.* 2009).

2.2.2. *MYCOBACTERIUM AVIUM*

Mycobacterium avium belongs to the *Mycobacterium avium* complex (MAC), along with *M. intracellulare*. Disseminated MAC was one of the first opportunistic infections detected on AIDS patients remaining until today a substantial cause of morbidity and mortality among these patients (Tortoli, E. 2009). HIV-infected patients with *M. avium* have serious

complications associated with debilitating symptoms and shortened survival. In 1994, in the United States, it was estimated that 37 000 people were infected with disseminated *Mycobacterium avium* complex, a greater number than the people infected by TB in this year also in the same country (Horsburgh, C.R. *et al.* 2001; Reed, C. *et al.* 2006). Infection of AIDS patients by *M. avium* occurs in advanced stages of the disease, when the levels of CD4+ T cells are very low. *M. avium* can also infect patients with other debilitating diseases, especially restrictive and obstructive pulmonary diseases, that compromise the immune system, such as chronic obstructive pulmonary disease, emphysema, chronic bronchitis and children with lymphadenitis (Appelberg, R. 2006b).

M. avium, similar to other mycobacteria, is a facultative intracellular pathogen residing mainly inside macrophages (figure 6). The pathogenicity of the mycobacteria is directly related to their ability to persist in macrophages circumventing the host immune response. After being phagocytozed by macrophages the mycobacteria live inside the phagosomes of the host cells (Pieters, J. 2001). Normally the phagosome undergoes maturation that consist of a progressive acidification and several fusion and fission events, leading to fusion with lysosomes forming the phagolysosome that has an acidic environment and contains proteolytic enzymes that degrade the content of the vacuole (Gomes, M.S. *et al.* 1999c). *M. avium* interferes with the intracellular trafficking of the macrophages, by inhibiting phagosome-lysosome fusion, escaping from harmful environments (*e.g.* acidic pH) and also by interacting with endosomes, allowing the bacteria to access nutrients that are required for their survival (Appelberg, R. 2006a; Appelberg, R. 2006b).

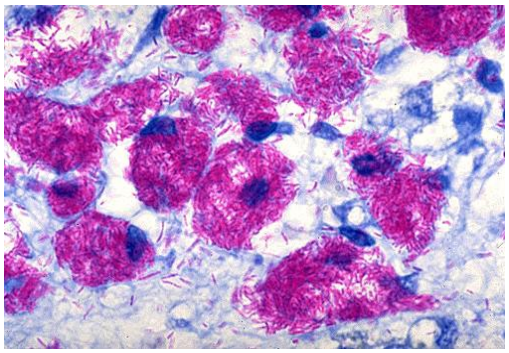

Figure 6 – *Mycobacterium avium*. Spleen macrophages (from an AIDS patient) infected with *M. avium* complex (pink) stained with the Ziehl-Neelsen method (WebPath: The Internet Pathology Laboratory).

The mechanism by which macrophages can inhibit the mycobacterial growth and the mechanisms used by mycobacteria to resist and live inside macrophages are partially understood. *M. avium* can grow exponentially inside non activated macrophages inhibiting

the production of superoxide, however this growth is more restrictive if the macrophages are activated with IFN-γ and TNF-α, independently of the production of nitrogen and oxygen reactive species (Gomes, M.S. & Appelberg, R. 2002; Gomes, M.S. *et al.* 1999b). Also mechanisms that lead to restriction of access to nutrients by mycobacteria and macrophage death pathways may contribute to the elimination of the pathogen (Appelberg, R. 2006a; Behar, S.M. *et al.* 2010).

2.2.3. TREATMENTS

Infections by mycobacteria are very difficult to treat due to a combination of factors, such as: i) the poor action of the antibiotics available, mainly due to the highly impermeable cell wall; ii) the fact that these are intracellular bacteria; iii) the long duration of the treatments, that much of the time the patients do not follow to the end and iv) the antibiotic resistance that is increasing worldwide.

The basic treatment of all pathogenic mycobacteria consists of a combination of different antibiotics taken for several months. In the case of tuberculosis the treatment varies according to the susceptibility of the isolated strain to the available drugs, going from the administration of antibiotics such as isoniazid, rifampicin, pyrazinamide and ethambutol to fluoroquinolone or second-line injectable drugs, such as amikacin, kanamycin and capreomycin, that are all ineffective against extensively-drug resistant TB (Koul, A. *et al.* 2011).

In the case of *Mycobacterium avium* current therapy consists of ethambutol with a macrolide that can be azithromycin or clarithromycin, and rifamycin derivatives (rifampicin and rifabutin) taken for six months to one year with an overall clinical success rate of no more than 60% in AIDS patients (Deshpande, D. *et al.* 2010). The administration of an effective antiretroviral therapy in immunocompromised patients reduces the number of people at risk of developing disseminated MAC by restoring the immune function of the patients essentially by elevating the number of CD4+ T cells (Horsburgh, C.R. *et al.* 2001).

The global control of mycobacterial infections will be achieved with new antimycobacterial drugs that follow some criteria: shorten treatment duration, target multidrug-resistant strains, a more simple treatment by reducing the daily pill burden and the dosing frequency, and the possibility of co-administration with anti-HIV drugs (Koul, A. *et al.* 2011).

Some alternative therapies consist in the combination of antibiotics with adjunctive immunomodulators – adjunctive immunotherapy – such as picolinic acid, that have shown

direct antimicrobial activity against both extracellular and intramacrophagic MAC organisms, alone and in combination with conventional antibiotics (Cai, S. *et al.* 2006). The observation that the growth of *M. avium* inside macrophages is directly proportional to the amount of iron available, and the fact that AIDS patients have increased iron deposition in different tissues favoring the growth of *M. avium*, makes the use of iron chelators, that deprives the mycobacteria from an essential nutrient for their survival, a promising road for the treatment of this disease. Recent studies have shown the efficacy of some iron chelators both *in vitro* and in animal models infected with *M. avium* (Fernandes, S.S. *et al.* 2010; Gomes, M.S. *et al.* 2001; Gomes, M.S. *et al.* 1999a).

2.2.4. MYCOBACTERIA AS A TARGET OF AMPS

Antimicrobial peptides, such as cathelicidins and defensins, like human neutrophil peptide (HNP) and human β-defensin 2 (HBD-2), have also shown activity against *M. tuberculosis* and *M.* avium (Jena, P. *et al.* 2011; Mendez-Samperio, P. 2008; Ogata, K. *et al.* 1992). The combination of HNPs with anti-tuberculosis drugs, like isoniazid and rifampicin, against intracellular mycobacteria resulted in a significant reduction in the mycobacterial load (Kalita, A. *et al.* 2004; Sharma, S. *et al.* 2000). This effect could be due to increased permeability of both mycobacterial cell wall and plasmatic membrane by AMPs increasing the access of the antibiotics to intracellular targets like DNA (Sharma, S. *et al.* 1999). By potentiating the effect of antibiotics, AMPs can allow the reduction of the therapeutic dosage of drugs to approximately half and also reduce the time of treatment (Kalita, A. *et al.* 2004).

3. ANTIMICROBIAL PEPTIDES (AMPS)

Considering the potential of antimicrobial peptides as new alternative therapies to fight infectious diseases, in the next sections, several aspects concerning AMPs will be addressed, namely: their structural characteristics, mechanisms of action, role in the immunity, and the problems, successes and challenges of introducing AMPs in the clinical practice.

The term “Antimicrobial peptide” refers to a large number of peptides first characterized on the basis of their activity against bacteria and fungi. They constitute a primitive immune defense mechanism and they were found, first in the 1980s, on a variety of eukaryotic organisms, from insects to humans (Nguyen, L.T. *et al.* 2011; Reddy, K.V. *et al.* 2004). Some of them were first isolated due to their antimicrobial activity, whereas others were discovered for various unrelated functions before their potential as antimicrobial peptides was recognized (Wiesner, J. & Vilcinskas, A. 2010). These peptides are multifunctional and act in concert with other immune mechanisms and have evolved in nature throughout centuries to protect their hosts against diverse pathogens, such as bacteria, fungi, virus, protozoa and even cancer cells (Nguyen, L.T. *et al.* 2011). So far, more than 1500 peptides from different sources have been reported in The Antimicrobial Peptide Database – APD (http://aps.unmc.edu/AP/main.php) and the majority of them are classified as antibacterial peptides. Virtually all human tissues can produce AMPs, either constitutively or induced by inflammatory stimulus, more frequently in body sites exposed to pathogens, such as the skin or mucosa, or in some blood cell types, such as neutrophils, eosinophils and platelets (Wiesner, J. & Vilcinskas, A. 2010).

Natural AMPs are gene-encoded and ribosome-synthesized peptides that derive from precursor peptides through one or more proteolytic activation steps. *De novo* design of synthetic peptides was introduced in the 1990s with the use of high-throughput combinatorial library screening, structure based modeling and predictive algorithms to determine the optimal active peptide. Although this approach produced interesting results, the need for a high initial investment in technology compared to traditional methods has delayed the widespread use of this approach. On the other hand, rational design of peptides using existent sequences as templates that are modified to obtain peptides with the desired properties (antimicrobial activity and selectivity) and the discovery of AMPs through the search within natural proteins (*e.g.* lactoferrin) have also led to the successful production of more potent antimicrobial peptides (Nguyen, L.T. *et al.* 2011).

3.1. STRUCTURAL CHARACTERISTICS

Antimicrobial peptides vary widely in many aspects, such as length, sequence, structure and source, but there are some common traits for most of them, that are important for their antimicrobial activity. They can be defined as short (<50 amino-acids), with a positive net charge (generally +2 to +9, due to the existence of lysine and arginine residues) that allows them to interact with negatively charged membranes, such as bacterial membranes, although anionic antimicrobial peptides have also been described. They also have a substantial proportion of hydrophobic residues. AMPs have a random structure in solution but when in a membranous environment they adopt an amphipatic structure, allowing them to bind more efficiently to phospholipid membranes (Shai, Y. *et al.* 2006). The full classification of AMPs is difficult due to their huge diversity but almost all authors divide and classify the peptides based on their secondary structure. One of those classifications divides AMPs into three groups (table 1 and figure 7): **α-helical peptides**, **β-sheet peptides** and **extended peptides** which do not fold into regular secondary structures and are often composed of a high number of certain amino-acids, like histidine, tryptophan and arginine (Nguyen, L.T. *et al.* 2011).

Table 1 – Overall characteristics of some AMPs (adapted from: Van 'T Hof, W. *et al.* 2001).

Group	Peptides	Organism	Source	Antimicrobial activity [a]	Reference
α-helical peptides	melittin	bee	venom (apitoxin)	G-; G+; V; H	(Reddy, K.V. *et al.* 2004)
	magainins	frogs	skin mucus	G-; G+; F	(Nguyen, L.T. *et al.* 2011)
	cecropins	insects: moth	hemocytes	G-; G+; F; P	(Reddy, K.V. *et al.* 2004)
	cathelicidins LL-37	humans	leukocytes, epithelia	G-; G+	(Guaní-Guerra, E. *et al.* 2010; Nguyen, L.T. *et al.* 2011)
	Dermaseptins	frogs	skin mucus	G-; G+; F	(Van 'T Hof, W. *et al.* 2001)
β-sheet peptides	lactoferricin	cow, humans	digested lactoferrin	G-; G+; V; F; P; T	(Chan, D.I. *et al.* 2006)
	defensins	humans	leukocytes, epithelia	G-; G+; V; F	(Guaní-Guerra, E. *et al.* 2010)
	protegrins	pig	leukocytes, epithelia	G-; G+; F	(Reddy, K.V. *et al.* 2004)
extended peptides	indolicin	cow	leukocytes	G-; G+; V; F; P	(Chan, D.I. *et al.* 2006)
	tripticin	pig	leukocytes	G-; G+; F	(Chan, D.I. *et al.* 2006)
	histatins	humans	saliva	G-; G+; F	(Van 'T Hof, W. *et al.* 2001)
	PR-39	pig	intestine	G-; G+;	(Chan, D.I. *et al.* 2006)

[a] G-: Gram-negative bacteria; G+: Gram-positive bacteria; F: fungi; P: protozoan; T: tumor; H: hemolytic.

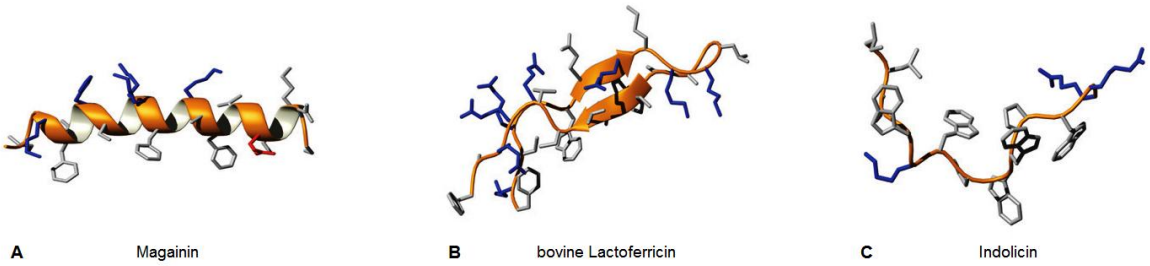

Figure 7 – The three major structures of AMPs. A) α-helical peptides **B)** β-sheet peptides **C)** extended peptides. Positively charged side chains are colored in blue, negatively charged side chains in red and remaining side chains in grey. PDB IDs: magainin 2: 2MAG; bovine lactoferricin: 1LFC; indolicidin: 1G89 (adapted from: Nguyen, L.T. *et al.* 2011).

3.2. Mechanisms of Action

The mechanism of action of antimicrobial peptides is not yet fully understood but it is well known that the biophysical properties mentioned above (secondary structure, overall charge and hydrophobicity) play important roles in their interaction with pathogen membranes. Other parameters are important for their action, such as the membrane lipid composition, the peptide-to-lipid molar ratio, and environmental conditions like ionic strength and pH (Lohner, K. & Blondelle, S.E. 2005). Due to the high diversity of AMPs and their properties there is no universal mechanism for the action of all AMPs as they can act in multiple ways making difficult to unravel all the molecular events resulting from the interaction between peptides and membranes. The activity of AMPs is most probably not related to specific membrane protein receptors since studies using all D-amino acids instead of L-amino acids in magainin, cecropin and melittin, showed that these D-peptides exhibited similar activities than the natural ones, suggesting that the interaction between peptides and membranes does not depend on chiral centers (Merrifield, E.L. *et al.* 1995; Wade, D. *et al.* 1990). There are some proposed mechanisms, providing explanations of possible ways for the peptides to disrupt the membrane leading to cell death. Overall, all the proposed mechanisms rely on the same main factors for initial action: adsorption of AMPs onto the membrane due to electrostatic interactions between the cationic peptides and the headgroups of anionic phospholipids, and then they vary in the next steps of action.

According to the **barrel stave model** (figure 8), peptides large enough (~ 22 amino acids) form transmembrane aqueous channels/pores by inserting into the hydrophobic core perpendicularly to the membrane plane where the hydrophobic region of the peptide is aligned with the acyl chains of the phospholipids while the peptide hydrophilic regions form the inner surface of the pore channel (figure 8). The continuous recruitment of

peptides to the membrane increases the pore size leading to leakage of the cells contents, membrane depolarization and thereby cell death (Nguyen, L.T. *et al.* 2011). The peptides arrangement in the membrane causes high repulsion due to their charges and this can culminate in the disintegration of the pore. This model is only possible for peptides with not too high charge and it is believed that the number of peptides acting by this mechanism is very low. An example is alamethicin from the fungus *Trichoderma viridis,* and perforin, produced by mammalian killer lymphocytes and complement component C9 (Wiesner, J. & Vilcinskas, A. 2010).

The **carpet model** (figure 8) proposes that the membrane surface is covered by the peptides, aligned parallel to the bilayer surface, in a “carpet like” manner, diminishing the fluidity of the membrane. When the concentration of the peptide reaches a threshold value the integrity of the membrane is lost leading to membrane micellization, toroidal pores or aggregated channels formation (see below) and cell death (figure 8) (Lohner, K. 2009).

The formation of **toroidal or wormhole pores** (figure 8) is the best characterized and the most consistent mechanism of action of AMPs. This model can be seen as representing one of the possible final stages of the previous carpet model, where the formation of pores leads to disruption of the membranes. In fact, these two models have been unified in the so called **“Shai-Matzusaki-Huang” model** that proposes that peptides bind parallel to the membrane with the apolar amino acids penetrating partly into the hydrophobic core, while the cationic residues interact with the headgroups of anionic phospholipids, inducing membrane thinning and a curvature strain. To release this strain the orientation of the peptides changes from parallel to perpendicular, inducing bending of the membrane interface towards the hydrophobic interior, maintaining the contact between the peptides and the charged headgroups from the phospholipids. These events lead to the formation of toroidal pores composed by the peptides and the lipid headgroups (figure 8). Upon disintegration of the pores some peptides can be translocated to the inner leaflet of the membranes reaching the cytoplasm or the membrane can be disrupted due to depolarization or micellization, resulting in cell death (Nicolas, P. 2009; Rivas, L. *et al.* 2009).

Other mechanisms of action have recently been suggested that do not imply the recruitment of a high concentration of peptides to the membrane, and they do not necessarily exclude each other (or even the models explained above) (figure 8). Some examples are, **lipid segregation**, where the lipids can move laterally in the membrane and thus form domains rich in anionic lipids (induced by the presence of the positively charged peptide), and this can induce small leakage of intracellular contents and/ or

membrane destabilization and **depolarization** due to dissipation of the membrane potential; **formation of non-bilayer intermediates** in the membrane and non-lamellar structures; and **membrane permeabilization** and/ or **depolarization** due to an efflux of small anions across the bilayer by coupling with peptides. (Nguyen, L.T. *et al.* 2011).

Whatever the mechanism is, peptide interaction with membranes can culminate in cell death by the dissipation of transmembrane electrochemical ion gradients, loss of metabolites, and eventually lysis of the cell (Wiesner, J. & Vilcinskas, A. 2010). If the peptides are translocated to the cytoplasm, with or without permeabilising the cytoplasmic membrane, some other effects could arise due to the existence of intracellular targets, such as DNA, RNA or protein synthesis and folding. Buforin is an example of a non-lytic peptide which although it has a high antimicrobial activity this does not correlate with lysis of the bacterial membrane. Instead the peptide translocates efficiently to the cytoplasm probably through the formation of short-lived toroidal pores, enabling this peptide to enter the cell and act on intracellular targets (Henriques, S.T. *et al.* 2006; Kobayashi, S. *et al.* 2004; Kobayashi, S. *et al.* 2000).

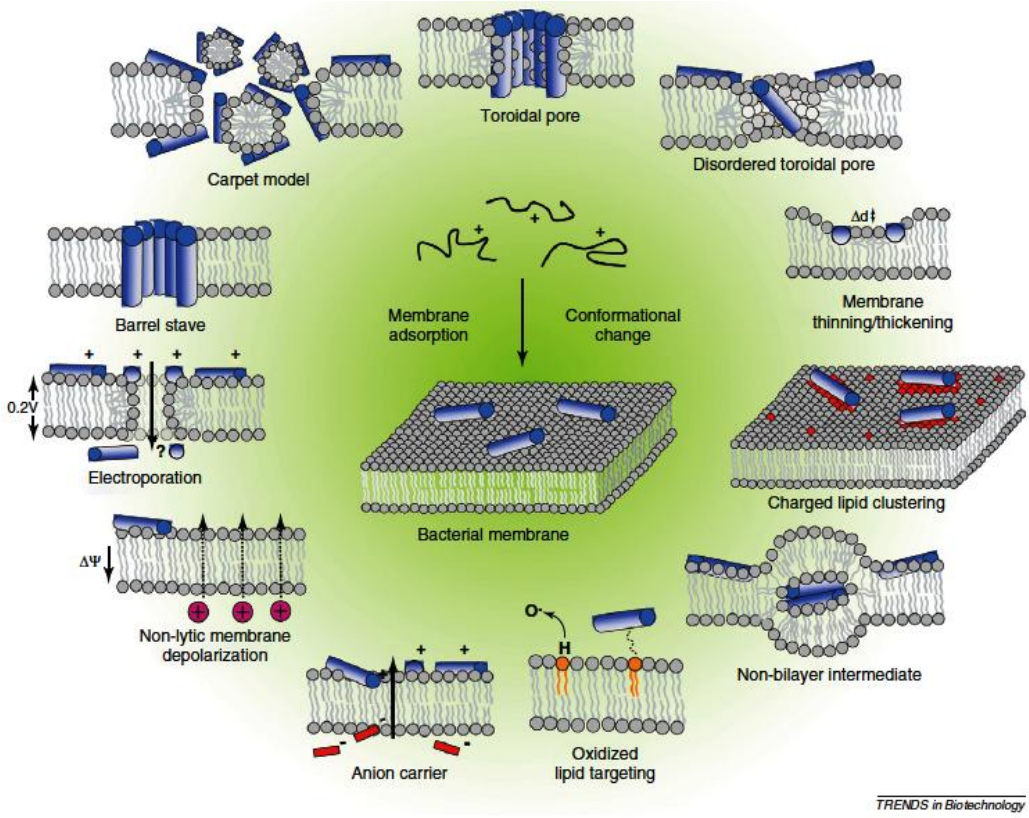

Figure 8 – Proposed mechanisms of action for AMPs. Illustration of some of the proposed events/mechanisms occurring at the cytoplasmic membrane after a initial interaction of antimicrobial peptides with the membranes (Nguyen, L.T. *et al.* 2011).

AMPs can also be cytotoxic against cancerous cells promoting apoptosis (*e.g.* lactoferrin proved to inhibit the tumor growth and metastasis in a rodent model with breast cancer, while lactoferrricin causes apoptosis in Jurkat T-leukemia cells) (Iigo, M. *et al.* 2009; Mader, J.S. *et al.* 2007; Wiesner, J. & Vilcinskas, A. 2010). It is believed that this effect is first due to lysis of the cellular membrane of the cancerous cells, that are recognized by some AMPs due to the unusually high amounts of negatively charged gangliosides and phosphatidylserine (PS) in the surface of these cells, a negative membrane potential and a higher membrane fluidity, allowing the peptides to insert into the membrane more easily (Hoskin, D.W. & Ramamoorthy, A. 2008). Afterwards, mitochondrial membranes can also be disrupted due to entrance of the peptide into the cell, apoptosis pathways can be activated and angiogenesis inhibited (Gifford, J.L. *et al.* 2005; Guaní-Guerra, E. *et al.* 2010).

3.3. SELECTIVITY / TOXICITY

Independently of how AMPs exert their activity against pathogens the peptides have to interact with the cellular membrane resulting either in disruption or traversing this barrier. This interaction must be as selective as possible regarding the distinction between mammalian cells (eukaryotic cells) and pathogen cells, such as bacteria (prokaryotic cells). Cytoplasmic membranes of mammalian cells are predominantly constituted by phospholipids and cholesterol. Phospholipids are asymmetrically distributed between the inner and the outer leaflet of the bilayer exposing predominantly zwitterionic phosphatidylcholine (PC) and sphingomyelin to the extracellular side. On the other hand, cytoplasmic bacterial membranes do not have cholesterol and are mainly composed of zwitterionic phosphotidylethanolamine (PE) and negatively charged phosphatidylglicerol (PG), diphosphatidylglicerol and cardiolipin, conferring an overall negative charge to the outer side of the cytoplasmatic membrane (Lohner, K. 2009; Lohner, K. & Blondelle, S.E. 2005). The main principle of peptide interaction with membranes relies on electrostatic interactions between cationic peptides and negatively charged membranes and thus considering the composition of both cell types, bacteria are a preferential target for AMPs. However, before reaching the cytoplasmic membrane of the bacteria, peptides have to overcome other barriers. In the case of Gram-positive bacteria, a thick peptidoglycan layer embedded with teichoic and lipoteichoic acids (which are negatively charged) is present around the cell, exterior to the lipid membrane (figure 9). Gram-negative bacteria also have a complex cell wall consisting of an outer lipid membrane layer, with a unique and highly asymmetrical composition, and a layer of peptidoglycan (much thinner than that of Gram-positive bacteria) between the inner and the outer membranes (figure 9) (Cabeen,

M.T. & Jacobs-Wagner, C. 2005). In Gram-negatives outer membrane, lipopolysaccharides (LPS – negatively charged) are located exclusively in the outer leaflet and phospholipids are confined to the inner leaflet (figure 9) (Lohner, K. 2009; Lohner, K. & Blondelle, S.E. 2005). In the case of Mycobacteria, an even thicker and more complex cell wall is also present (see section 2.2.1). Even so, in normal conditions the overall charge of a bacterial cell wall is negative, increasing the chances of interaction with antimicrobial peptides. (Guenin-Mace, L. *et al.* 2009; Prescott, L.M. *et al.* 2002).

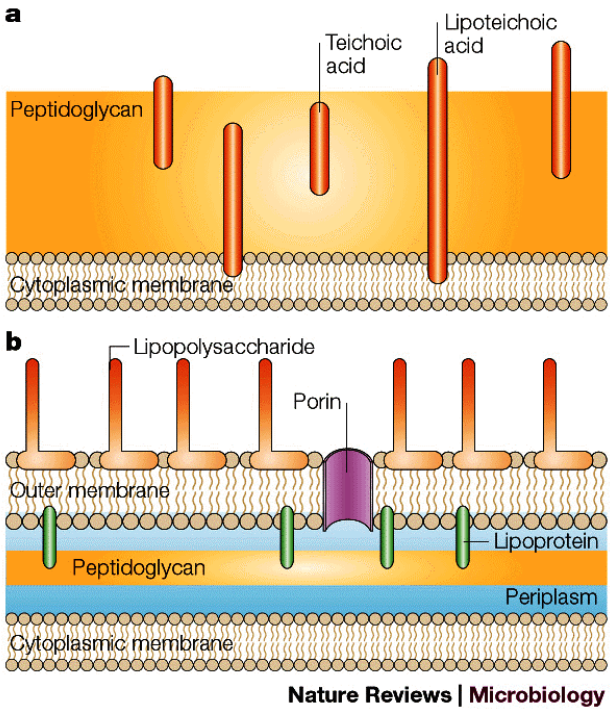

Figure 9 – Bacteria cell wall. Schematic representation of the cell wall of **a)** Gram-positive and **b)** Gram-negative bacteria (Cabeen, M.T. & Jacobs-Wagner, C. 2005).

The membrane potential can also contribute for the selectivity of AMPs since in eukaryotes it is less negative than in prokaryotes (Van 'T Hof, W. *et al.* 2001).

Antimicrobial peptides can also kill eukaryotic pathogens, such as fungi and protozoa, raising the question of what are the features of the pathogens, either eukaryotes or prokaryotes that allows some AMPs to discriminate them from mammal cells. These specificity seems to rely on charge, since pathogens seems to have a higher percentage of anionic phospholipids than mammal cells, thus explaining the specificity of the cationic AMPs (Lohner, K. 2009; Lohner, K. & Blondelle, S.E. 2005). However some peptides do not distinguish host and pathogen cells being able to disrupt mammalian cells (*e.g.* melittin) and thus cannot be used in the clinic. The reasons behind this activity are not completely understood and are most probably due to the properties of the peptides, like a

high positive charge, too large hydrophobic surfaces, between others (Nguyen, L.T. *et al.* 2011). In the evaluation of the activity of AMPs the cytotoxicity of these peptides towards mammalian cells should always be addressed. There are many different cytotoxicity assays that can be performed but the outcome of each of them strongly depends on many factors such as the origin and life storage of the mammal cells, the peptide-to-cell ratio, the medium used, between others, so the results must be carefully analyzed (Van 'T Hof, W. *et al.* 2001). Based on the results of cytotoxicity assays together with the antimicrobial activity of AMPs, the selectivity index, defined as the cytotoxicity activity divided by the antimicrobial activity, can be determined being a useful tool to predict the potential of a given AMP as a therapeutic agent. However, the characteristics of these peptides and the prediction of their action, either antimicrobial activity or cytotoxicity, is not straightforward and so they should be analyzed on a case-by-case basis.

3.4. AMPs IN THE IMMUNITY

Besides the role of AMPs as endogenous antibiotics (directly killing the pathogens), they can also participate in multiple aspects of immunity and for that they can also be called host defense peptides (HDPs). Combination of these different but complementary functions is essential for the effective control of infections in the host organism. AMPs seem to actively participate in the immune system either by immunomodulation, neutralization of endotoxins, enhancement of phagocytosis, induction of both angiogenesis and wound repair, leukocyte chemotaxis and synergism with cytokines (Yang, D. *et al.* 2002; Zaiou, M. 2007). Thus, in response to an infection, antimicrobial peptides can promote bacterial clearance not only through direct killing but also through the establishment of immune cell circuits (Auvynet, C. & Rosenstein, Y. 2009; Guaní-Guerra, E. *et al.* 2010).

As a consequence of the actions stated above these peptides are also involved in autoimmunity. The abnormal concentration, processing or signaling of AMPs is associated with a growing list of autoimmune diseases (figure 10), in which these peptides usually act, not as a single direct cause but as an important factor that influences the outcome of the diseases (Guaní-Guerra, E. *et al.* 2010).

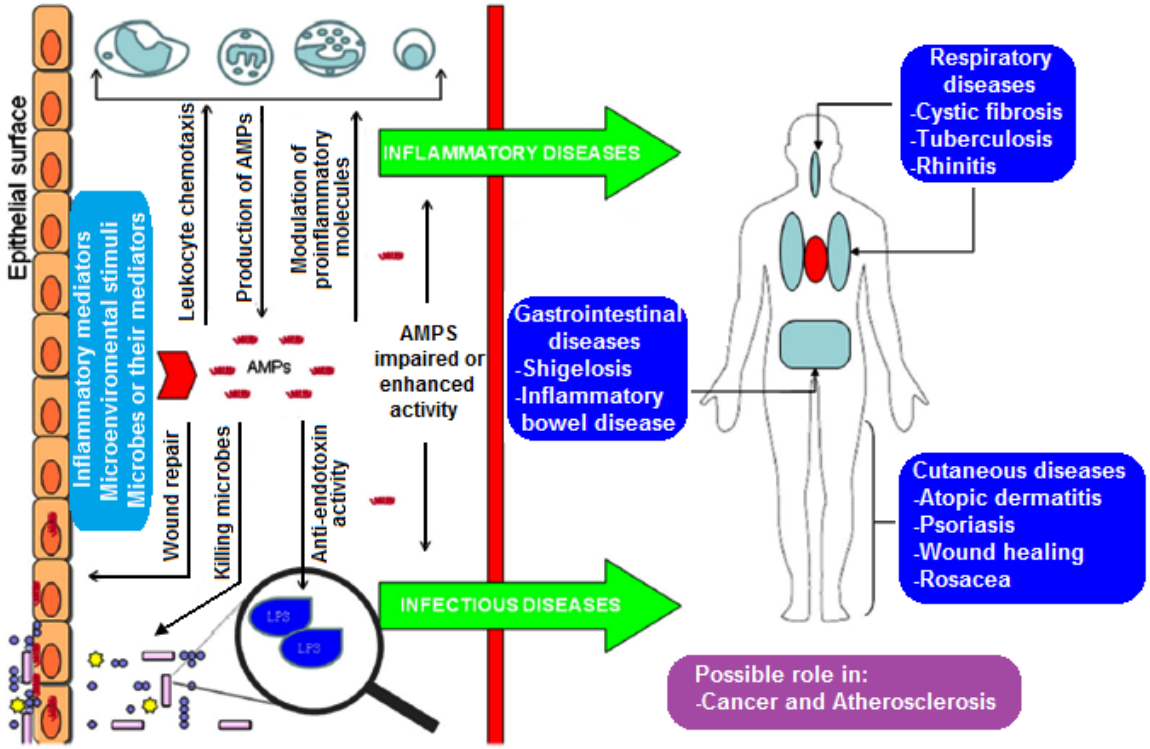

Figure 10 – Role of AMPs in the immune system. Exemplification of some of the immune processes and inflammatory diseases where AMPs are involved (Guaní-Guerra, E. *et al.* 2010).

3.5. Resistance to AMPs

Induction of resistance is less likely for AMPs when compared to conventional antibiotics, due to the fast kinetics of the antimicrobial process and to the nature of the target – plasma membrane – since it would require an overall reorganization of the cell membrane structure, namely the phospholipid composition, affecting simultaneously the pleiade of transport systems and the enzymes embedded in the phospholipid matrix. Although in principle acquiring resistance to AMPs is more difficult, it has been pointed that it might have more severe effects if it led to cross-resistance to innate human antimicrobial peptides (Hancock, R.E. & Sahl, H.G. 2006).

The most common mechanisms of bacterial resistance to AMPs described so far consist in the modification of the bacterial envelope leading to charge reduction and the proteolytic cleavage of peptides (Wiesner, J. & Vilcinskas, A. 2010). These mechanisms include the production of secreted proteins or cell-surface proteins that irreversibly binds or cleaves AMPs and glycopolymeric matrices that trap the peptides preventing their access to the bacterial cytoplasmic membrane (figure 11). Electrostatic repulsion of AMPs can also arise from several modifications such as substitutions of lipid A of LPS by aminoarabinose and ethanolamine, modification of phosphatidylglicerol by ligation of a L-

lysine, or esterification of theicoic acids (WTA and LTA) by D-alanine, culminating in the reduction of the bacterial negative surface charge. In addition, increased resistance is achieved by changing membrane fluidity by modification of fatty acid acylation pattern of lipid A of LPS (figure 11) (Koprivnjak, T. & Peschel, A. 2011).

For these mechanisms and modifications to take place, bacteria have to sense and respond to the presence of AMPs by up regulating the genes responsible for their resistance mechanism. Discovery of these mechanisms could point towards new and attractive targets for the development of antimicrobial drugs.

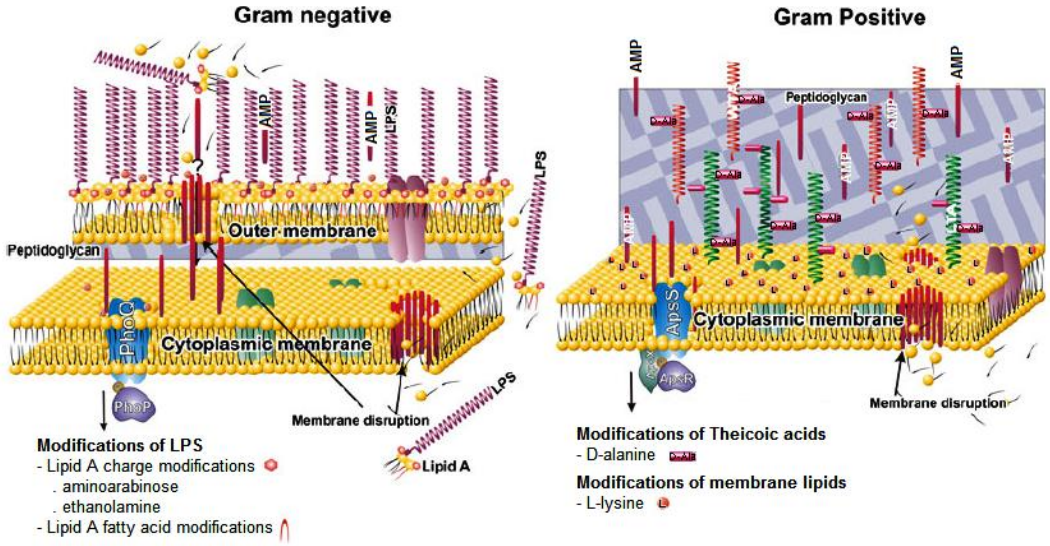

Figure 11 – Sensing and responding to AMPs in Gram-negative and Gram-positive bacteria. Exemplification of some of the bacterial resistance mechanism to AMPs. WTA and LTA – theicoic acids; LPS – lipopolysaccharide (adapted from: Koprivnjak, T. & Peschel, A. 2011).

3.6. AMPs IN THE CLINIC: SUCCESS, PROBLEMS AND CHALLENGES

In the last years, since the discovery of the potential of antimicrobial peptides as new therapies to fight infectious diseases, there has been a tremendous effort to try to get these peptides into clinical trials and eventually use them in the clinic. Cationic peptides such as polymyxin B and gramicidin S have been used for a long time in the clinic as topical agents, and the lantibiotic nisin, produced by fermentation using *Lactococcus lactis* is used as an antimicrobial food addictive (Hancock, R.E. & Sahl, H.G. 2006).

Several new generation antimicrobial peptides are in different stages of clinical trials but only a few are in the late stages phase III (table 2) (Godballe, T. *et al.* 2011). As drug candidates AMPs present some disadvantages due to their peptidic nature, including reduced activity in presence of salts and divalent cations, susceptibility to pH changes and

to protease and other plasma components' activity, resulting in low metabolic stability and bioavailability, and reduced *in vivo* half-lives (Rotem, S. & Mor, A. 2009). The root of administration is also a problem since they would not be reabsorbed from the intestinal tract if administered orally, and if injected they could trigger immune responses that neutralize the active component or induce allergic reactions. So they should be administered as topical drugs for the treatment of skin and wound infections, limiting their applicability (Wiesner, J. & Vilcinskas, A. 2010). Other safety considerations should be taken into account. They can cross-react with receptors for neuropeptides and peptide hormones, and the rapidly degradation of AMPs could lead to unwanted levels of amino acids for which some patients are sensitive, like glutamate (Chinese restaurant syndrome) and phenylalanine (phenylketonuria) (Van 'T Hof, W. *et al.* 2001).

The high cost of production associated with peptide synthesis is another drawback in the clinical application of these peptides. If the peptides have to be applied as topical formulations, the amount of peptide applied needs to be much higher and therefore the costs will be higher as well. Even when AMPs successfully finished several phase III trials, in most of the cases, they were not able to demonstrate superior protection when compared to traditional therapies (Godballe, T. *et al.* 2011).

Several approaches have been adopted to try to overcome these problems, keeping in mind that the requirements for antimicrobial activity do not rely on a defined secondary structure or even in a consensus sequence, which was not identified when several sequences of natural AMPs deposited in the Antimicrobial Sequence Database – AMSDb (http://www.bbcm.univ.trieste.it/~tossi/amsdb.html) were analyzed (Rotem, S. & Mor, A. 2009). The only common trait to all of them, as mentioned above (3.1) is the occurrence of both hydrophobic and positively charged amino acids. In this context some solutions have been proposed: i) the use of D-amino acids (rather than L-amino acids) which are resistant to proteases but have higher costs when compared with L-amino acids; ii) the use of nonpeptidic backbones (peptidomimetics); iii) formulation to improve stability, for example in liposomes; iv) pro-drug molecules, among others (Hancock, R.E. & Sahl, H.G. 2006).

Peptidomimetics (mimics of AMPs) deserves a special word, since this approach conserves the important features mentioned before (hydrophobicity and charge), and the antimicrobial activity, trying to solve some of the problems associated with natural peptides (costs, susceptibility to proteases, etc.) (Godballe, T. *et al.* 2011). Peptidomimetics refers to any oligomeric sequence designed to mimic a peptide structure

and/ or function but whose backbone is not solely based on α-amino acids (Rotem, S. & Mor, A. 2009).

Table 2 – Antimicrobial peptides and mimetics in clinical development (adapted from: Godballe, T. *et al.* 2011; Guaní-Guerra, E. *et al.* 2010; Hancock, R.E. & Sahl, H.G. 2006; Wiesner, J. & Vilcinskas, A. 2010).

Peptide	Peptide class	Clinical Use	Outcome
hLF 1-11	Lactoferrin	Bacterial hospital infections	Safety in humans established
P-113	Histatins	Used as mouth rinse for treatment of plaques and gingivitis	Improvement in certain dose groups and certain populations
Pexiganan	Magainins	Diabetic foot ulcers	Comparably effective to ofloxacin
Iseganan	Protegrins	Stomatitis associated with chemotherapy; oral mucositis associated with radiotherapy; ventilator-associated pneumonia	No efficacy
Omiganan	Indolicin	Central catheter-related bloodstream infections	Significantly superior to povidone iodine in reduction of catheter colonisation and catheter-related local site infections
Lytixar™ (LTX-109)	Defensin mimetic	Nasal decolonisation of methicillin-resistant *S. aureus* (MRSA); Skin infections caused by Gram-positive bacteria	Bactericidal and effective against wild-type as well as drug-resistant bacteria such as MRSA
CeraShield™	Ceragenins mimetic	Multi-drug resistant strains	Positive results in preclinical studies

3.7. CECROPIN A-MELITTIN-DERIVED PEPTIDES

Cecropin A, with 37 amino acids, was the first antimicrobial peptide from an insect to be reported, and it is active on Gram-negative and Gram-positive bacteria and forms a nearly perfect amphipatic α-helix (Andreu, D. *et al.* 1992; Boman, H.G. *et al.* 1989). This peptide interacts with lipid membranes forming voltage-dependent channels that collapse the ionic gradients (Diaz-Achirica, P. *et al.* 1994).

Melittin, from the bee venom toxin, has a variety of toxic properties namely a high hemolytic activity. This peptide adopts an α-helical conformation aggregating into tetramers and is used as a model peptide to monitor lipid–protein interactions using a variety of biophysical techniques (Raghuraman, H. & Chattopadhyay, A. 2007).

These two peptides, cecropin A and melittin, are composed by hydrophilic and hydrophobic domains separated by a flexible hinge region, and therefore they adopt an α-helix – hinge – α-helix conformation, the only difference is the reverse polarity. The hydrophobic region of cecropin A is localized on the C-terminal whereas in melittin is localized on the N-terminal (Boman, H.G. *et al.* 1989). In an attempt to obtain antimicrobial peptides with strong bactericidal activity and lower hemolytic properties, H.G. Boman, *et al* in 1989 synthesized for the first time **cecropin A-melittin hybrids**, and they found that these hybrids had better antimicrobial properties than the parental compounds (Boman, H.G. *et al.* 1989). In particular a hybrid formed by the first 8 amino acids of the cationic region of cecropin A and the first 18 amino acids from the hydrophobic and nonhemolytic region of mellitin (CA(1-8)M(1-18)) exhibited a wider spectrum of activity and improved potency relative to cecropin A without the cytotoxic effects of melittin (Boman, H.G. *et al.* 1989). In the continuation of this work, D. Andreu *et al* in 1992 synthesized shorter hybrids (15 aa) that retained a significant activity when compared to the larger versions of the hybrid, especially CA(1-7)M(2-9) (Andreu, D. *et al.* 1992).

The mechanism of action of these hybrids is thought to be membrane disruption due to the formation of ionic channels (larger hybrids) or disintegration of the membrane due to a detergent-like action (shorter hybrids) or even the formation of toroidal pores (Abrunhosa, F. *et al.* 2005; Andreu, D. *et al.* 1992; Bastos, M. *et al.* 2008; Diaz-Achirica, P. *et al.* 1994). D-enantiomers of all the hybrids were synthesized and tested whether they required interaction with receptors to exert their antimicrobial activity. The antimicrobial activity of the D-enantiomers was quantitatively equivalent to that of the L-enantiomers. This is interpreted to mean that the peptides do not act by tight interactions with chiral receptors, enzymes or lipids in the lysis and killing of the pathogens (Merrifield, E.L. *et al.* 1995; Wade, D. *et al.* 1990).

Although the aim of the synthesis of these hybrids was the reduction of hemolytic activity together with a potent antimicrobial activity, the first goal was not successfully achieved. The hybrids CA(1-8)M(1-18) and CA(1-7)M(2-9), especially the last one, are able to interact and disrupt zwitterionic model membranes (composed by phosphatidylcholine - PC), a model for erythrocytes, and toxicity towards erythrocytes was also described for them (Abrunhosa, F. *et al.* 2005; Fernandez-Reyes, M. *et al.* 2010). In an attempt to preserve the high antimicrobial activity of CA(1-7)M(2-9) but decreasing the hemolytic activity, some additional modifications where performed in this hybrid peptide.

Peptide optimization by residue-specific modifications is one of the most effective and used strategies to obtain more active peptides with lower hemolytic activity. There are

some modifications that play important roles *in vivo*, like methylation, acetylation, ubiquitination, sumoylation or citrullination (Fernandez-Reyes, M. *et al.* 2010). Previous studies showed that upon methylation of all the amino groups of melittin (all the hydrogen atoms of the amino groups are replaced by methyl groups) the peptide entirely lost its hemolytic activity (Ramalingam, K. & Bello, J. 1992). The decrease of the hemolytic activity is probably due to the loss of hydrogen bonding ability of the amino groups and for the introduction of some steric effects as a result of the bulky of trimethylammonium groups. However this peptide still retains the same global positive charge so the antimicrobial activity should not be comprised (Ramalingam, K. & Bello, J. 1992). In fact, recently, a systematic study to assay the effect of lysine N^{ε}-trimethylation (figure 12) on the hemolytic and the antimicrobial activity of a cecropin A-mellitin hybrid, CA(1-7)M(2-9), shows that all trimethylated peptides have a significantly lower hemolytic activity when compared to the parental hybrid peptide, where more resistant to proteolysis, and some of them retain a high antimicrobial activity both against *Leishmania* parasites and Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Acinetobacter baumannii*) bacteria (Fernandez-Reyes, M. *et al.* 2010). In this study the single trimethylated peptides on a lysine at position 6 (K6) and 7 (K7) (figure 13) revealed to have the better selectivity index (defined as the ratio between the hemolytic activity and the antimicrobial activity) of all the peptides and antimicrobial activities in the range of micromolar (Fernandez-Reyes, M. *et al.* 2010). In a biophysical approach K6 and K7 retain the ability to interact with partially negatively charged model membranes (mainly by segregation mechanisms) whereas they do not interact significantly with zwitterionic model membranes of PC (model membranes for erythrocytes) at odds with the parental hybrid peptide that disturbs all these model membranes (Teixeira, V. *et al.* 2010). The combination of these findings has highlighted the potentiality of lysine trimethylation as a powerful tool to overcome some of the problems for the applicability of AMPs as pharmacological agents, such as toxicity and susceptibility to proteases.

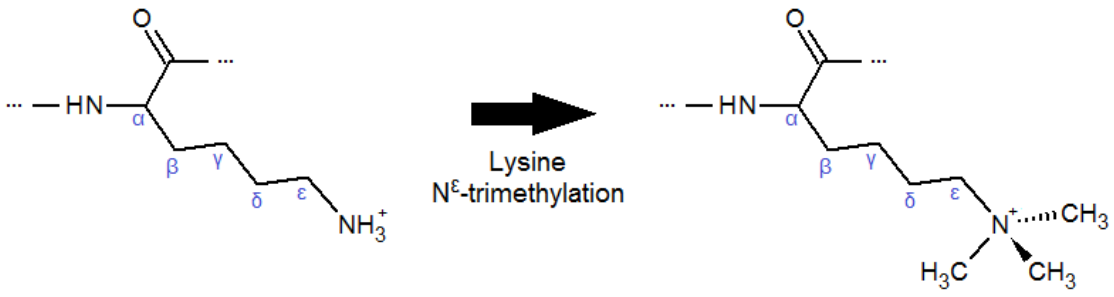

Figure 12 – Lysine N^{ε}-trimethylation. Trimethylation of the amino group on the ε-carbon of a lysine.

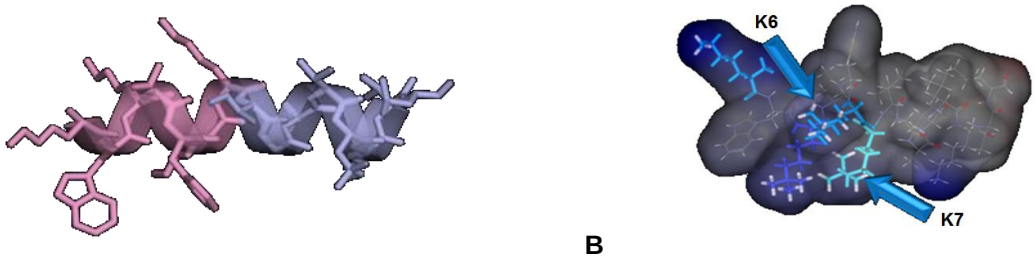

Figure 13 – Cecropin A-melittin hybris. A) Secondary structure of CA(1-7)M(2-9) (the corresponding part of cecropin A in purple and melittin in lilac). **B)** Positions of the trimethyl groups on K6 and K7 peptides (adapted from: Teixeira, V. *et al.* 2010).

3.8. LACTOFERRIN-DERIVED PEPTIDES

Lactoferrin (LF) is a mammalian iron-binding glycoprotein of 80 kDa, that belongs to the transferrin family (Pierce, A. & Legrand, D. 2009). Contrarily to transferrin, that appears primarily in the bloodstream delivering iron to the cells, lactoferrin is mostly found in exocrine secretions, like milk, tear fluid and seminal plasma, and in neutrophil granules playing an important role in maternal and innate immunity (Wiesner, J. & Vilcinskas, A. 2010). This is a multifunctional protein that has antibacterial, antiviral, antifungal and antiparasitic activities and is also implicated in protection against cancer development and metastasis owing to its immunomodulatory potential (Pierce, A. & Legrand, D. 2009; Wiesner, J. & Vilcinskas, A. 2010). In the beginning, it was thought that the antibacterial activity of lactoferrin was only due to its ability to sequester iron, an essential nutrient, from bacteria (Jenssen, H. & Hancock, R.E. 2009). Now it is known that allied to that, the existence of particular domains, such as the highly cationic N1 terminal domain, where two antimicrobial peptides are found namely, lactoferricin and lactoferrampin, are crucial for the antimicrobial activities of lactoferrin (figure 14).

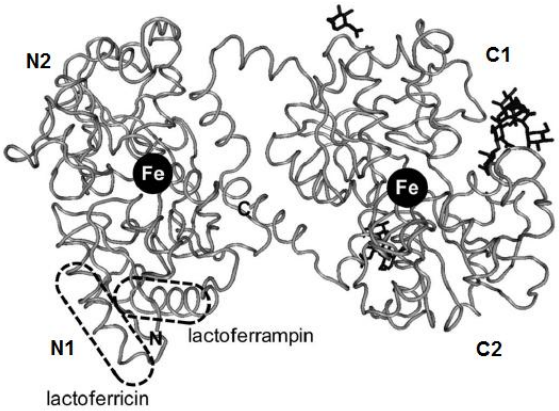

Figure 14 – Bovine lactoferrin. Structural model representing the polypeptide backbone of bovine lactoferrin (PDB ID: 1BLF). The positions of the two bond Fe^{3+} ions, lactoferricin and lactoferrampin, these last two in the N1 domain, are indicated (adapted from: Wiesner, J. & Vilcinskas, A. 2010).

Lactoferricin is obtained by pepsin digestion of lactoferrin, and has been extensively studied. The bovine peptide is constituted by 25 amino acids, corresponding to the amino acids 17-41 in the native protein (bovine lactoferrin) it has a disulfide bond between two cysteine residues, and forms a β-sheet structure (Chan, D.I. *et al.* 2006; Gifford, J.L. *et al.* 2005). Antimicrobial activity of lactoferricin ranges from several Gram-negative to Gram-positive bacteria, (Jenssen, H. & Hancock, R.E. 2009; Oo, T.Z. *et al.* 2010; Sanchez-Gomez, S. *et al.* 2011) fungi, protozoan and virus. Lactoferricin has been shown to have antitumor effects, like inhibition of tumor metastasis, suppression of tumor-induced angiogenesis and significant reduction of solid tumor (fibrosarcomas, melanomas, coloncarcinomas) size in mice, without affecting erythrocytes or fibroblasts (Eliassen, L.T. *et al.* 2002; Gifford, J.L. *et al.* 2005; Yoo, Y.C. *et al.* 1997a; Yoo, Y.C. *et al.* 1997b). This peptide can also have immunomodulatory properties, playing a role in the innate and adaptive immune system, and it was also proposed that it inhibits septic shock by binding to endotoxins (Chan, D.I. *et al.* 2006; Yamauchi, K. *et al.* 1993). J. Groenink, *et al* in 1999 synthesized shorter peptides with sequences homologous to the N-terminal domain of bovine and human lactoferricin, and found that of the peptides tested, bovine lactoferricin containing amino acids 17-30 (**LFcin17-30**) had the higher number of positively charged residues and the highest antimicrobial activity against both Gram-positive and Gram-negative bacteria (Groenink, J. *et al.* 1999). The corresponding human peptide, hLFcin with the first 11 amino acids from human lactoferrin (**hLFcin1-11**) was demonstrated to be active against a variety of bacteria, such as *Acinetobacter baumanii* and *Stahphylococcus aureus* and fungi, especially *Candida albicans*. A clinical phase I trial using this peptide to combat bacterial hospital infections has been completed, and the overall safety was proved in preclinical and clinical studies (table 2) (Wiesner, J. & Vilcinskas, A. 2010) (www.am-pharma.com).

Recently, another peptide in the N1 domain of bovine lactoferrin was identified by the group of Jan G. Bolscher, namely **lactoferrampin** containing the amino acids 268-284 (LFampin268-284). This peptide exhibited antimicrobial activity against a broad range of pathogens, different from the activity of lactoferricin, especially against *Candida albicans* and several bacteria, (Van Der Kraan, M.I. *et al.* 2004). Systematic studies, using the initial sequence of lactoferrampin to obtain other peptides by truncation and/ or extension, have shown that lactoferrampin 265-284 (**LFampin265-284**) was the shortest and most active peptide, especially against *C. albicans* (Van Der Kraan, M.I. et al. 2005a; Van Der Kraan, M.I. et al. 2005b). The three extra amino acids (Aspartic acid-Leucine-Isoleucine) did not confer any additional positive charge (one of the most important characteristics for AMPs) since the first is negatively charged and the other two are uncharged. Instead, this

N-terminal sequence endowed lactoferrampin with a high tendency to adopt a more stable amphipatic α-helix, enhancing in this way its microbicidal activity (Haney, E.F. *et al.* 2009; Van Der Kraan, M.I. *et al.* 2005a; Van Der Kraan, M.I. *et al.* 2006).

Lactoferricin and lactoferrampin are spatially close in lactoferrin, making it plausible that they can cooperate in many of the beneficial properties of this protein. To test if these peptides would form a functional unit, a chimeric peptide (**LFChimera**) containing **LFcin17-30** and **LFampin265-284** was synthesized by Jan G. Bolscher (Bolscher, J.G.M. *et al.* 2009). To try to mimic the spatial topology of these two peptides in lactoferrin, LFcin17-30 and LFampin265-284 are coupled by their C-terminals to the α- and ε-amino groups, respectively, of an additional lysine leaving the two N-terminals as free ends (figure 15). **LFChimera** displays a strong activity against a wide variety of pathogens such as *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio parahaemolyticus, Entamoeba histolytica*, which is maintained in different physiological conditions (including high ionic strength and rich growth medium) (Bolscher, J.G.M. *et al.* 2009; Flores-Villasenor, H. *et al.* 2010; Leon-Sicairos, N. *et al.* 2009; Lopez-Soto, F. *et al.* 2010).

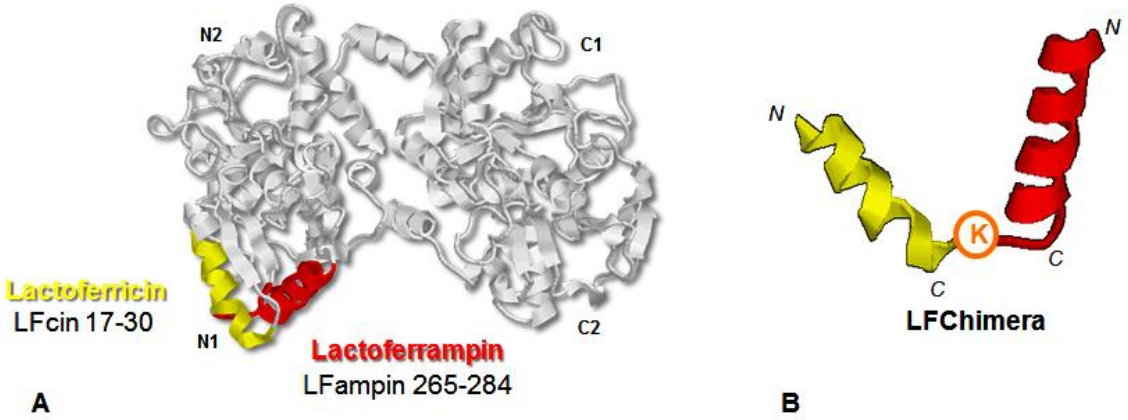

Figure 15 – Lactoferrin-derived peptides. A) Ribbon diagram of bovine lactoferrin with LFcin17-30 (yellow) and LFampin265-284 (red). **B)** Designed of LFChimera composed by LFcin17-30 and LFampin265-284 coupled by an additional lysine (K) (adapted from: Bolscher, J.G.M. *et al.* 2009).

4. LIPIDS IN BIOLOGICAL AND MODEL MEMBRANES

Biological membranes are mainly composed by a lipid matrix that surrounds all other components, like proteins, glycoproteins and glycolipids that performs crucial functions to the life of the cell. Phospholipids, the building blocks of the cellular compartments, are amphiphilic molecules composed by a hydrophilic headgroup and a hydrophobic tail that consist in the hydrocarbon chains. The lipid composition is different between organisms, and even cell types of the same organism. The phospholipid distribution between the inner and outer leaflets of the lipid bilayer is asymmetrical, and some lipids are richer in one leaflet than the other (Pozo Navas, B. *et al.* 2005).

Although, in the past, the widespread opinion was that lipids are passive components in biological membranes and that only proteins have specific physiological functions, it is now known that lipid molecules are dynamic and actively involved in various biological processes being not only a scaffold for proteins (Haney, E.F. *et al.* 2010; Luzzati, V. 1997).

4.1. LIPID POLYMORPHISM

The way that lipids are structurally organized when in contact with water, is highly variable, depending on their intrinsic properties, on the composition of the medium (pH, ionic strength, additives, etc), as well as on temperature and water content, giving rise to a complex phase-diagram (figure 16).

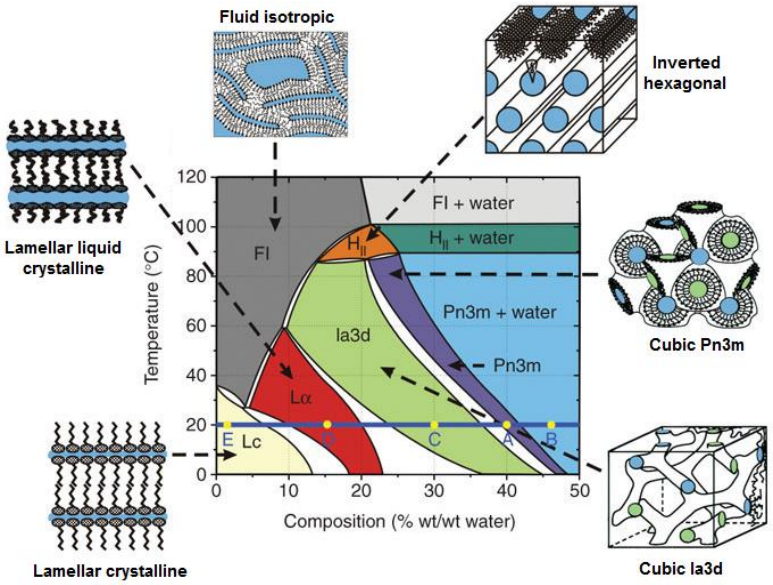

Figure 16 – Temperature-composition phase diagram of monoolein. The phases formed by the lipid monoolein depend on temperature and hydration as illustrated in this phase diagram (adapted from: Qiu, H. & Caffrey, M. 2000).

This high polymorphism allows them to self-assemble into different phases and three-dimensional ordered structures, that are usually classified as lamellar, and non-lamellar (such as hexagonal and cubic) phases. These non-lamellar phases were first characterized by Luzzati and coworkers using X-ray scattering techniques to analyze the three-dimensional arrangement of numerous membranes (Haney, E.F. *et al.* 2010).

Different lipids have different propensity to form the different lipid phases mainly due to the relative size of their polar headgroup and apolar tails, that are responsible for determining the size and shape of the lipid molecule (figure 17) (Tresset, G. 2009). In cases where the headgroup and the hydrocarbon chains have similar cross-sectional areas, the molecule has a cylindrical shape (*e.g.* phosphatidylcholine - PC and phosphatidylserine). Lipids with a small headgroup like phosphatidylethanolamine (PE) have the structure of a truncated cone. In contrast when the hydrophobic part occupies a relatively smaller surface area, the molecule has the shape of an inverted cone (*e.g.* lysophosphatidylcholine) (figure 17) (Sprong, H. *et al.* 2001).

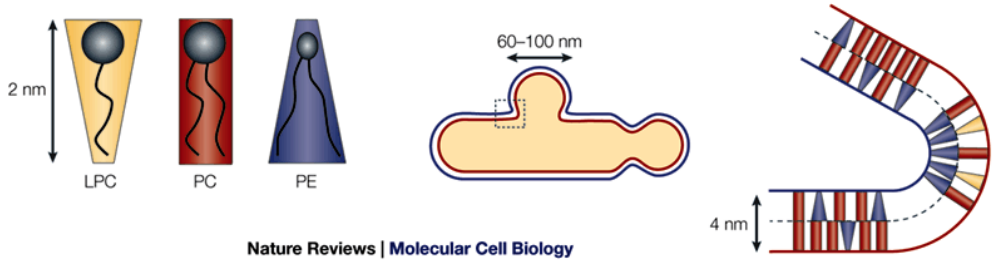

Figure 17 – Phospholipids molecular shapes: inverted cone (LPC - lysophosphatidylcholine), cylindrical (PC - phosphatidylcholine) and truncated cone (PE - phosphatidylethanolamine), and their role in the shape of the cellular membrane (adapted from: Sprong, H. *et al.* 2001).

The functional structure of a biological membrane is a lamellar lipid phase that acts as a fundamental permeability barrier. Lamellar phases are characterized by two opposing lipid monolayers with the hydrocarbon chains facing each other. Two types of lamellar phases can occur, a liquid crystalline or fluid lamellar phase (L_α) that occurs at higher temperatures, where the hydrocarbon chains are in a melted and fluid state allowing the membrane to spontaneously reorganize upon external stimulus, and a gel lamellar phase (L_β) (lower temperatures) in which the hydrocarbon chains are extended and rigid (figure 18) (Haney, E.F. *et al.* 2010). The typical state in biological membranes is the liquid crystalline or fluid lamellar state. Lamellar phases typically occur for lipids with a packing parameter (*p*) close to 1, which means that an individual molecule fits to a cylinder (*e.g.* PC) (figure 17) (Tresset, G. 2009).

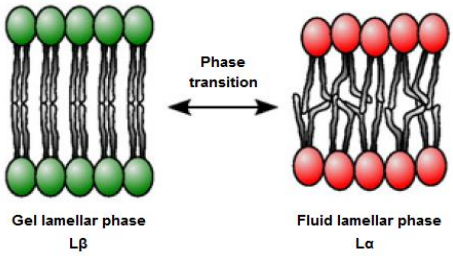

Figure 18 – Lamellar phases. Schematic representation of the structure and orientation of the phospholipids hydrocarbon chains in the two lamellar phases, gel (L_β) and fluid (L_α) (adapted from: Tresset, G. 2009).

Although the maintenance of a stable bilayer is essential for normal membrane function, it is well known that in some cases, membranes containing high amounts of non-lamellar phase-forming lipids (*e.g.* phosphatidylethanolamine) have the ability to form more complex 3D morphologies, non-lamellar structures such as hexagonal and cubic phases, that are believed to play important roles in biological processes (Lohner, K. 2009; Lohner, K. & Blondelle, S.E. 2005).

Hexagonal phases are made of lipids that all together adopt a cylindrical shape. When the phospholipids headgroups are oriented towards the core of the cylinder, that is filled with water, the structure is called inverted hexagonal phase – H_{II}, whereas in a normal/ micellar hexagonal phase – H_I, the centers of the cylinders consist of the hydrocarbon chains or phospholipid tails (figure 19). The H_{II} is the simplest non-lamellar structure implied in membrane fusion processes. Very few lipids adopt an H_I phase (Haney, E.F. *et al.* 2010; Tresset, G. 2009).

The transition from lamellar to hexagonal phase occurs with increasing temperature as the interfacial area expands leading to unfavorable contact between aqueous and hydrophobic regions resulting in the formation of inverted hexagonal phases (Rappolt, M. *et al.* 2003).

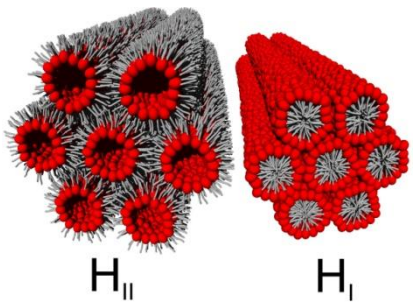

Figure 19 – Hexagonal phases. Adopted orientation of phospholipids in the different hexagonal phases H_I and H_{II}. In red the headgroups of phospholipids (hydrophilic region) and in grey the hydrocarbon chains (hydrophobic region) (Tresset, G. 2009).

Other important non-lamellar structures are the three-dimensional cubic phases. The cubic symmetry is attributed not only to a lipid phase but to a large family of phases that are complex and diverse (Luzzati, V. *et al.* 1997). Several cubic phases are known and they can be divided essentially into two classes, bicontinuous and micellar phases. The bicontinuous phases are unique and consist on a single bilayer folded into three-dimensional cubic network separating two disjointed water compartments with continuous regions of both polar (hydrophilic headgroups) and non-polar (hydrocarbon chains) structures (Ia3d, Pn3m, Im3m) whereas the micellar phases are impervious to water-soluble components and the structure is made of disjointed micelles with different sizes allowing a more efficient packing on a cubic lattice (*e.g.* Fd3m, Pm3n) (figure 20) (Luzzati, V. 1997; Tresset, G. 2009).

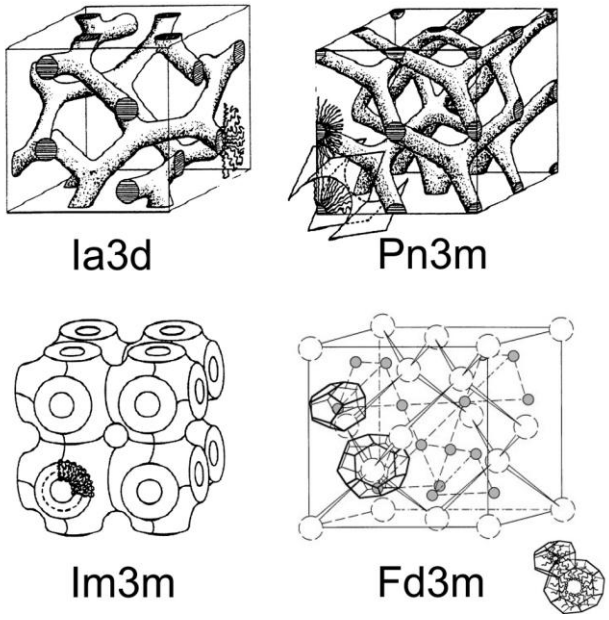

Figure 20 – Cubic phases of lipids. Bicontinuous cubic phases: Im3m, Pn3m and Ia3d; and a micellar cubic phase (Fd3m) (adapted from: Tresset, G. 2009).

Growing evidences show that cubic phases are ubiquitous in the biological world as they have been detected in the plasma membrane of archaebacteria, as well as in the endoplasmic reticulum and mitochondria of mammalian cells. These phases are also involved in biological processes such as membrane fusion, fat digestion and in the reorganization of cell membrane composition (Almsherqi, Z.A. *et al.* 2009; Erbes, J. *et al.* 1994; Tresset, G. 2009).

4.2. AMP INTERACTION WITH LIPID MEMBRANES AS REVEALED BY MODEL STUDIES

Regarding the interaction of antimicrobial peptides with membranes it is crucial to understand if AMPs are capable of altering the phospholipid phase behavior and in that case to correlate these possible changes in lipid polymorphism with models for the mechanism of action of AMPs. Using model membranes, namely liposomes (concentric bilayer vesicles) with different phospholipid compositions, and analyzing them by using different techniques (*e.g.* calorimetry, X-ray diffraction, spectroscopy), can give the desired information that enables the understanding of these changes and how they affect the overall structure and stability of the membrane. In this way the mechanism of action of AMPs could be clarified, and lead to a fine-tuning of new peptides in order to obtain a better therapeutic agent. The induction of cubic phases by AMPs, mostly by lowering the lamellar to non-lamellar phase boundary of lipid membranes, could mean that the peptide induces membrane disruption. Without disruption of the membrane, the formation of non-lamellar phases can also alter the lipid domains in the cellular membrane that are important for many biological processes such endocytosis (induced by lipid rafts) or even the activity of membranar proteins that most of the time depend on these domains culminating into impairment of the membrane function (Lohner, K. 2009; Lohner, K. & Blondelle, S.E. 2005).

Model membranes can be composed of different phospholipids, and as such their characteristics can be fine-tunned to the desired purpose of study. The compositions most widely used for the study of AMPs are: zwitterionic membranes of PC that mimics the erythrocytes membranes; the combination of PC and PG or PE with PG to try to mimic the cytoplasmic bacterial membrane that has a global negative charge. Recently much emphasis has been put on the use of POPE as the major class of phospholipids in the bacterial membrane whereas the amount of PC is more important in fungus. These are very simplified models of more complex structures, where the main aspect relies on changing the charge balance. Nevertheless, it is more and more clear that the AMPs are sensitive to the actual membrane composition, and thus efforts are also being made in biophysical studies to vary model membrane composition. More elaborated models can be used by the introduction of sphingomyelin and sterols for eukaryotic membranes and cardiolipin for prokaryotic membranes, or even by using membrane extracts of the bacteria. It should nevertheless be stressed that the main paradigm of model studies is to “keep it simple”, in order to enable discriminate analysis of interaction effects.

PART II

OBJECTIVES

OBJECTIVES

The objectives of this work were:

1- To study the effects of the lactoferrin-derived peptides listed in table 4 (below) on *Leishmania* growth and viability.
2- To study the effects of some lactoferrin-derived peptides listed in table 4 and of cecropin A-mellitin hybrid derivatives listed in table 3 (below) on the growth of *Mycobacterium avium*, both axenically and inside macrophages.
3- To characterize the interaction of cecropin A-melittin derivatives CAM, K6 and K7 (table 3) and lactoferrin-derived peptides LFcin17-30, LFampin265-284 and LFChimera (table 4) with lipid membranes by X-ray diffraction, using 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) model membranes.

The work on the antimicrobial activity of AMPs against *Leishmania* was carried out at Centro Investigaciones Biológicas CIB/CSIC, Madrid, Spain, under the supervision of Luis Rivas.

The work on *Mycobacterium avium* was carried out at Instituto de Biologia Molecular e Celular (IBMC), Porto, Portugal, under the supervision of Salomé Gomes.

The studies on interaction with model membranes by X-ray diffraction were carried out at Faculdade de Ciências da Universidade do Porto, Portugal and Hasylab, DESY, Hamburg, Germany, under the supervision of Margarida Bastos.

The cecropin A-melittin-derived peptides (table 3) were provided by David Andreu, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona, Spain (CA(1-7)M(2-9), K6 and K7), and by Paula Gomes, CIQ(UP), Faculdade Ciências da Universidade do Porto, Porto, Portugal (CA(1-7)M(2-9)).

Table 3 – Properties of cecropin A-melittin-derived peptides.

Peptide	Sequence[a]	#AA	Charge[b]
CA(1-7)M(2-9)	KWKLFKKIGAVLKVL	15	5+
K6	KWKLF**K**(Me_3)KIGAVLKVL	15	5+
K7	KWKLFK**K**(Me_3)IGAVLKVL	15	5+

[a] The C-terminals in all peptides are amidated; [b] Estimated net charge at neutral pH.

The peptides derived from lactoferrin (table 4) were provided by Jan Bolscher, ACTA, Department of Oral Biochemistry, Amsterdam, The Netherlands – LFampin265-284, LFcin17-30, LFChimera, and LFChimera surrogates. Paula Gomes, CIQ(UP), Faculdade Ciências da Universidade do Porto, Porto, Portugal, provided human lactoferricin 1-11 (hLFcin1-11).

The set of LFChimera surrogates (table 4) is composed by: LFChimera-R that differs from the parental hybrid peptide (LFChimera) in the order of the constituent peptides, instead of having LFcin17-30 bond to the α-carbon of an additional lysine and LFampin265-284 bond to the ε-carbon, we have the opposite situation; Di-LFcin and Di-LFampin, where the peptides are formed by two lactoferricins 17-30 and two lactoferrampins 265-284, respectively, also couple by an additional lysine in the same way as in LFChimera; and LFcin–LFampin and LFampin–LFcin, that are linear hybrids of lactoferricin 17-30 and lactoferrampin 265-284 coupled by a normal peptide bond, the only difference being the order of the peptides.

Table 4 – Properties of lactoferrin-derived peptides.

Peptide	Sequence	#AA	Charge[a]
LFcin17-30	FKCRRWQWRMKKLG	14	+6
hLFcin1-11	GRRRRSVQWCA	11	+4
LFampin265-284	DLIWKLLSKAQEKFGKNKSR	20	+4
LFChimera	FKCRRWQWRMKKLG—K | DLIWKLLSKAQEKFGKNKSR	35	+12
LFChimera-R	DLIWKLLSKAQEKFGKNKSR | FKCRRWQWRMKKLG—K	35	+12
Di-LFcin	FKCRRWQWRMKKLG—K | FKCRRWQWRMKKLG	29	+14
Di-LFampin	DLIWKLLSKAQEKFGKNKSR—K | DLIWKLLSKAQEKFGKNKSR	41	+10
LFcin – LFampin	FKCRRWQWRMKKLG–DLIWKLLSKAQEKFGKNKSR	34	+10
LFampin – LFcin	DLIWKLLSKAQEKFGKNKSR–FKCRRWQWRMKKLG	34	+10

[a] Estimated net charge at neutral pH.

PART III

EXPERIMENTAL METHODS AND RESULTS

III.1 – Antimicrobial activity of lactoferrin-derived peptides against *Leishmania*

1. MATERIAL AND METHODS

Peptides

The lactoferrin-derived peptides tested for their antimicrobial activity against *Leishmania* were lactoferricin (LFcin17-30), lactoferrampin (LFampin265-284), LFChimera, and LFChimera surrogates described on table 4 of page 56.

Leishmania donovani promastigotes

Promastigotes of strain MHOM/SD/00/1S-2D (WT TURCO) of *L. donovani* were grown at 26 ºC in RPMI 1640 (Gibco) pH=6.8, supplemented with 10% heat-inactivated fetal calf serum (HIFCS), 25 mM HEPES, 2 mM L-glutamine, 10 µg/mL gentamicin and 20 units/mL unicilin.

Promastigotes of the WT TURCO-derived 3-Luc strain of *L. donovani*, which express a cytoplasmic form of luciferase (Luque-Ortega, J.R. *et al.* 2001), were grown in the same conditions as the wild-type strain, except for the addition of 30 µg/mL geneticin to the medium.

Leishmania pifanoi amastigotes

Amastigotes MHOM/VE/60/Ltrod of *L. pifanoi* were grown at 32 ºC in M199 pH=7.2, supplemented with 20% HIFCS, 5% trypticase, 13.8 mM glucose, 76 µM hemin and 48 µg/mL gentamicin.

Leishmania harvesting

The parasites were collected at late exponential growth phase by centrifugation (2000 rpm, 10 min, 4 ºC). The supernatant was discarded carefully and the cells were washed twice with Hank's Balanced Salt Solution (HBSS: 137 mM NaCl, 5.3 mM KCl, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$ and 0.4 mM Na_2HPO_4, pH=7.2, supplemented with 10 mM D-glucose) at 4ºC. Cells were resuspended in the same buffer at final density of $4x10^7$ cells/mL or $2x10^7$ cells/mL, and kept on ice until use.

Determination of antimicrobial activities

Principles

The antimicrobial activity of the different peptides was determined by colorimetric measurement of the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to blue insoluble formazan by mitochondrial reductases. When cell viability is impaired, this leads to a decrease in formazan formation; by the same rule, when tested on parasite proliferation, the amount of formazan is proportional to the number of viable cells.

This method can be applied to assess the impact of an antimicrobial peptide on cell viability (short-term effect) and on the cell proliferation (long-term effect), using the same sample, and determine parameters like the IC_{50} (the concentration of AMP that inhibits 50% of MTT reduction at the end of 4h) and the LD_{50} (lethal concentration that causes 50% inhibition of parasite proliferation at the end of 3 or 5 days, for promastigotes and amastigotes, respectively) (Luque-Ortega, J.R. & Rivas, L. 2010).

Method

Parasite suspension ($4x10^7$ cells/mL) was distributed (60 µL/well) in a sterile 96-well microplate; 60 µL of the corresponding peptide solution (all the solutions and suspensions in HBSS + 10 mM glucose) was added to the respective well, and incubated for 4h at 26 ºC or 32 ºC, for promastigotes and amastigotes, respectively.

Long-term effect:

- For promastigotes, after 4h of incubation 20 µL of each well were transferred into a new 96-well microplate with 130 µL/well of RPMI 1640 without phenol red + 10% HIFCS, and incubated at 26 ºC, for 3 days, to allow parasite proliferation. At the end of the incubation, 50 µL of 2 mg/mL MTT solution was added to each well and further incubated at 26 ºC (protected from light) until colour development. Finally, to stop the reaction and to dissolve the blue formazan crystals, 50 µL of SDS 10% was added to each well. Absorbance was read at 600 nm in a microplate reader (Bio-Rad model 680) fitted with a 595 nm filter.

- For amastigotes, in the end of the incubation, 20 µL of each well were transferred to a new 96-well microplate with 130 µL/well of M199 + 10% HIFCS, and incubated at 32 ºC, for 5 days, to allow parasite proliferation. Afterwards, the content of each well was transfer into 1.5 mL microcentrifuge tubes, and wells were additionally washed with 100 µL of HBSS. The tubes were centrifuged and washed again with 1 mL of HBSS. The

pellet was resuspended in 100 µL of 0.5 mg/mL MTT solution, and the whole content of the tubes was transferred into a new 96-well microplate and MTT reduction carried out as described previously.

For the short-term assay, after incubation with the respective peptide, 100 µL of MTT (1 mg/mL) solution were added to each well of the plate incubated for 4h, and proceeded as described before.

The parameters IC_{50} and LD_{50} were calculated using the SigmaPlot® software, and the results represented as the media of at least two independent experiments (conditions tested in triplicates) with the corresponding standard deviation (SD) indicated.

Real-time monitoring of in vivo changes in intracellular levels of ATP in *L. donovani* promastigotes

Principles

A strain of *L. donovani* promastigote, 3-Luc that express a cytoplasmic form of luciferase was used to monitor the levels of intracellular ATP. This assay is based on the reaction, catalyzed by luciferase that converts D-luciferin into D-oxoluciferin in the presence of O_2, with consumption of one molecule of ATP and emission of one foton. To overcome the low permeability of the cellular membrane to D-luciferin at physiological pH, a caged ester analogue of luciferin, DMNPE-D-luciferin (D-luciferin, 1-(4,5-dimethoxy-2-nitrophenyl) ethyl ester), free-permeable to the membrane, was used. Once inside the cytoplasm D-luciferin is released by intracellular esterases. In this case, luminescence is directly related to the cytoplasmic levels of free-ATP, the limiting substrate for the reaction (Luque-Ortega, J.R. *et al.* 2003).

When the cells are intact, and in the presence of luciferase substrate, cells emit light. However in the presence of a peptide that induces membrane damages, the loss of ionic gradient or, for larger lesions, leakage of ATP, with subsequent depletion in the cytoplasm, will lead to the decrease of the light emitted (Luque-Ortega, J.R. *et al.* 2003).

Method

Parasites, *L. donovani* 3-Luc promastigotes, were resuspended at $2x10^7$ cells/mL in HBSS + 10 mM glucose, incubated at 26 ºC with DMNPE-D-luciferin (25 µM, final concentration), and 90 µL of the suspension was distributed into a black 96-well microplate. When luminescence reached a plateau, 10 µL of the respective peptide was added (t = 0) and

the decay on the luminescence was monitored in a BMG Polarstar Galaxy microwell reader (Öffenburg, Germany) equipped with luminescence setting. Luminescence data were referred as percentage of luminescence corrected with the corresponding values of untreated promastigotes.

Plasma membrane permeabilization of *L. donovani* promastigotes

Principles

The presence of a peptide may induce lesions into the cytoplasmic membrane large enough to allow entrance of the cationic vital dye, Sytox Green (MW = 600 Da), to which intact cells are impermeable. Once the dye gains access to the intracellular space, it binds to nucleic acids with high-affinity, close to irreversibility in practical terms. This enhances its fluorescence more than 500-fold (Luque-Ortega, J.R. & Rivas, L. 2010). So the increase in fluorescence stands for the severity of the damage to the parasites membranes.

Method

Two different assays were performed to assess the entrance of the vital dye:

- First, the parasites *L. donovani* promastigotes (90 µL at $2x10^7$ cells/mL) were incubated with Sytox Green (1 µM, final concentration) in HBSS + 10 mM glucose and fluorescence changes were monitored at λ_{ex} = 504 nm and λ_{em} = 524 nm in a BMG Polarstar Galaxy microwell reader (Öffenburg, Germany), immediately after adding 10 µL of the corresponding peptide solution (t = 0). The results were expressed as percentage relative to full permeabilized cells achieved by the addition of 0.1% Triton X-100.

- Second, to assess the incorporation of the dye into individual parasites, *L. donovani* promastigotes and antimicrobial peptides were incubated at the same conditions as described above, except that the Sytox Green was added only after 4h of incubation at 26 ºC, and the fluorescence was measured by cytofluorometry in a Beckman-Coulter FC-500 Analyzer. Maximal permeation was achieved by 15 µM of digitonin in order to preserve parasite shape.

AMPs or CPPs: Confocal Microscopy

To evaluate whether the peptides enter the cells without permeabilizing the membrane and extrapolate if these peptides had an internal target to account for their lethal action, in other words, whether they behaved or not as Cell-Penetrating Peptides (CPPs) *L. donovani* promastigotes and *L. pifanoi* amastigotes ($4x10^7$ cells/mL) were incubated with the peptides labeled with FITC (fluorescein isothiocyanate) in HBSS + 10 mM glucose for 4h at 26 ºC and 32 ºC for promastigotes and amastigotes, respectively. After 4h of incubation propidium iodide, PI, (50 µg/mL, final concentration) was added to the cells. A 15 µL drop of each sample was placed in microscope slides previously coated with poly-lysine (to favour parasite attachment and immobilization) and parasites were observed and photographed in a Confocal Laser Scanning Microscope Leica TCS – SP2 ABOS.

Ultrastructural alterations: Transmission Electron Microscopy

L. donovani promastigotes or *L. pifanoi* amastigotes suspension ($4x10^7$ cells/mL) in HBSS + 10 mM glucose were incubated with the peptides for 4h at 26 ºC or 32 ºC, for promastigotes and amastigotes, respectively. After the incubation, cells were washed with PBS and resuspended in a 3% glutaraldehyde solution in PBS for 1-2h, for cell fixation, and then incubated with 2.5% osmium tetroxide (OsO_4) for about the same time (1-2h), with PBS washes between steps. Cells were dehydrated by successive incubations with increasing concentrations of ethanol: 30% and 50% for 30 min; 70% overnight; 90% and 100% for 30 min. At the end, parasites were incubated with propylene oxide and afterwards, propylene oxide was successively replaced by a mix of resins 3A / 7B (resin A: 6.88 mL of Epon-812 epoxy resin + 11.11 mL of dodecenyl succinic anhydride (DDSA) – resin B: 22 mL of Epon-812 epoxy resin + 19.8 mL of methyl -5-norbornene-2,3-dicarboxylic anhydride (NMA)). The protocol consisted in successive incubations with different ratios of a mixture between propylene oxide and the resins: 3:1 and 1:1 for 30 min, 1:3 overnight and 0:1 for 30 min. The inclusion of cells into the resins was completed with a polymerization step (70ºC for 48h), catalyzed by the addition of 1.8% 2,4,6-tri(dimethylamino methyl) phenol (DMP30) as accelerator. The samples were distributed into electron microscopy capsules (Size 3, Agar Scientific, Stanted, England) that were trimmed and sliced with an ultramicrotome LKB. Sections were placed into formvard-carbon-coated copper grids and stained with 25 mM lead citrate. The samples were observed and photographed in a transmission electron microscope JEOL-1230.

Colocalization of antimicrobial peptides and *L. pifanoi* amastigotes in peritoneal macrophages: Confocal microscopy

Three days (day 0) prior to macrophage harvesting, BALB/c mice were intraperitoneally (i.p) injected with 1 mL of 10% thioglycolate. On the 3rd day, mice were sacrificed by cervical dislocation, and macrophages were obtained by washing the peritoneum twice with warm PBS. 100 000 cells/well were seeded in a Lab-Tek 16-well chamber slide system, with RPMI 1640 + 10% HIFCS. Macrophages were incubated for 2-3h at 37 ºC to allow cell attachment to the glass bottom of the chamber slide.

When the macrophages were well attached to the coverslides, the medium was replaced with a suspension of *L. pifanoi* amastigotes in fresh medium, at a final ratio amastigote:macrophage of 3:1 and incubated for at least 4h at 32 ºC. Next, non phagocytized parasites were removed by washing each well twice with warm PBS, and substituted for new media (RPMI 1640 + 10% HIFCS) with or without peptides at different concentrations. To ascertain whether FITC-labeled peptides, have any privileged subcellular accumulation inside the infected macrophages, including the parasitophorus vacuole where the *Leishmania* amastigotes grow, macrophages, previously seeded in a Lab-Tek 16-well chamber slide system, were incubated with the labeled peptides overnight. After that, wells were washed twice with warm PBS. The plastic wells, and any trace of silicone gasket, were removed from the slide carefully according to the manufacturer instructions. Before covering the slide with a coverslide, a drop of PBS was placed in the middle of each preparation to avoid cell drying. Finally macrophages were observed and photographed in a Confocal Laser Scanning Microscope Leica TCS – SP5 ABOS.

Determination of macrophage viability

To measure macrophage viability in the presence of different peptide concentrations, mouse peritoneal macrophages were seeded (100 000 cells/well) into a 96-well plate. After adherence to the plate, macrophages were incubated, for 4h in 100 µL of HBSS + 10 mM glucose with or without peptides at different concentrations. After peptide incubation, 100 µL of 1 mg/mL MTT solution was added to each well, and MTT reduction was measured as described before.

2. RESULTS

2.1. Determination of the antimicrobial activity of lactoferrin-derived peptides against *L. donovani* promastigotes and *L. pifanoi* amastigotes

Lactoferrin-derived peptides, LFcin17-30, LFampin265-284 and LFChimera were all leishmanicidal (determined by MTT reduction) against *L. donovani* promastigotes below 50 μM (table 5). LFChimera was the most active, with IC_{50} and LD_{50} values around 4 μM (table 5). All the peptides were less active against amastigotes than promastigotes. LFcin and LFampin had IC_{50} and LD_{50} values above 50 μM against amastigotes while the LFChimera was once again more active, with IC_{50} and LD_{50} values below 10 μM (table 5). *Leishmania* parasites were not able to recover from the damage induced by the peptides after 4h of incubation (represented by IC_{50}), as the LD_{50} was always similar to the IC_{50}, for both promastigotes and amastigotes (table 5).

The inhibitory activity of LFChimera was always higher than the sum of its separated components (figure 21), stressing the importance of having the peptides bond to each other.

Table 5 – Leishmanicidal activity of LFcin17-30, LFampin265-284, LFChimera and LFChimera surrogates against *L. donovani* promastigotes and *L. pifanoi* amastigotes. The values correspond to the concentration causing 50% inhibition of MTT reduction. Each value represents the average and standard deviation of at least two independent experiments.

AMP	*L. donovani* promastigotes		*L. pifanoi* amastigotes	
	IC_{50} / μM	LD_{50} / μM	IC_{50} / μM	LD_{50} / μM
LFcin17-30	21.4 ± 1.3	25.7 ± 1.4	> 50	> 50
LFampin265-284	29.1 ± 1.7	33.2 ± 1.5	> 50	> 50
LFChimera	3.7 ± 0.4	3.6 ± 0.1	6.0 ± 1.1	4.7 ± 1.1
LFChimera-R	3.5 ± 0.1	2.6 ± 0.4	4.2 ± 0.3	3.6 ± 1.1
Di-LFcin	2.4 ± 0.1	2.1 ± 0.2	3.4 ± 0.6	3.2 ± 0.6
Di-LFampin	4.4 ± 0.1	4.0 ± 0.4	4.8 ± 0.8	5.9 ± 1.4
LFcin – LFampin	3.9 ± 0.1	3.4 ± 0.2	4.8 ± 0.6	4.9 ± 1.2
LFampin – LFcin	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.3	0.73 ± 0.02

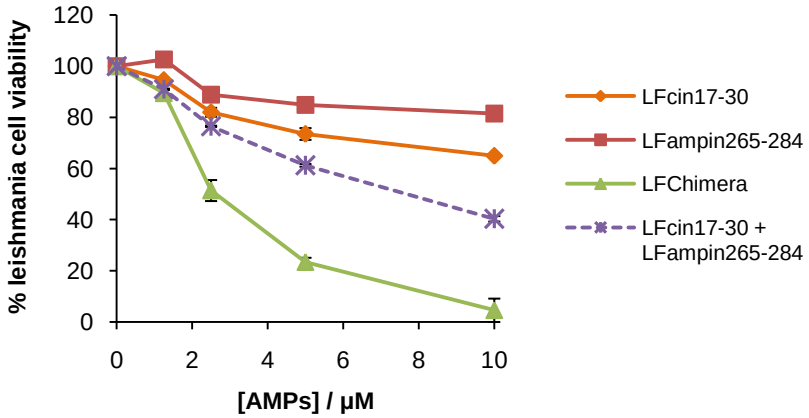

Figure 21 – Effect of lactoferrin-derived peptides on the viability of *L. donovani* promastigotes. Parasites were incubated with different concentrations of LFcin17-30, LFampin265-284, LFChimera, and with its two constituent peptides together, for 4h in HBSS + 10 mM glucose. The viability of the parasites was measured by MTT reduction. The results of one representative experiment out of three are shown.

In order to determine structure-activity relationships for LFChimera, a set of LFChimera surrogates was tested along with the parental hybrid peptide. In preliminary experiments, the IC_{50} and LD_{50} of these peptides against *L. donovani* promastigotes and *L. pifanoi* amastigotes were determined by MTT reduction. All the surrogates tested had an activity similar to that of LFChimera, except for two peptides that were even more active, Di-LFcin and LFampin-LFcin (table 5). This last one was active below 2 µM against both forms of the parasite (table 5). An interesting fact was that all peptides had similar activities on promastigotes and on amastigotes, which was not observed for LFChimera. So, the attempt to reach structure-activity relationships is not yet fully accomplished, as the experiments so far were limited to the small number of surrogates available, and further experiments with different surrogates to obtain deeper information about structure-activity relationships are already planned. Nevertheless, some preliminary conclusions can be drawn: i) it seems that the presence of two lactoferricins 17-30 (Di-LFcin) improves the activity of the peptide due to a higher charge (+14 compared to +12 of LFChimera and to +10 of Di-LFampin); ii) the fact that a linear hybrid, LFampin-LFcin, has higher activity than LFChimera suggest that the link between the LFcin and LFampin through a additional lysine is not crucial for the antimicrobial activity of the parental hybrid peptide. Moreover in these linear peptides it seems important the order of the constituent peptides, as LFampin-LFcin was more active than LFcin-LFampin. Due to the finding of at least two very promising peptides, that could be used in the future to fight leishmaniasis, further experiences will be performed with these LFChimera surrogates to uncover their mechanism of action and cytotoxicity.

2.2 Evaluation of *Leishmania* plasma membrane permeabilization by lactoferrin-derived peptides

In order to test whether the lethal mechanism of lactoferrin-derived peptides against *Leishmania* correlates with a plasma membrane permeabilization process, a set of permeabilization assays were performed. These experiments can elucidate on how these peptides act, the time-frame of action, what is the main target, among others.

First the intracellular levels of free-ATP in living parasites, *L. donovani* promastigotes, were monitored. Immediately after peptide addition (t=0) there was a fast and dose-dependent bioenergetic collapse of the parasites (figure 22). This means that the presence of the peptide disturbs the membrane, impairing the production of ATP by the parasites, due to loss of the ionic gradient or leakage of ATP, leading to their death. Once again the effect of LFChimera was stronger, as for much lower concentrations (between 10 and 1.25 μM) a similar or even higher effect was observed, when compared with the other two peptides.

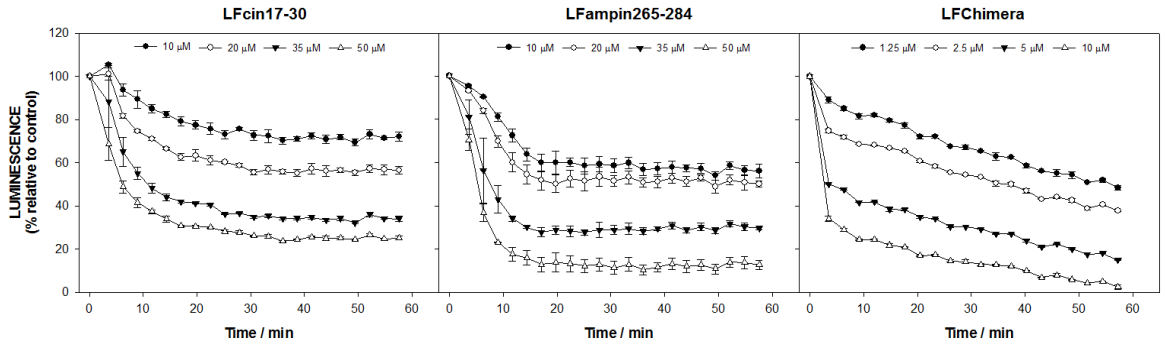

Figure 22 – Real-time depletion of intracellular ATP levels upon peptide addition in living *L. donovani* 3-Luc promastigotes. Measurement of intracellular levels of ATP, by luminescence, in *L. donovani* 3-Luc promastigotes after peptide (LFcin17-30, LFampin265-284 and LFChimera) addition at t = 0 (luminescence = 100%). The results of one representative experiment out of two are shown as percentage relative to control parasites (without the addition of AMPs).

With the above results, it was recognized that these peptides induce membrane damages. However how severe were those damages? Are they big enough to let a cationic molecule, Sytox Green, with 600 Da to enter inside the cytoplasm? To answer these questions the entrance of Sytox Green was monitored, after adding the peptide, by the fluorescence enhancement, due to the binding of the dye to nucleic acids. The results obtained were similar and in agreement with the luminescence results. It was possible to see a fast and dose-dependent uptake of the dye that reaches saturation minutes after peptide addition

(figure 23). Once more, LFChimera was much more active than its separated components, reaching 100% of dye entrance (all cells are dead) at 10 μM, whereas the other two peptides at this concentration only cause approximately 20% of dye entrance.

The kinetic behavior of LFcin17-30 was very peculiar, and different from the other peptides (figure 23). The entrance of the dye was much slower, revealing that the fast bioenergetic collapse of the parasites when in contact with this peptide is probably due first to loss of the ionic gradient and only later to leakage of ATP, which requires larger membrane damages, the same ones that allows Sytox entrance to the cells.

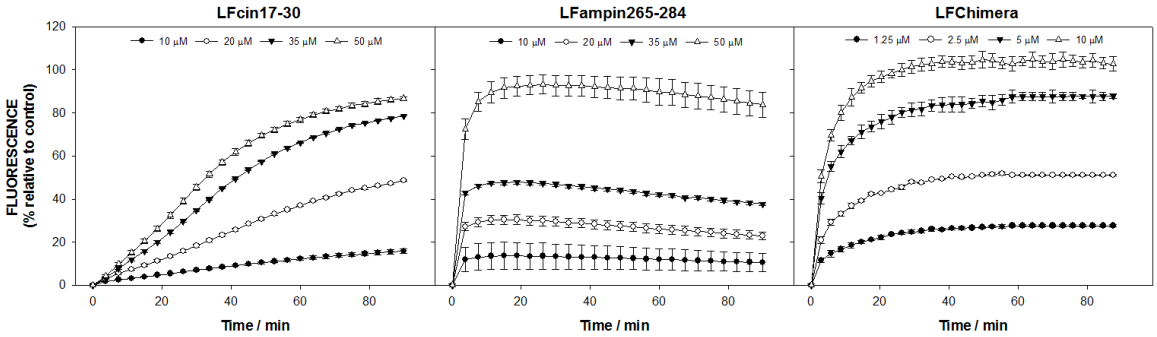

Figure 23 – Permeabilization of the plasma membrane of *L. donovani* promastigotes by lactoferrin-derived peptides measured by the entrance of a vital dye. Kinetics of Sytox Green entrance after peptide (LFcin17-30, LFampin265-284 and LFChimera) addition (t = 0; fluorescence = 0), measured by the increase of fluorescence after binding of the dye to intracellular nucleic acids (λ_{ex} = 504 nm; λ_{em} = 524 nm). The results of one representative experiment out of two are shown and the values were expressed as percentage relative to full permeabilized cells (with 0.1% of Triton X100).

The binding of Sytox Green to nucleic acids is practically irreversible, so once the saturation is reached, even if the cell recovers from the damages this will not be reflected on the levels of fluorescence (if the assay is conducted under the above conditions). So, another assay was performed with this dye, but instead of adding at time zero, Sytox Green was added after 4h of incubation of cells with the peptides at different concentrations. In figure 24, it can be seen that the accumulation was not due to transitory disruptions of the membrane, as fluorescence is cumulative, but that the lesion was present and parasites were not able to recover from the damages induced in the first minutes (both assays of uptake of vital dyes are highly concordant) and the amount of labeled cells were in agreement with results obtained for the IC_{50} (also determined at the end of 4h).

A deeper insight into the results, shows that parasites treated with LFcin17-30 seem to form aggregates, as two different populations that incorporated the dye (populations that are shifted to the right in comparison with the control population – 0 μM – in green), aside from the one unlabeled, were distinguished (figure 24).

LFChimera, according to the previous results, was much more active. The results showed two different types of populations (figure 24), a minor one, essentially unlabeled, that diminishes as the concentration of LFChimera increases, and a major one, which increases with peptide concentration, but without shift of its fluorescence levels (abscissa's axis) a situation suggesting an all-or-none effect of the peptide.

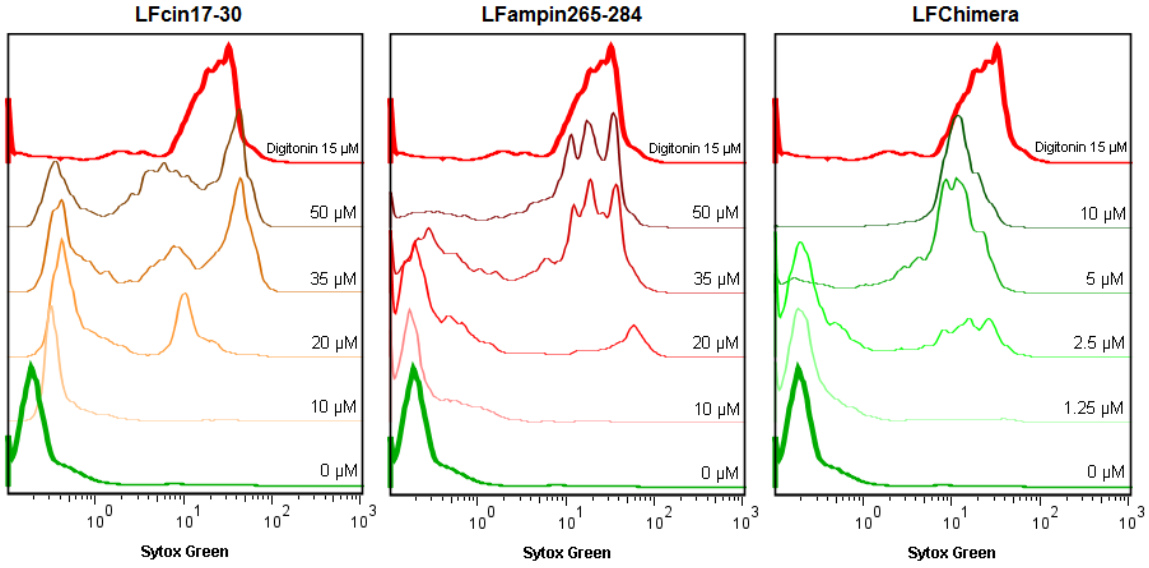

Figure 24 – Permeabilization of *L. donovani* promastigotes plasma membrane by lactoferrin-derived peptides measured by the entrance of vital dyes. Cell incorporation of Sytox Green, after 4h of incubation, with different concentrations of the peptides LFcin17-30, LFampin265-284 and LFChimera, as measured by cytofluorometry. Full permeabilization was achieved with 15 μM of digitonin. The results of one representative experiment out of three are shown.

To evaluate whether these peptides may act as CPPs (entering the cells without killing them and acting in an intracellular target) the incorporation of labeled peptides with FITC into parasites was evaluated by confocal microscopy. *L. donovani* promastigotes and *L. pifanoi* amastigotes were incubated for 4h with LFcin17-30-FITC, LFampin265-284-FITC and LFChimera-FITC. At the end of the experiment, propidium iodide, that only enters into severely damaged or dead cells, was added. For both forms of the parasites, the conclusion was the same, all cells labeled by the respective peptide (green) were also dead (red) (figure 25 and 26). In other words, it was not possible to spot green labeled cells alive. This observation was quite evident for *L. pifanoi* amastigotes, as for the first

two peptides, both live and dead cells are seen, but only the dead cells are labeled in green (figure 26). So these peptides act with a mixed mechanism by which membrane permeabilization appears to be the main effect, although the presence of peptides inside the cells may suggest the existence of possible intracellular targets.

For LFcin17-30 cell aggregates were visible (figure 25), which reinforces the theory that this peptide induces cellular aggregation (already seen by cytofluorometry) probably due to is charge (+6) and the anionic character of *Leishmania* membrane (Bolscher, J.G.M. *et al.* 2009; Wassef, M.K. *et al.* 1985).

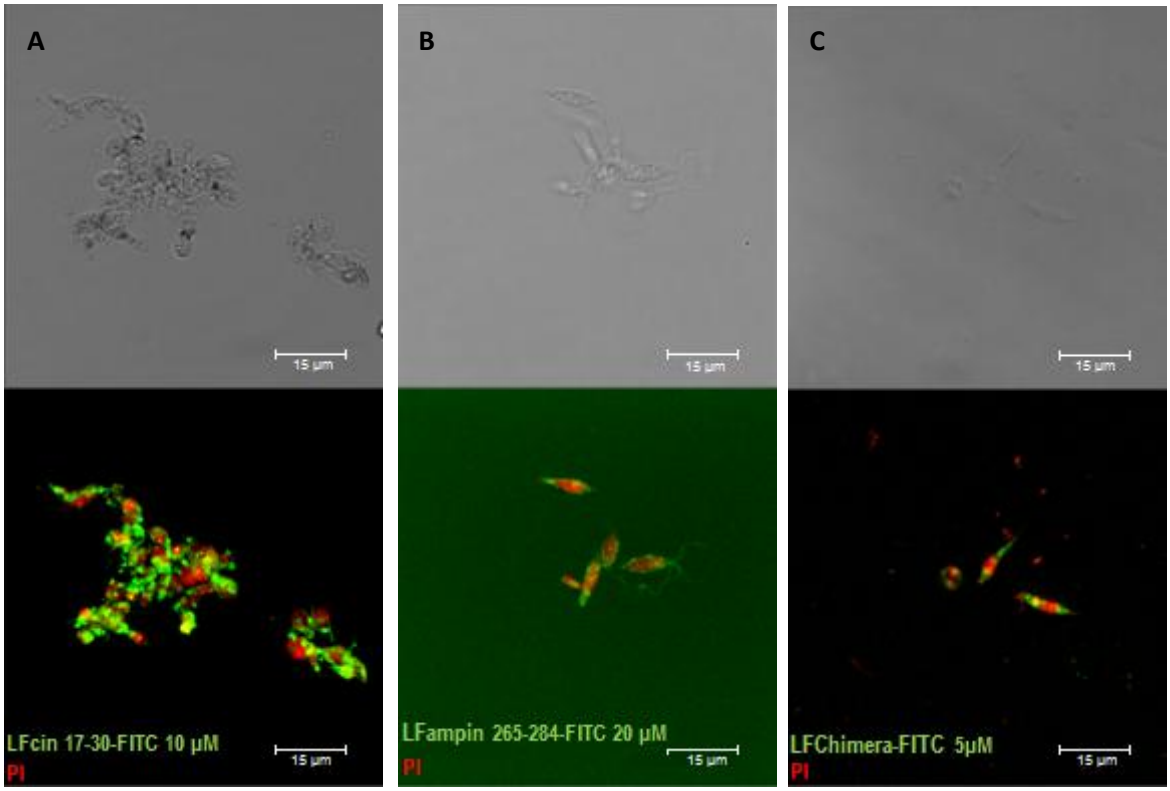

Figure 25 – Permeabilization of *L. donovani* promastigotes plasma membrane by lactoferrin-derived peptides assessed by fluorescence microscopy. *L. donovani* promastigotes were incubated for 4h with lactoferrin-derived peptides labeled with FITC and at the end of the incubation, propidium iodide (PI) was added. Cells were photographed in a Confocal Laser Scanning Microscope Leica TCS – SP2 ABOS with final magnification of 630x, and representative pictures are shown. Upper panels show phase contrast and bottom panels the corresponding fluorescence image. **A)** 10 µM LFcin17-30-FITC, **B)** 20 µM LFampin265-284-FITC and **C)** 5 µM LFChimera-FITC.

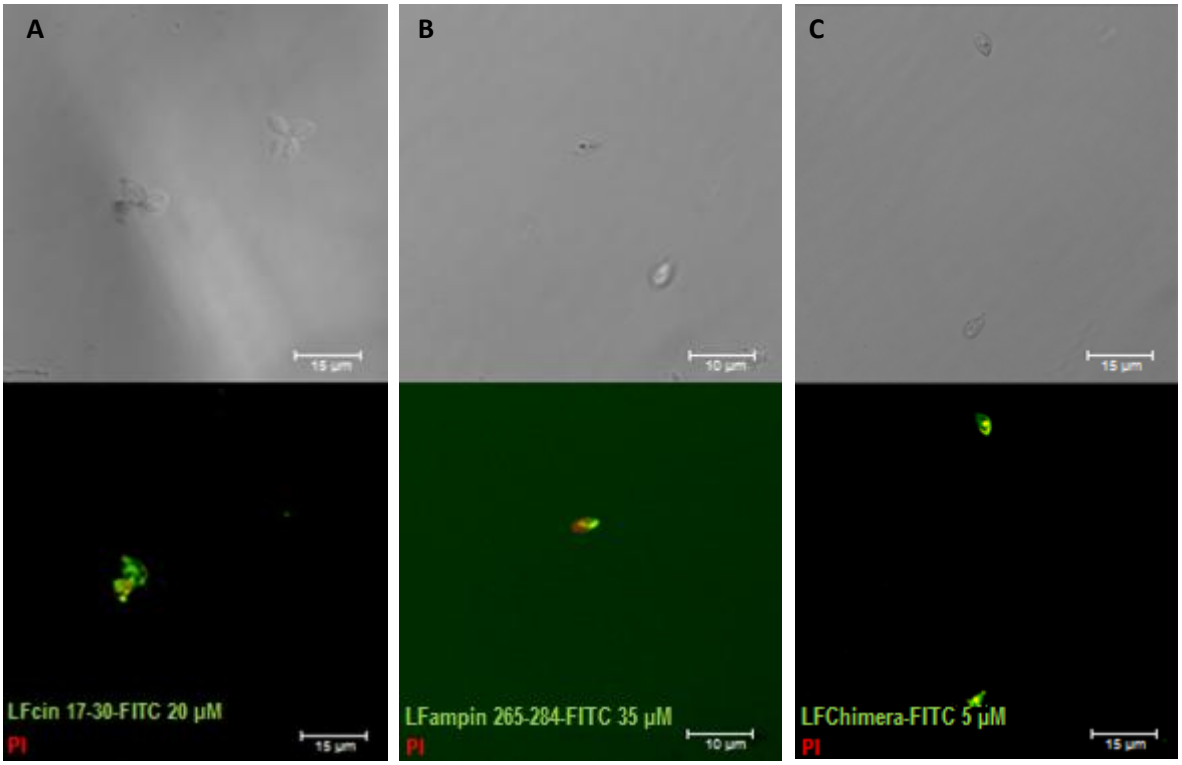

Figure 26 – Permeabilization of *L. pifanoi* amastigotes plasma membrane by lactoferrin-derived peptides assessed by fluorescence microscopy. *L. pifanoi* amastigotes were incubated for 4h with lactoferrin-derived peptides labeled with FITC and finally propidium iodide (PI) was added. Cells were photographed in a Confocal Laser Scanning Microscope Leica TCS – SP2 ABOS with final magnification of 630x, and representative pictures are shown. Upper panels shows phase contrast and bottom panels the corresponding fluorescence image. **A)** 20 µM LFcin17-30-FITC, **B)** 35 µM LFampin265-284-FITC and **C)** 5 µM LFChimera-FITC.

Transmission electron microscopy (TEM) is a useful technique to identify morphological damages on the parasites after their incubation with peptides. In this work, *L. donovani* promastigotes were incubated with 5 µM LFChimera and cells were observed in a transmission electron microscope. By analyzing the obtained pictures (figure 27), it may be concluded that the peptide induced in most of the parasites, cytoplasmic vacuolation, loss of definition of the organelles, and a massive leakage of intracellular material (figure 27 – red arrows), whereas some cells appear healthy with no visible damages (figure 27 – green arrows), suggestion once again an all-or-none effect.

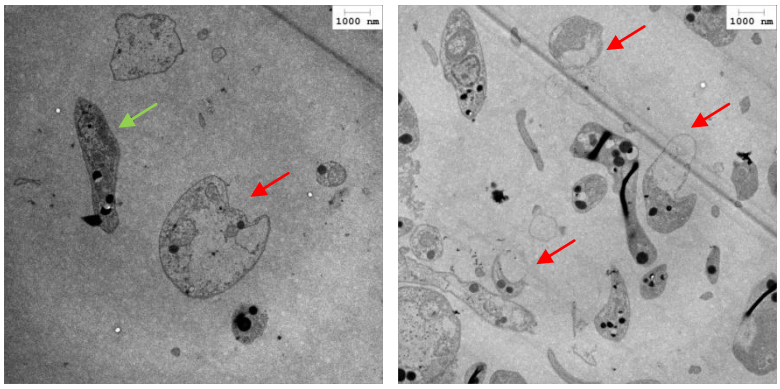

Figure 27 – Assessment of morphological damages of *L. donovani* promastigotes incubated with LFChimera. Parasites incubated with 5 µM LFChimera for 4h were observed and photographed in a transmission electron microscope. Representative pictures are shown. Red arrows – damaged cells; green arrows – healthy cells.

2.3 Effect of lactoferrin-derived peptides on *L. pifanoi*-infected macrophages.

Leishmania parasites, namely amastigotes, live inside mononuclear phagocytic cells of a wide variety of mammals. So, it is important to know what are the effects of lactoferrin-derived peptides on macrophages.

The toxicity of the three peptides towards non-infected peritoneal macrophages, was determined by MTT reduction, when the cells were incubated with the peptides for 4h in buffer (HBSS + 10 mM glucose). LFChimera showed a high cytotoxicity activity when compared with the other two peptides which did not reach 50% of loss of cell viability up to 50 µM (figure 28).

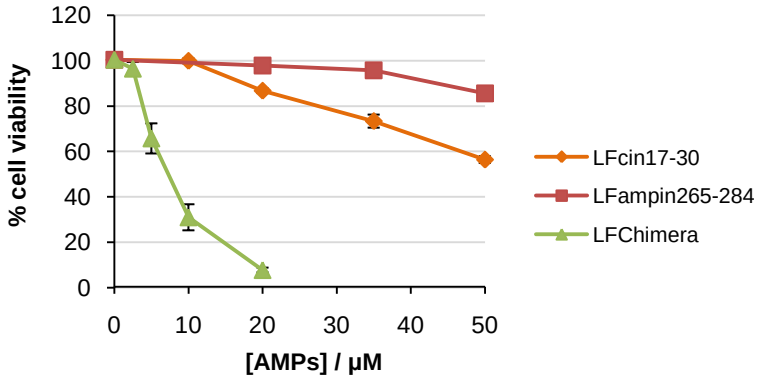

Figure 28 – Toxicity of lactoferrin-derived peptides towards peritoneal macrophages. Non-infected peritoneal macrophages were treated with different concentrations of LFcin17-30, LFampin265-284 and LFChimera, for 4h in HBSS + 10 mM glucose, and macrophage viability was determined by MTT reduction.

The ability of the peptides to reach intracellular amastigotes (which live inside macrophage vacuoles) as well as their overall subcellular localization, was determined by incubating macrophages infected with *L. pifanoi* amastigotes, with LFcin17-30-FITC,

LFampin265-284-FITC and LFChimera-FITC. Due to a limited number of macrophages, only one concentration of each peptide was tested. The choice was made according to the results of toxicity by choosing the highest concentration with lower toxicity (preferably below 20%). LFcin17-30 at 20 µM colocalized with the intracellular amastigotes, whereas LFampin265-284 at 50 µM and LFChimera at 5 µM, were able to enter into the mammalian cell but do not colocalize with the parasites (figure 29). Further experiences will be done to evaluate the colocalization with different concentration of peptides and with different times of incubation. Interestingly, in these experiments the peptides did not seem to be toxic to the macrophages at the tested concentrations, as the cell morphology was not affected (figure 29). When macrophages were treated with peptides for localization experiments, the assays were performed in full-medium (RPMI 1640 + 10% HIFCS) and not in buffer, as was the case with MTT reduction assays. Because the full-medium, contains HIFCS, as well as proteolytic enzymes, the activity of the peptides, may be distinct from that in HBSS buffer. Preliminary experiments were performed to assess toxicity of the peptides in full-medium but no conclusive results were obtained.

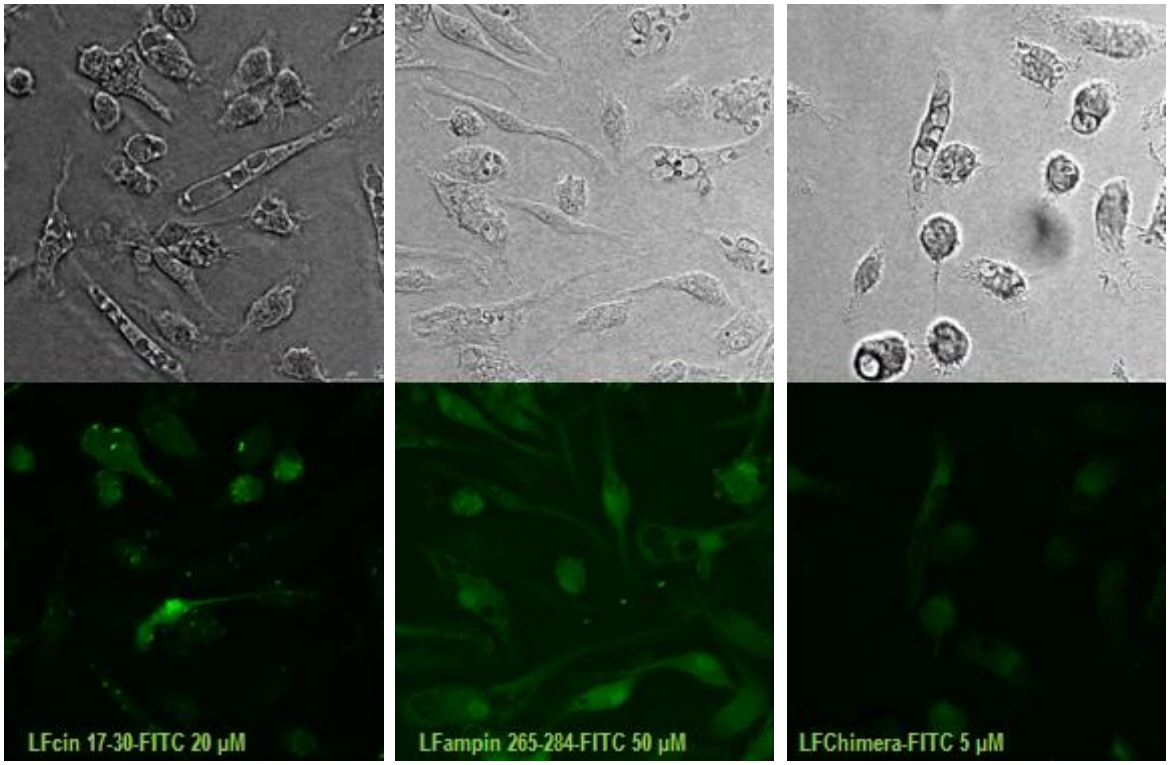

Figure 29 – Colocalization of lactoferrin-derived peptides and *L. pifanoi* amastigotes in peritoneal macrophages. Infected macrophages with *L. pifanoi* amastigotes and incubated, overnight, with lactoferrin-derived peptides labeled with FITC were photographed in a Confocal Laser Scanning Microscope Leica TCS – SP5 ABOS with final magnification of 630x, and representative pictures are shown. Upper panels show phase contrast, and bottom panels the corresponding fluorescence image. **A)** 20 µM LFcin17-30-FITC, **B)** 50 µM LFampin265-284-FITC and **C)** 5 µM LFChimera-FITC.

III.2 – Activity of cecropin A-mellitin-derived and lactoferrin-derived peptides against *Mycobacterium avium*

1. MATERIAL AND METHODS

Peptides

In the evaluation of the activity against *Mycobacterium avium,* the following peptides were used: cecropin A-melittin derivatives CA(1-7)M(2-9) or CAM, K6 and K7 (described in table 3, page 55) and lactoferrin-derived peptides hLF1-11, LFcin17-30, LFampin265-284 and LFChimera (described in table 4, page 56).

Bacteria

M. avium strain 2447, smooth transparent variant (SmT) was isolated from an AIDS patient and provided to us by Dr. F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium).

Mycobacteria were grown to mid-log phase in Middlebrook 7H9 medium (Difco, Sparks, MD) containing 0.05% of Tween 80 (Sigma, St. Louis, MO) and 10% of ADC supplement (Albumin-Dextrose-Catalase) at 37 ºC. Bacteria were harvested by centrifugation, washed twice with saline containing 0.04% Tween 80, resuspended in the same solution and briefly sonicated in order to disrupt bacterial clumps. The suspension was stored in aliquots at -80 ºC until use. Just before use, an aliquot was quickly thawed and diluted to the appropriate concentration.

Effect of antimicrobial peptides on the viability of *M. avium* in axenic cultures

Principles

To determine the direct effect of the antimicrobial peptides on *M. avium* 2447 SmT in axenic cultures (bacteria growing in liquid medium), three different methods were used. First the bacterial growth was monitored by optical density at 600 nm. When they were in the exponential phase of growth, the viability of *M. avium* was quantified by resazurin reduction and by colony forming units (CFUs).

Resazurin is a blue oxidation-reduction indicator (Palomino, J.C. *et al.* 2002) which is non-fluorescent until it is reduced to the pink colored and highly fluorescent resorufin. Resazurin reduction can be used to measure cellular viability, as only viable cells can reduce this dye and the level of resazurin reduction is proportional to the number and viability of the cells present. This is a simple and non-destructive assay, that can be

applied both to adherent and non-adherent cells, and it complements and gives comparable data with the colony forming units (CFUs) assay (Anoopkumar-Dukie, S. *et al.* 2005).

Method

M. avium 2447 SmT was grown in Middlebrook 7H9 medium to exponential phase. 10^7 CFU of bacteria were seeded per well into 96-wells flat bottom plates and incubated with the peptides in a final volume of 200 µL. Each peptide concentration was tested in triplicates. The plates were incubated at 37 ºC in a humid atmosphere. At the 6^{th} day of culture, 10 µL of 2.5 mM resazurin was added to each well and after 24h of incubation the fluorescence was measured in a Gemini XM plate fluorometer (Molecular Devices) (λ_{ex} = 560 nm; λ_{em} = 590 nm). The results were expressed as percentage of the fluorescence obtained in control wells without peptides.

$$\% \text{ mycobacterial cell viability} = \frac{\text{relative fluorescence intensity in experimental wells}}{\text{relative fluorescence intensity in control wells}} \times 100$$

In some of the assays, after 7 days of incubation, the viability of the bacteria was also measured by a colony forming units (CFUs) assay (using the same wells as the resazurin assay). The bacterial suspension in each well was serially diluted in water containing 0.04% Tween 80. The dilutions were plated in Middlebrook 7H10 agar medium (Difco, Sparks, MD), supplemented with OADC (Oleic acid-Albumin-Dextrose-Catalase) and the number of colonies was counted after 7 days at 37 ºC. The difference, in terms of $\log_{10}$ CFU/mL between the first and the last day of incubation was designated “log increase”.

$$\text{log increase CFU/mL} = \log_{10} \text{CFU/mL (day 7)} - \log_{10} \text{CFU/mL (day 0)}$$

Collection, infection and treatment of Bone Marrow derived Macrophages (BMMØ)

Macrophages were derived from the bone marrow of BALB/c INF-γ knock-out mice. Each femur and tibia was flushed with 5 mL of Hank’s Balanced Salt Solution (HBSS, Gibco, Paisley, U.K.). The cell suspension was centrifuged and resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Paisley, U.K.) supplemented with 10 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 10% Fetal Bovine Serum (FBS, Gibco, Paisley, U.K.) and 10% of L929 cell conditioned medium (LCCM) as a source of Macrophage Colony Stimulating Factor (M-CSF).

Cells were cultured overnight at 37 ºC in a 7% CO_2 atmosphere to remove fibroblasts. Non-adherent cells were collected with cold HBSS medium, washed and seeded at the concentration of $5x10^5$ cells/well in 24-wells culture plates (Nunc, Gibco). In some cases LPS-free round glass coverslips were placed at the bottom of culture wells, so that macrophages could adhere to this coverslip and later be observed by confocal microscopy. The plates were incubated at 37 ºC in a 7% CO_2 atmosphere. Four days after seeding, 10% of LCCM was added to the culture medium and on the 7^{th} day, the medium was renewed. At day 10 of culture, when the cells are fully differentiated into macrophages, each well was incubated with 10^6 CFU of *M avium* 2447 SmT in DMEM, for 4h at 37 ºC in a 7% CO_2 atmosphere. After incubation, cells were washed several times with warm HBSS to remove non-internalized bacteria, and re-incubated with new medium, DMEM with 10% FBS and 10% LCCM, with or without different concentrations of peptides alone or in combination with different antibiotics. Each condition was tested in triplicates.

After 4 or 7 days of infection, the intracellular growth of *M. avium* 2447 SmT was evaluated by colony forming units (CFUs). Macrophages were lysed, using 0.1% saponin. The bacterial suspensions were serially diluted in water containing 0.04% Tween 80 and plated in Middlebrook 7H10 agar medium supplemented with OADC. The number of colonies was counted after 7 days at 37 ºC. The difference, in terms of $\log_{10}$ CFU/well between the first and the last day of culture was designated “log increase”.

$$\text{log increase CFU/well} = \log_{10} \text{CFU/well (day 4 or 7)} - \log_{10} \text{CFU/well (day 0)}$$

Determination of macrophage viability

Principles

The viability of infected macrophages was determined by resazurin reduction (see above) and by the use of Vibrant™ Cytotoxicity Assay Kit V-23111 (Molecular Probes) (both assays can be performed using the same wells).

The cytotoxicity assay kit monitors the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) from damaged cells into the surrounding medium. This enzyme is detected in cell culture supernatants by a two-step enzymatic process that leads to the reduction of resazurin (figure 30), and the resulting fluorescence signal is proportional to the amount of G6PD released which in turn correlates with the number of dead cells in the sample.

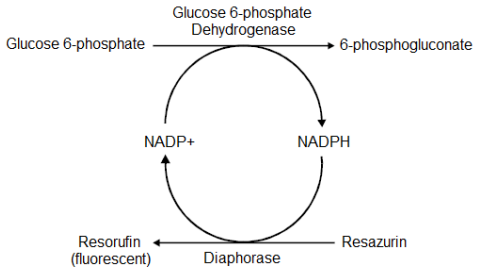

Figure 30 – Principle of the coupled enzymatic assay for detection of glucose 6-phosphate dehydrogenase activity. Oxidation of glucose 6-phosphate by glucose 6-phosphate dehydrogenase results in the generation of NADPH, which in turn leads to the reduction of resazurin by diaphorase to yield fluorescent resorufin (Molecular Probes).

Method

Resazurin reduction as a measure of cell viability: After 4 or 7 days of infection and peptide treatment, 100 µL of resazurin 1.25 mM was added to each well. The plates were incubated for 4h at 37 ºC in a 7% CO_2 atmosphere. In the end, the fluorescence was measured in a Gemini XM plate fluorometer and expressed as percentage of the fluorescence obtained in control wells (non-treated infected macrophages).

$$\% \text{ macrophage viability} = \frac{\text{relative fluorescence intensity in treated infected macrophages}}{\text{relative fluorescence intensity in non-treated infected macrophages}} \text{x100}$$

Vibrant™ Cytotoxicity Assay Kit to measure cell death: After 2 days of infection and peptide treatment 50 µL of the supernatant of each well was transferred into a new 96-wells flat bottom plate. An enzymatic mixture was prepared according to manufactor's instructions and added to the wells. Fluorescence readings (λ_{ex} = 530 nm; λ_{em} = 590 nm) were taken at 5 minutes intervals at 37 ºC. The relative fluorescence intensity obtained for experimental cells (treated infected macrophages) and for fully lysed cells, was corrected by subtracting the values obtained from the untreated infected macrophages. The relative cytotoxicity was determined by dividing the corrected fluorescence intensity of experimental cells by the corrected fluorescence intensity of fully lysed cells.

$$\% \text{ cytotoxicity} = \frac{\text{corrected fluorescence intensity of experimental cells}}{\text{corrected fluorescence intensity of fully lysed cells}} \text{ x 100}$$

Confocal Microscopy

At day 3rd of infection, macrophages that were cultured in glass coverslips, were washed with warm PBS and fixed with 4% paraformaldehyde (Merck) in PBS supplemented with 120 mM sucrose for 30 min at room temperature. For fluorescence quenching, cells were incubated with PBS containing 50 mM of NH_4Cl, for 10 min. Cells were washed again with PBS and incubated with Auramine O stain kit (Polysciences, Inc.). Briefly, cells were incubated with a few drops of Auramine O for 15 minutes (without letting the surface dry and protected from light), then with fluorescent decolorizer, for 30-60 seconds, and finally with potassium permanganate for 2 minutes. Between incubations cells were washed with distilled water. Slides were mounted using Mowiol (Polyscience) and sealed with nail polish. Macrophages were observed and photographed in a Laser Scanning Confocal Microscope Leica SP2 AOBS SE (Leica Microsystems, Germany).

2. RESULTS

2.1. Cecropin A-melittin-derived peptides

2.1.1. Effect on *M. avium* viability in axenic cultures

Cecropin A-melittin hybrid, CA(1-7)M(2-9) or CAM, and the respective trimethylated derivatives K6 and K7, had a direct effect against *M. avium*, significantly inhibiting its growth in liquid medium above 50 µM. However, even at four-fold higher concentration (200 µM) the growth inhibition did not reach 50% (figure 31). This direct effect on *M. avium* viability was determined, in three independent experiments, by resazurin reduction, and confirmed by CFUs. The results obtained by the two techniques are highly concordant.

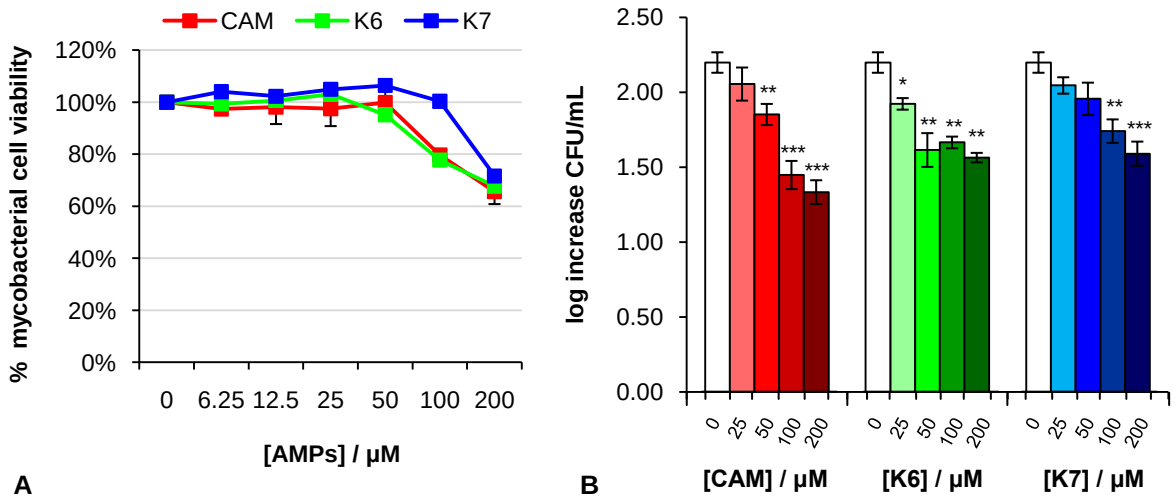

Figure 31 – Effect of cecropin A-melittin-derived peptides on *M. avium* viability. *M. avium* was cultured in liquid medium, in the presence of 0, 6.25, 12.5, 25, 50, 100 and 200 µM of CAM, K6 and K7 for 7 days. The viability was measured by **A)** resazurin reduction, where the results were expressed as percentage of cell viability relative to control wells, and **B)** colony forming units (CFUs) assay. Statistical analysis was performed using the two-tailed Student's t-test: * p<0,05; ** p<0,005; *** p<0,0005. The results of one representative experiment out of three are shown.

2.1.2. Effect on *M. avium* growing inside macrophages and macrophage toxicity

Recently, the hemolytic activity of CAM, K6 and K7 was determined (Fernandez-Reyes, M. *et al.* 2010), and the analogues K6 and K7 showed a significantly lower hemolytic activity compared to the parental hybrid peptide. However, when we tested the same peptides on macrophage cultures infected with *M. avium*, they revealed a high toxicity, assessed by resazurin reduction (results not shown) and by the use of Vibrant™ Citotoxicity Kit Assay

(figure 32). These results suggest that, although erythrocytes and macrophages are both mammalian cells, they are different enough, in this case probably at the plasmatic membrane composition, for their differential susceptibility to these peptides.

K7 was the peptide with lower toxicity, and at 10 μM had a citotoxicity below 10% (figure 32), but at this concentration no effect on the mycobacterial intramacrophagic growth was observed (results not shown).

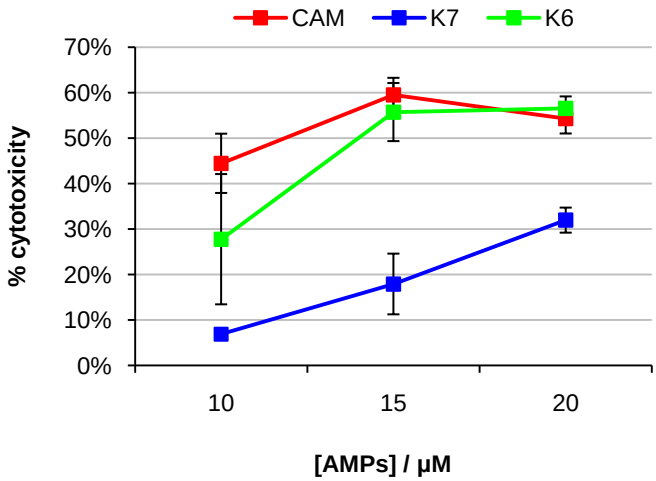

Figure 32 – Toxicity of cecropin A-melittin-derived peptides on infected BMMØ. The loss of viability of BMMØ infected with *M. avium* and treated with 10, 15 and 20 μM of CAM, K6 and K7 was determined by the use of Vibrant™ Citotoxicity Kit Assay, after 2 days of incubation. The results are presented as percentage of dead cells relative to fully lysed macrophages.

2.2. Lactoferin-derived peptides

2.2.1. Effect on *M. avium* viability in axenic cultures

The direct effect of four lactoferrin-derived peptides, human and bovine lactoferricin (hLFcin1-11 and LFcin17-30), bovine lactoferrampin (LFampin265-284) and LFChimera, on *M. avium* was first assessed by resazurin reduction (figure 33). The first two peptides were tested once, and the last two, were tested twice. The results are presented as percentage relative to the corresponding untreated wells. Lactoferricins were the most active peptides, the bovine peptide being more active than the human (figure 33). Lactoferrampin had no effect, and LFChimera, at higher concentrations (50 and 100 µM) seemed to promote the growth of *M. avium* (figure 33).

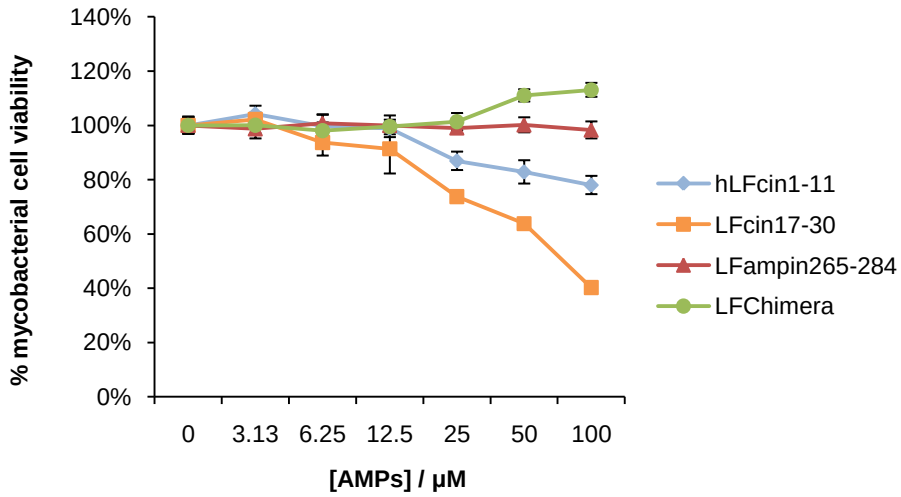

Figure 33 – Effect of lactoferrin-derived peptides on *M. avium* viability. *M. avium* was grown in Middlebrook 7H9 with hLFcin1-11, LFcin17-30, LFampin265-284 and LFChimera at 0, 3.13, 6.25, 12.5, 25, 50 and 100 µM of final concentration for all the peptides. Mycobacterial viability was measured by resazurin reduction after 7 days of incubation. The results of one experiment (hLFcin1-11 and LFcin17-30) and one representative experiment out of two (LFampin265-284 and LFChimera) are shown and presented as percentage of viable cells relative to the corresponding non-treated wells (control wells).

Since the lactoferricins appear to be the most interesting peptides, as they are active at 25 µM and above, the quantification of mycobacteria by CFUs was performed, and again, the results were in agreement with the ones obtained by the resazurin reduction assay (figure 34). Bovine lactoferricin (LFcin17-30) showed a higher inhibitory activity than human lactoferricin (hLFcin1-11).

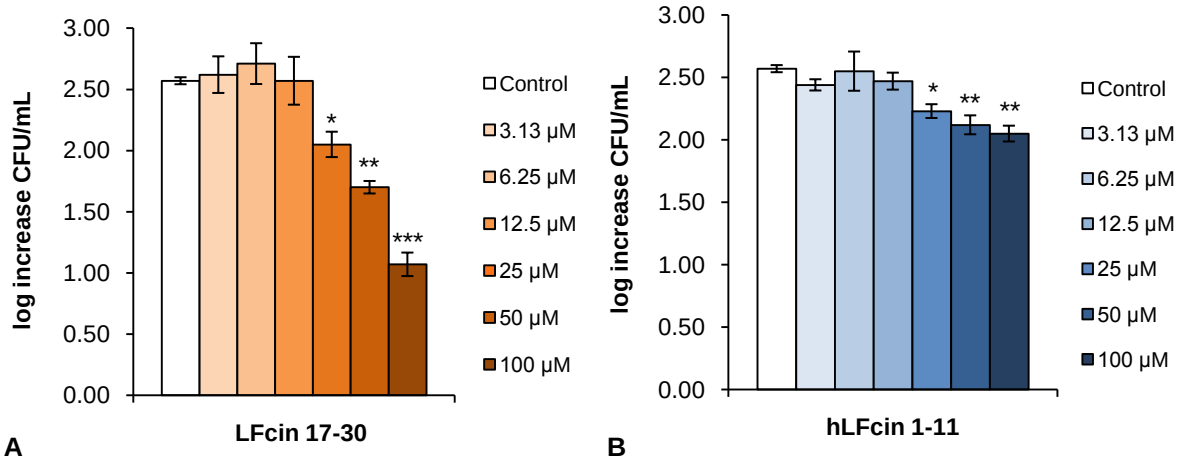

Figure 34 – Effect of lactoferricin on *M. avium* viability. *M. avium* was incubated with **A)** bovine and **B)** human lactoferricin (LFcin17-30 and hLFcin1-11) at 0, 3.13, 6.25, 12.5, 25, 50 and 100 µM. *M. avium* viability was determined by CFUs, after 7 days of incubation. Statistical analysis was performed using the two-tailed Student's t-test: * p<0,05; ** p<0,005; *** p<0,0005. The results of one experiment are shown.

2.2.2. Effect on *M. avium* growing inside macrophages and macrophage toxicity

As *M. avium* is an intracellular pathogen, that lives, mainly, inside macrophages, the next step was to test the same peptides on *M. avium* growing inside macrophages.

It had been described before that this family of antimicrobial peptides could act as an adjuvant of conventional antibiotics potentiating their therapeutic effect (Leon-Sicairos, N. *et al.* 2006; Oo, T.Z. *et al.* 2010; Sanchez-Gomez, S. *et al.* 2011). In this work, lactoferrin-derived peptides were combined with ethambutol, an antibiotic used to treat *M. avium* infections, to evaluate a potential synergistic effect against *M. avium* growing inside macrophages.

Lactoferrampin at 10 µM had no effect in the growth of *M. avium* inside macrophages, even when combined with ethambutol (results not shown).

Lactoferricins alone, human and bovine, were not effective against *M. avium* growing inside macrophages, at 20, 30 or 40 µM (figure 35 and 36). However, when each of the peptides was combined with ethambutol 4 µg/mL, which was also not effective alone (at this concentration) against *M. avium* growing inside macrophages, there was a synergistic effect (first determined for the lowest concentration of peptide – 20 µM – and then confirmed for this condition and tested for the other two, 30 and 40 µM) (figure 35 and 36), suggesting that the AMP increases the access of the antibiotic to the mycobacteria, when they are inside its host cell. Also in this case, as happened in the axenic assays, the

bovine peptide, LFcin17-30, was the most active, inducing a significant decrease on *M. avium* growth at 20 µM, when combined with ethambutol (figure 35).

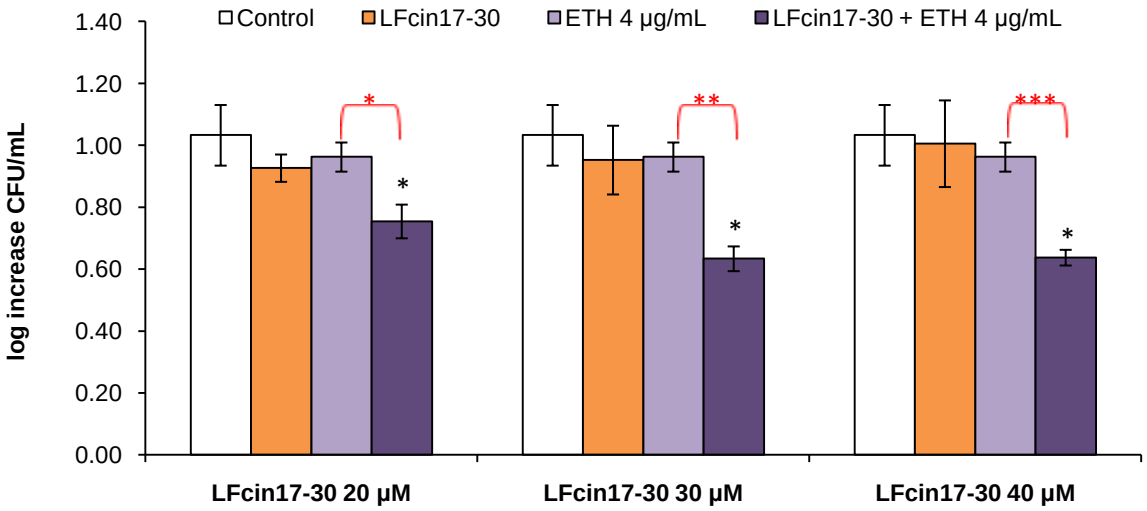

Figure 35 – Effect of bovine lactoferricin on the viability of *M. avium* growing inside macrophages. BMMØ infected with *M. avium* were treated with 0, 20, 30 and 40 µM of LFcin17-30 in the presence or absence of ethambutol 4 µg/mL. Intramacrophagic growth of *M. avium* was determined by CFUs after 7 days of incubation. Statistical analysis was performed using the two-tailed Student's t-test: * p<0,05; ** p<0,005; *** p<0,0005. The results of one experiment are shown.

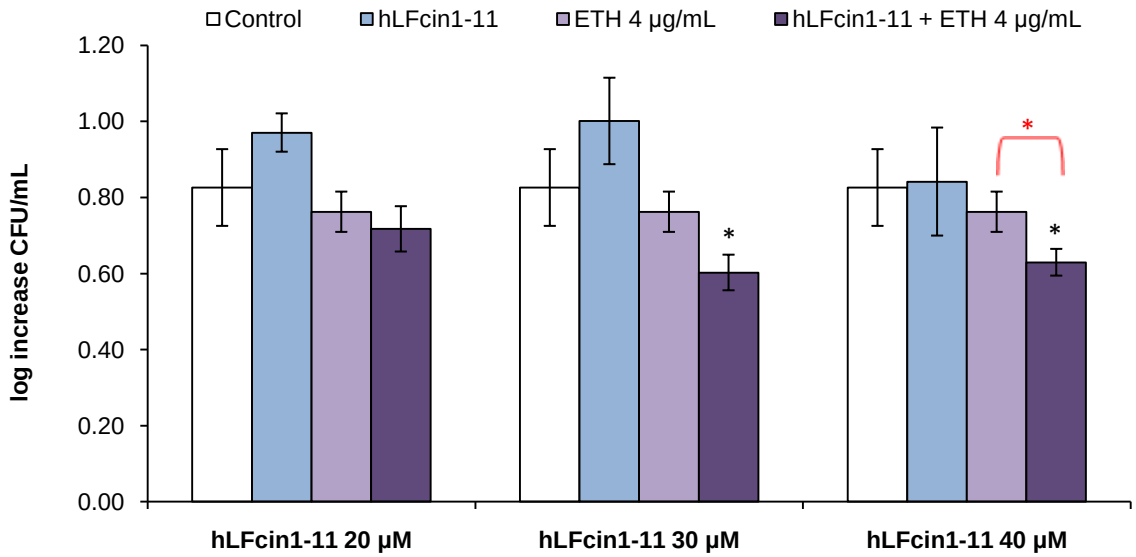

Figure 36 – Effect of human lactoferricin on the viability of *M. avium* growing inside macrophages. BMMØ infected with *M. avium* were treated with 0, 20, 30 and 40 µM of hLFcin1-11 in the presence or absence of ethambutol 4 µg/mL. *M. avium* viability was measured by CFUs after 7 days of incubation. Statistical analysis was performed using the two-tailed Student's t-test: * p<0,05. The results of one experiment are shown.

The toxicity of lactoferricins towards macrophages was determined by resazurin reduction after 7 days of incubation (results are presented as percentage relative to non-treated infected macrophages). None of the peptides was significantly toxic for macrophages up to 40 μM, since the cells maintained their viability in all concentrations for both AMPs (figure 37).

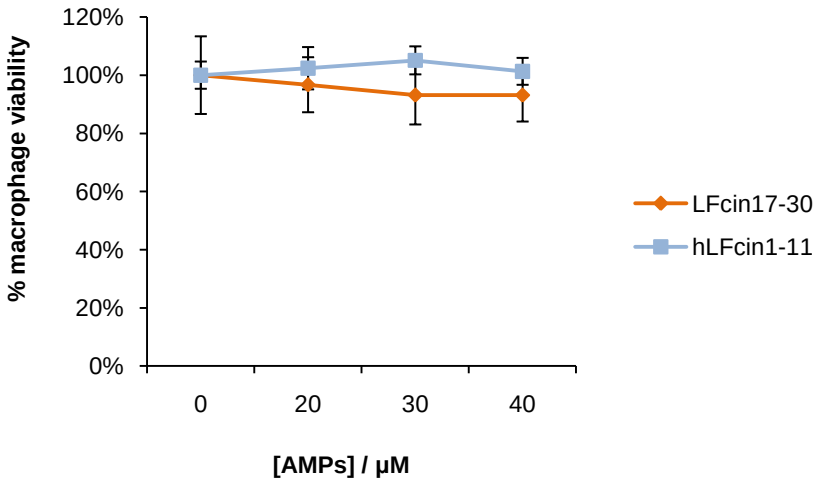

Figure 37 – Lactoferricin toxicity on infected BMMØ. Macrophages infected with *M. avium* were incubated with 0, 20, 30 and 40 μM of LFcin17-30 and hLFcin1-11. Macrophage viability was determined after 7 days of incubation, by resazurin reduction. The results are presented as percentage of viable cells relative to the corresponding non-treated infected wells.

Contrarily to what happens with other AMPs, we found that 10 μM LFChimera significantly promoted the growth of *M. avium* inside macrophages (figure 38). This assay was repeated four times, all giving the same result. To try to understand what is the mechanism behind this increase in proliferation, different concentrations of LFChimera (2.5, 5, 10 and 15 μM) at different time points (4 and 7 days) were tested. The LFChimera effect was dose-dependent and already visible after 4 days of incubation (figure 38).

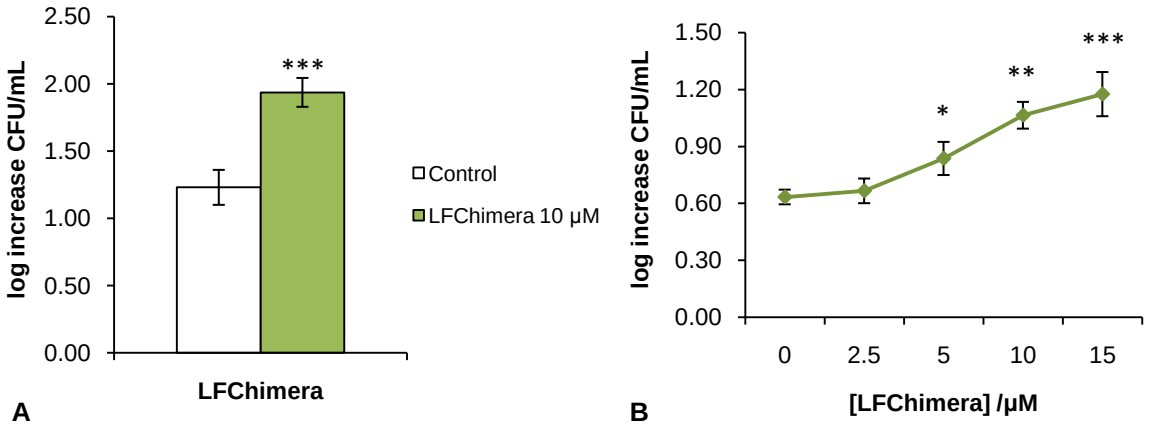

Figure 38 – Effect of LFChimera on the viability of *M. avium* growing inside macrophages. Intramacrophagic growth of *M. avium* was assessed by CFUs **A)** after 7 days of incubation when infected BMMØ were treated with 10 µM LFChimera, and **B)** after 4 days, when macrophages were incubated with 0, 2.5, 5, 10 and 15 µM of LFChimera. Statistical analysis was performed using the two-tailed Student's t-test: * p<0,05; ** p<0,005; *** p<0,0005. The results of one representative experiment out of **A)** four and **B)** two are shown.

Could this effect be an artifact due to AMP toxicity that is killing the macrophages and releasing the bacteria to the surrounding medium, where they grow faster than inside the host cell? To answer this question the toxicity of LFChimera towards macrophages in the same concentrations and times of incubation was measured. LFChimera was not toxic for infected BMMØ at any of the concentrations tested (figure 39). The results are presented as percentage relative to non-treated infected cells in each time-point. On day 7 post-infection, the relative viability of macrophages actually seemed to increase with the increase in LFChimera concentration. In fact, in terms of absolute values what we observed was a decrease of viability in the untreated macrophages whereas this decrease was less marked in LFChimera-treated macrophages. Also, the amount of extracellular bacteria growing in the surrounding medium was determined being less than 20% of the total amount of bacteria per well (results not shown).

To confirm that macrophages incubated with LFChimera were intact and with a higher number of *M. avium,* after 3 days of incubation infected macrophages grown in glass coverslips were fixed and stained with Auramine O, a green dye which stains mycobacteria. In figure 54, representative pictures of phase contrast and fluorescence microscopy of control cells, without AMP, and cells treated with 10 µM LFChimera, are shown. Although quantitative data were not obtained, the relative number of bacteria per macrophage in treated cells appears to be higher than in control cells and macrophages

seem to be healthy as the morphology of the cells, as far as we can see and assess, was not affected (figure 40).

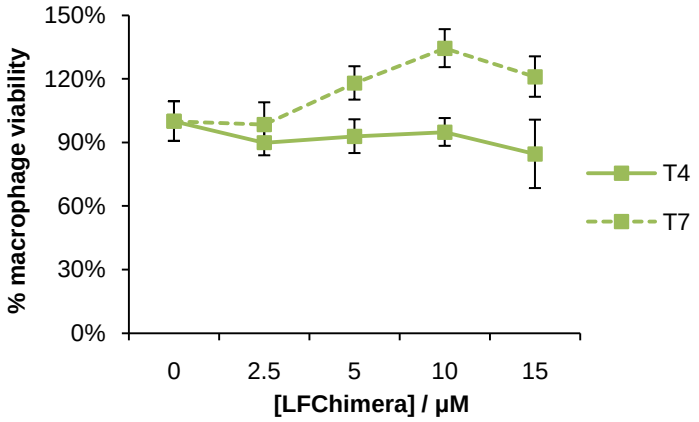

Figure 39 – Toxicity of LFChimera on infected BMMØ. After 4 and 7 days of incubation of infected-macrophages with 0, 2.5, 5, 10 and 15 µM of LFChimera, cellular viability was determined by resazurin reduction. The results of one representative experiment out of two are shown and presented as percentage of viable cells relative to non-treated infected wells in each time-point.

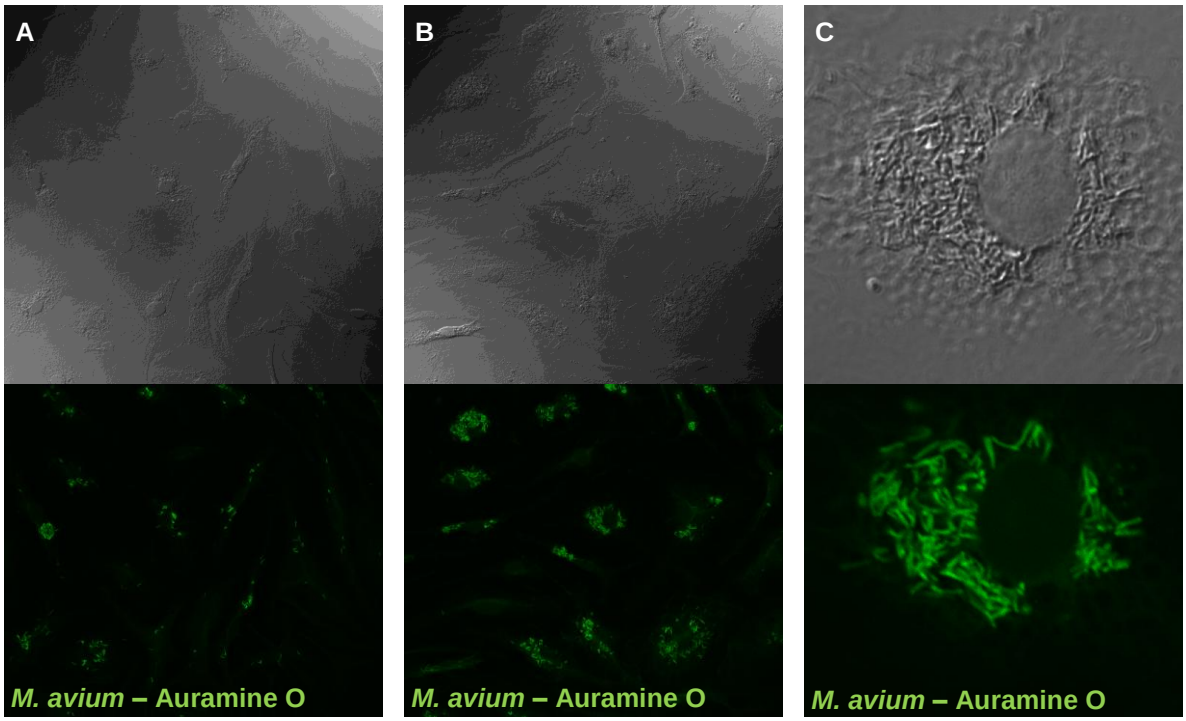

Figure 40 – Effect of LFChimera on the intramacrophagic growth of *M. avium*. Macrophages infected with *M. avium* for 3 days were fixed and mycobacteria were stained with Auramine O. Cells were photographed in a Laser Scanning Confocal Microscope Leica SP2 with final magnification of 630x, and representative pictures are shown. Upper panels show phase contrast and lower panels the corresponding fluorescence image. **A)** Non-treated infected macrophages. **B)** Infected macrophages treated with 10 µM LFChimera. **C)** Higher magnification of one macrophage from picture B.

III.3 – Characterization of peptide-lipid interaction by X-ray diffraction

1. MATERIAL AND METHODS

Lipids

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE or 16:0-18:1 PE) lipid was obtained from Avanti Polar Lipids (Alabaster, AL), and used without further purification.

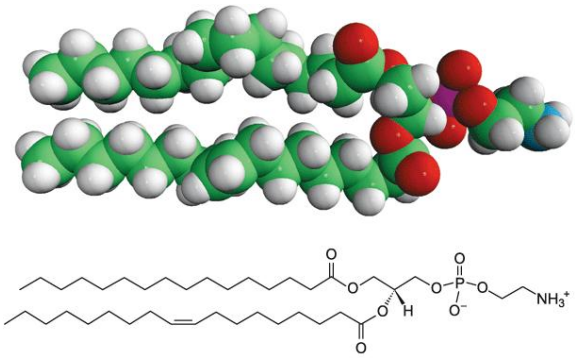

Figure 41 – Structural representation of POPE (Avanti Polar Lipids).

Peptides

To assay the peptide-lipid interaction on model membranes by X-ray diffraction, cecropin A-melittin derivatives (CAM, K6 and K7) and lactoferrin-derived peptides (LFcin17-30, LFampin265-284 and LFChimera) were used (see tables 3 and 4 on pages 55 and 56).

Preparation of liposomes

To obtain multilamellar vesicles (MLVs) of POPE, 2 methods were used:

1. Appropriate amounts of the lipid were dissolved in a small volume of chloroform, and transferred to a round flask, where a film was obtained by drying the chloroform under a slow nitrogen flow. The film was thereafter kept under vacuum for 3h to remove all traces of organic solvents. The lipid film was hydrated with PBS (150 mM NaCl, pH 7.2) and warmed for about 1h in a thermostated bath at 35 ºC, above the main transition temperature (T_M = ~25 ºC) and below the lamellar to hexagonal transition (T_H = ~71 ºC) to guarantee that all lipids are in the fluid lamellar state. The resulting vesicles (MLVs) were frozen in liquid nitrogen and thawed in the thermostated bath, and this process was repeated 5 times.
2. Appropriate amounts of the lipid were hydrated with PBS (150 mM NaCl, pH 7.2), and incubated for 30 min in a water bath at ~35 ºC, above the main transition temperature,

for the same reasons as stated above. After this, a large number of short cycles of alternating vortex at room temperature and heating at 35 ºC were performed.

An aliquot of each liposome suspension was stored at -20 ºC for determination of the exact lipid concentration. The remaining lipid was kept at 4 ºC until use.

Peptide-lipid mixture's preparation

Peptide solution in the same buffer used for preparing the liposomes was added to the liposome suspension at different peptide-to-lipid (P:L) ratios, and the mixtures were incubated for 30 min at 35 °C. The samples were then centrifuged at 13 000 rpm in a microcentrifuge at least for 15 min, and transferred thereafter into glass capillaries (Spezialglas Markröhrchen 1.5 mm capillaries, Glass Technik & Konstruktion – Müller & Müller OHG, Germany). The capillaries were sealed by flame, and stored at 4 ºC, at least one day before use.

X-Ray diffraction

Principles

X-ray diffraction is a powerful tool to characterize the structure of lipid phases and polymorphic changes in the lipid bilayer. Lipid dispersions in water form structures with long-range periodic order that can be analyze by X-ray techniques, in particular by diffraction methods, as these periodic structures will diffract the X-rays in a particular pattern depending on the existent phase and the respective space group. This is described by Bragg's law

$$m\lambda = 2d\sin\theta$$

where m is the diffraction order, λ is the wavelength of the diffracted radiation, d is the lattice spacing and 2θ is the scattering angle. Measurements of the intensity of the scattered radiation as a function of the scattering angle allows the determination of the lattice parameter (Hanulóva, M. 2008).

The radiation can be diffracted at small angles, up to 3º – Small Angle X-ray Diffraction (SAXD) or in larger angles, up to 20º – Wide Angle X-ray Diffraction (WAXD) (Hanulóva, M. 2008). Therefore, by SAXD we can get information about distances in large structural repetitions that are in the order of 10 to several 100 Å, like the lattice parameter (*e.g.*

bilayer spacing), whereas WAXD provides information about repeating structural patterns at small distances (2 to 9 Å), like the hydrocarbon chain lattice, if they are not in a liquid disordered state. Ideally, these two distinct regions of the diffraction pattern should be measured simultaneously to characterize completely the structure present.

Lamellar, hexagonal and cubic phases can be detected and characterized by X-ray diffraction. The first two phases are normally present in microscopic crystalline domains, randomly oriented throughout the sample, whereas cubic phases can grow to large dimensions, in some cases of the size of the sample container. With the exception of the last one (cubic phase), a few diffractions are enough to unequivocally describe the type of symmetry (*e.g.* lamellar or hexagonal) and to obtain the repeat distances (d or a).

When we measure the intensity of the scattered radiation as a function of the scattering angle in the SAXD region (the SAXD pattern), we obtain peaks, which for a gel (L_β) and a fluid lamellar phase (L_α) are equidistant (figure 1) and appear at $s_h = h/d$ (h – Miller index), reflecting the bilayer spacing (or lattice parameter of lamellar phase – d in figure 42). In WAXD a sharp peak appears for the gel lamellar phase (L_β) and only a broad diffuse peak for the liquid lamellar phase, as in the gel lamellar phase the lipid chains are more extended and rigid, and thus have order reflections and thus this can be used to safely assess the presence of a gel lamellar phase (Hanulóva, M. 2008).

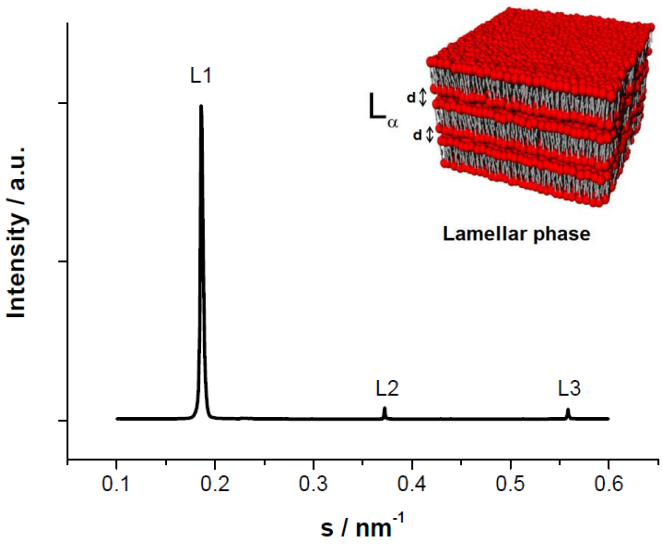

Figure 42 – SAXD pattern of the fluid lamellar phase (L_α) of POPE membranes. The corresponding structure (adapted from: Tresset, G. 2009), is shown in the inset with the respective lattice parameter (d).

The diffraction pattern of a hexagonal phase (H_{II}) in the SAXD region contains a series of peaks that appear at positions $s_{hk} = (2\sqrt{h^2+k^2+hk} / \sqrt{3}a_h)$ (h, k – Miller indices), where a_h is the distance between the centers of the neighboring lipid cylinders (figure 43). In WAXD, similar to the fluid lamellar phase, only a broad peak appears (Hanulóva, M. 2008).

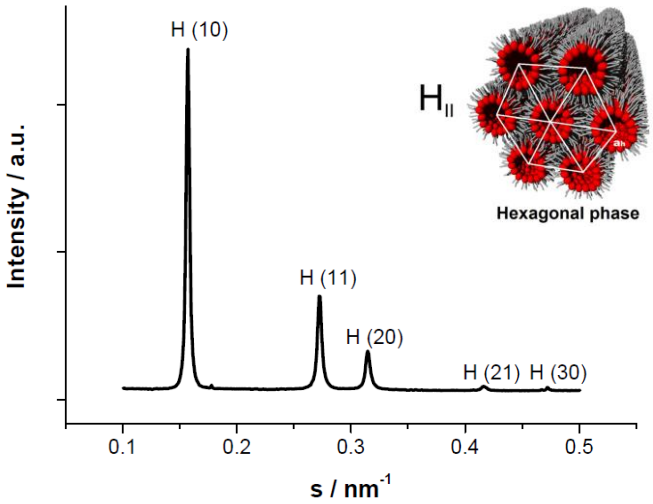

Figure 43 – SAXD pattern of the inverse hexagonal phase (H_{II}) of POPE membranes. The corresponding structure (adapted from: Tresset, G. 2009), is shown in the inset with the respective lattice parameter (a_h).

The SAXD diffraction pattern of a cubic phase shows a number of peaks at positions given by $s_{hkl} = (\sqrt{h^2+ k^2+ l^2} / a_c)$ (h, k, l – Miller indices), where a_c is the cubic lattice parameter (figure 44). The more intense peaks of the cubic phase appear at small *s* values (reciprocal spacing), reflecting the large repeating units of this phase. In the WAXD region a broad diffuse peak is seen (Hanulóva, M. 2008).

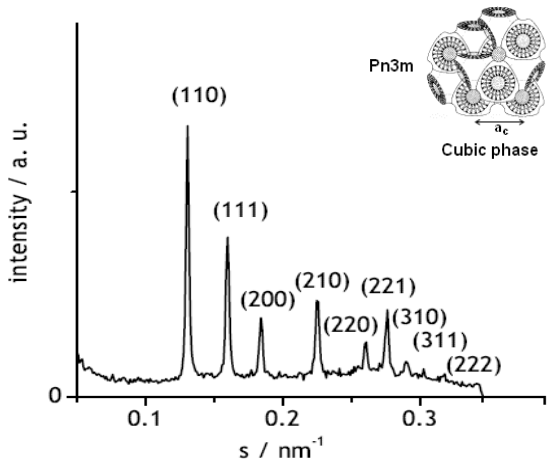

Figure 44 – SAXD pattern of the cubic phase Pn3m. The corresponding structure is shown in the inset with the respective lattice parameter (a_c) (Hanulóva, M. 2008).

Typically, depending on the lipid and temperature, liposomes can form lamellar or hexagonal phases. However the presence of an antimicrobial peptide can induce structural changes, either altering only the lattice parameters or leading to the formation of cubic phases. Understanding the type of polymorphic structure that is present in a peptide-lipid mixture can lead to a deeper knowledge of the mechanism of action of the peptide (Haney, E.F. *et al.* 2010).

Method

SAXD and WAXD experiments were performed at the synchrotron soft condensed matter beamline A2 in HASYLAB at Deutsches Elektronen Synchrotron (DESY), Hamburg, Germany, using a monochromatic radiation of λ = 0.15 nm wavelength.

Figure 45 – Beamline A2 in Hasylab. A) A general view of the instrument is shown. **B)** In detail, the capillary holder and the two detectors for SAXD and WAXD.

Up and down temperature scans were performed for each sample, all at a scanning rate of 1 ºC/min, over the temperature range 20 - 80 ºC. Diffractograms were taken with 10 s of exposure time at each temperature. The heating and cooling of the sample was regulated by a thermocouple connected to the temperature controller JUMO IMAGO 500 (JUMO GmbH & Co. KG, Fulda, Germany). Each SAXD diffraction pattern is presented as normalized diffraction intensity in arbitrary units versus the reciprocal spacing *s*. The SAXD patterns were analysed in two different ways: i) by use of the A2tool program as a first approach to the (qualitative) analysis of the results; and ii) quantitatively by analyzing each difractogram in detail, where the diffraction peaks were fitted by Lorentzians above a linear background by use of the Origin software, in order to derive the lattice parameters (d or a) that characterize the different phases present. The positions of the peaks plotted *vs*. h for a lamellar phase, $\sqrt{h^2+hk+k^2}$ for a hexagonal phase or $\sqrt{h^2+k^2+l^2}$ for a cubic

phase (h, k, l – Miller indices), should fall on a straight line, passing through the origin (0,0), and the lattice parameter d, a_h or a_c, for lamellar, hexagonal and cubic phase, respectively, is given by the inverse of the slope. WAXD measurements were used to verify the existence of the gel phase (L_β) (not shown).

2. RESULTS

2.1. Cecropin A-melittin-derived peptides

2.1.1. Phase behavior of POPE membranes

The diffraction profile of a lipid changes with temperature. In the case of pure POPE membranes, as the temperature increases there is a transition from gel lamellar phase (L_β) to fluid lamellar phase (L_α) at 26 °C, and from fluid lamellar (L_α) to hexagonal phase (H_{II}) at 71 °C (figure 47).

When the peptides (CAM, K6 or K7) were added to the lipid system, the transition from gel to fluid lamellar phase was not significantly affected, neither in terms of transition temperature nor in lattice value, but some changes were observed in the transition from fluid lamellar to hexagonal phase. This difference was already apparent in the temperature scan of each peptide/lipid mixture (figure 46) and later was confirmed by the determination of the lattice parameters (figure 47). The studied peptides have somewhat different effects:

1. CAM does not alter the transition temperature to hexagonal phase.
2. For both K6 and K7 we observed the coexistence of hexagonal phase (figure 46 – green arrows) together with fluid lamellar phase (figure 46 – red arrows), from about 10 °C below the T_H of pure POPE transition. This suggests that these two peptides destabilize the lamellar phase and have better compatibility with the hexagonal phase, as it appears at lower temperatures than in the absence of the peptide. Thus, part of the mixture moves to the hexagonal phase (peptide/POPE) earlier, whereas the remaining, presumably not affected by the peptide, remains in the lamellar phase.

After the transition to hexagonal phase, it is possible to observe, for the three peptides, the superimposition of two hexagonal phases with close periodicity (figure 47). Similarly to the early appearance of the hexagonal phase, these two hexagonal phases are most probably due to the existence of distinct domains within the membrane - the peptides bind and destabilize only part of the membrane, this being reflected in a different lattice spacing of one hexagonal phase as compared to the one composed of pure lipid. For instance, at 80 ºC the lattice parameter of the hexagonal phase for pure POPE is 7.09 nm, whereas in the presence of CAM, one of the hexagonal phases has a value of 7.12 nm (similar to the pure lipid) and the other 6.89 nm, the same happening for the other two peptides (K6 – 7.11 and 6.85 nm; K7 – 7.10 and 6.88 nm).

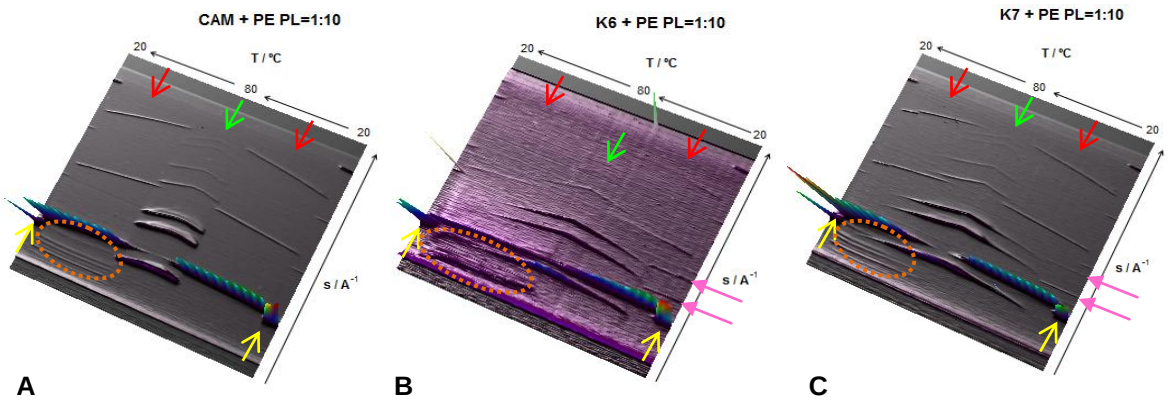

Figure 46 – SAXD pattern of temperature scans of POPE membranes with cecropin A-melittin derivatives. Graphical representation of all the diffractograms from up-and-down temperature scans (20-80ºC) of peptide/lipid mixtures (P:L=1:10) using the A2tool software **A)** CAM, **B)** K6, **C)** K7. The different phases present are marked by: yellow – gel lamellar phase (L_β); red – fluid lamellar phase (L_α); green – hexagonal phase (H_{II}); orange circle – cubic phases.

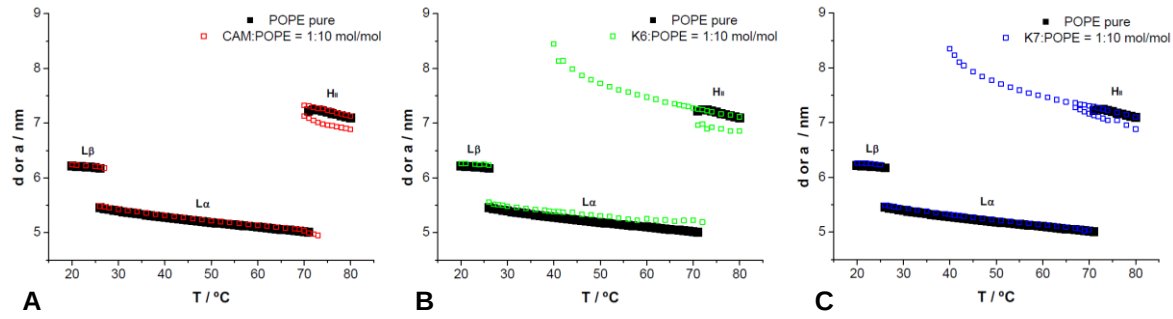

Figure 47 – Phase behavior of POPE membranes with cecropin A-melittin derivatives. Lattice parameters (d or a) for the lamellar (L_β and L_α) and hexagonal (H_{II}) phases observed for the pure POPE and for peptide/lipid mixtures (P:L=1:10) with **A)** CAM, **B)** K6 e **C)** K7. Data derived from up-scans from 20 - 80 ºC.

With K6 and K7 it was also possible to detect the presence of two extra peaks, as marked in figure 46 by the pink arrows, together with the lamellar phase that do not belong either to this or to the hexagonal phase (figure 46 – pink arrows). With only two peaks it is impossible to identify unequivocally a cubic phase, but we would like to stress that these two peaks are always present in both samples and they are consistent with an Im3m cubic phase (figure 48). This could be an indication that this phase is present, already in the heating process. Nevertheless, further analysis with different P:L ratios (higher peptide content) are necessary to characterize this possible phase, leading to safer conclusions.

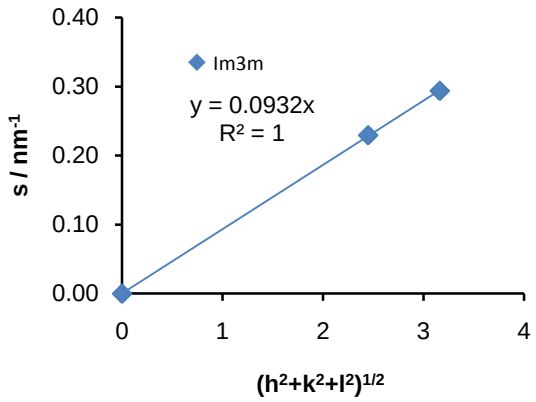

Figure 48 – Evidence for a cubic Im3m phase induced by K6 and K7 in POPE membranes. A plot of s = f ($\sqrt{h^2+k^2+l^2}$) for the observed reflections at 23 ºC that do not fit with lamellar or hexagonal phase.

In the cooling process, the temperature scan indicated, with all the peptides, the presence of structures of higher symmetry (several peaks observed at small *s* values) (figure 46 – orange circle), most probably a cubic phase. Further analysis was thus performed with these down-scans, to try to determine what was the phase present. At this moment, we chose to analyze in detail for all peptides the diffraction patterns at one temperature, namely at 50 ºC (on cooling) (tables 6-8, figure 49). The diffraction patterns are compatible with the coexistence of **two bicontinuous cubic phases**, of Im3m and Pn3m symmetry, and a fluid lamellar phase. It should be stressed that in some cases, due to very few reflections (less than 4-5) it is not possible to make a safe assignment to a particular lattice. For the parental hybrid peptide, CAM, the Pn3m phase is better defined as compared with the Im3m phase, as more peaks are observed for the first one. With the trimethylated peptides (K6 and K7) more peaks corresponding to an Im3m phase are visible, especially at higher *s* values. The lattice parameters determined have similar values between the same phases present in the three peptide/lipid mixtures (tables 6-8). For example the fluid lamellar phase (L_α) present in each of the three peptide/lipid mixtures have similar values between them (d_{CAM} = 5.24 nm; d_{K6} = 5.28 nm; d_{K7} = 5.23 nm).

Table 6 – SAXD data for POPE membranes with cecropin A-melittin derivatives, CAM, K6 and K7 (P:L=1:10), at 50 ºC indexed to a fluid lamellar phase (L_α). The respective lattice parameter (d) is indicated.

H	L_α (s / nm^{-1})		
	CAM	**K6**	**K7**
1	0.19043	0.18638	0.19076
2	0.38225	0.38031	0.38233
d / nm	**5.24**	**5.28**	**5.23**

Table 7 – SAXD data for POPE membranes with cecropin A-melittin derivatives, CAM, K6 and K7 (P:L=1:10), at 50 ºC indexed to a Im3m cubic phase. The respective lattice parameter (a_c) is indicated.

Hkl	$\sqrt{h^2+k^2+l^2}$	Im3m (s / nm^{-1})		
		CAM	**K6**	**K7**
110	√2	0.10906	0.10547	-
200	√4	0.15662	0.14796	0.15016
211	√6	0.19043	-	-
220	√8	-	0.20833	-
222	√12	-	-	0.25916
400	√16	-	-	0.29894
420	√20	-	-	0.32935
332	√22	-	0.34421	-
a_c / nm		**12.85**	**13.59**	**13.44**

Table 8 – SAXD data for POPE membranes with cecropin A-melittin derivatives, CAM, K6 and K7 (P:L=1:10) at 50 ºC indexed to a Pn3m cubic phase. The respective lattice parameter (a_c) is indicated.

Hkl	$\sqrt{h^2+k^2+l^2}$	Pn3m (s / nm^{-1})		
		CAM	**K6**	**K7**
110	√2	0.08851	0.08220	0.08962
111	√3	0.10906	0.10547	-
200	√4	0.12834	0.12440	0.12195
211	√6	0.15662	0.15460	0.015016
221	√9	0.19043	0.18638	-
310	√10	-	-	0.19076
311	√11	-	0.20833	-
222	√12	-	0.21784	-
321	√14	0.23547	-	-
400	√16	-	0.25490	-
a_c / nm		**15.80**	**15.92**	**16.39**

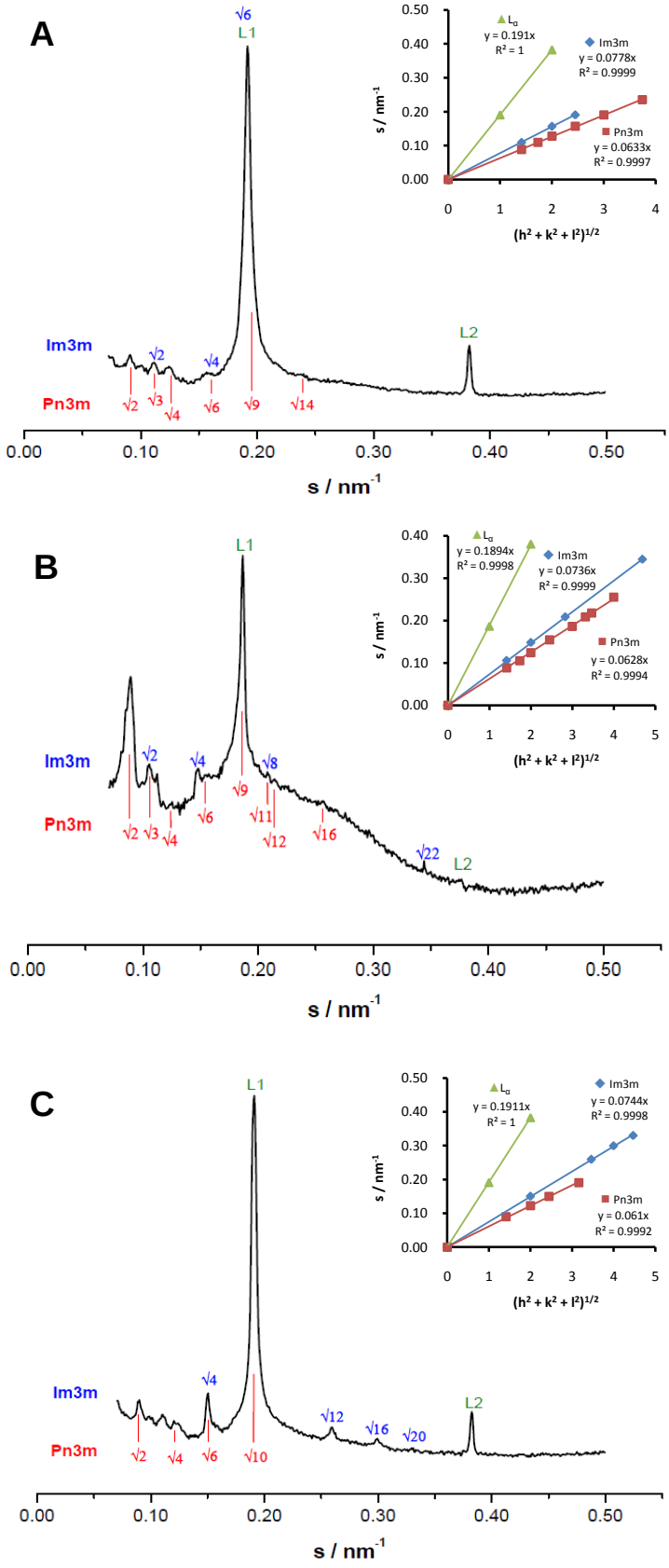

Figure 49 – Cubic phases (Im3m and Pn3m) in POPE membranes with cecropin A-melittin derivatives. SAXD patterns for peptide/lipid mixtures (P:L=1:10) at 50 ºC on cooling. **A)** CAM, **B)** K6, **C)** K7. Insert: A plot of s = f($\sqrt{h^2+k^2+l^2}$) for all observed reflections is shown.

2.2. Lactoferrin-derived peptides

2.2.1. Phase behavior of POPE membranes

In the case of all the lactoferrin-derived peptides, LFcin17-30, LFampin265-284 and LFChimera, no changes in parameters or transition temperatures from gel to fluid lamellar phase or in the transition from fluid lamellar to hexagonal phase were observed in the heating process (figure 50-51). The only difference was the prevalence of the first peak of the fluid lamellar phase after the transition to hexagonal phase when the sample was heated (in a small content meaning, only the first peak is visible and with a low intensity) in the presence of LFChimera at PL=1:18, in the heating process. However, and as for the first family of peptides, the presence of other structures with peaks at small *s* values, were observed when the sample was cooled down (figure 50 – orange circles).

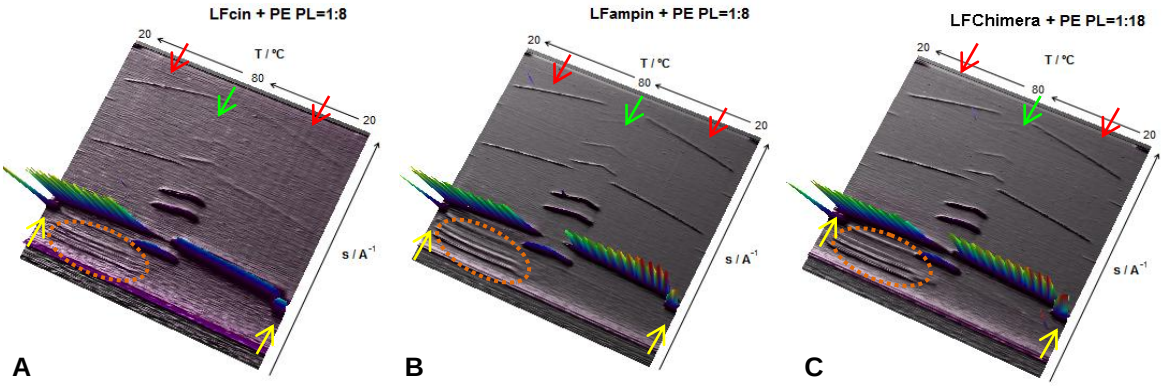

Figure 50 – SAXD pattern of temperature scans of POPE membranes with lactoferrin-derived peptides. Graphical representation of all diffractograms from up-and-down temperature scans (20-80ºC) of peptide/lipid mixtures using the A2tool software **A)** LFcin17-30 at PL=1:8, **B)** LFampin265-284 at PL=1:8 and **C)** LFChimera at PL=1:18. The different phases present are marked by: yellow arrows – gel lamellar phase (L_β); red arrows – fluid lamellar phase (L_α); green arrows – hexagonal phase (H_{II}); orange circles – cubic phases.

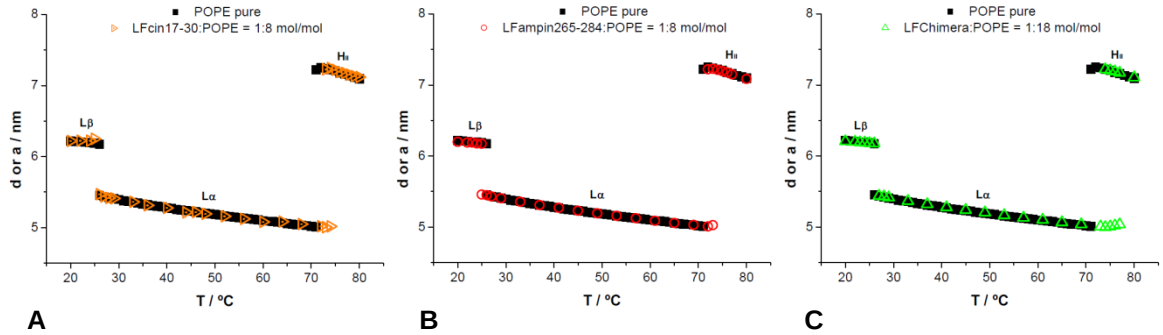

Figure 51 – Phase behavior of POPE membranes with lactoferrin-derived peptides. Lattice parameters (d or a) for the lamellar (L_α and L_β) and hexagonal (H_{II}) phases observed for the pure POPE and for peptide/lipid mixtures. **A)** LFcin17-30 at PL=1:8, **B)** LFampin265-284 at PL=1:8 and **C)** LFChimera at PL=1:18. Data derived from up-scans from 20 - 80 ºC.

The peaks observed at small *s* values were also analyzed in detail (at 50 ºC on the cooling scan) in the same way as before (for cecropin A-melittin peptides). They also suggest the coexistence of two bicontinuous cubic phases (Im3m and Pn3m), together with a fluid lamellar phase (tables 9-11; figure 50). The lattice parameters are again similar between the same phases present in the three lipid/ peptide mixtures, and with the values obtained for the other peptide family, with the exception of LFampin265-284 that presents a larger cubic lattice spacing (tables 10 and 11).

Table 9 – SAXD data for POPE membranes with lactoferrin-derived peptides: LFcin17-30 (PL=1:8), LFampin265-284 (PL=1:8) and LFChimera (PL=1:18) at 50 ºC indexed to a fluid lamellar phase (L_α). The respective lattice parameter (d) is indicated.

H	L_α (s / nm^{-1})		
	LFcin	**LFampin**	**LFChimera**
1	0.19105	0.19182	0.19147
2	0.38362	0.38464	0.38372
d / nm	**5.22**	**5.20**	**5.21**

Table 10 – SAXD data for POPE membranes with lactoferrin-derived peptides: LFcin17-30 (PL=1:8), LFampin265-284 (PL=1:8) and LFChimera (PL=1:18) at 50 ºC indexed to a Im3m cubic phase. The respective lattice parameter (a_c) is indicated.

Hkl	$\sqrt{h^2+k^2+l^2}$	Im3m (s / nm^{-1})		
		LFcin	**LFampin**	**LFChimera**
110	√2	0.10659	0.099021	0.10642
200	√4	0.15303	0.14008	0.14725
211	√6	-	0.17561	-
310	√10	-	-	0.2368
400	√16	-	-	0.29612
330	√18	0.31718	-	0.31485
420	√20	-	0.32043	-
a_c / nm		**13.32**	**14.00**	**13.46**

Table 11 – SAXD data for POPE membranes with lactoferrin-derived peptides: LFcin17-30 (PL=1:8), LFampin265-284 (PL=1:8) and LFChimera (PL=1:18) at 50 ºC indexed to a Pn3m cubic phase. The respective lattice parameter (a_c) is indicated.

Hkl	$\sqrt{h^2+k^2+l^2}$	Pn3m (s / nm^{-1})		
		LFcin	**LFampin**	**LFChimera**
110	√2	0.087868	0.079632	0.087068
111	√3	0.10659	0.094663	0.10642
200	√4	0.12030	0.11129	0.12390
211	√6	0.15303	0.13350	0.015259
310	√10	-	0.17561	0.19147
222	√12	-	0.19182	-
a_c / nm		**16.23**	**18.08**	**16.29**

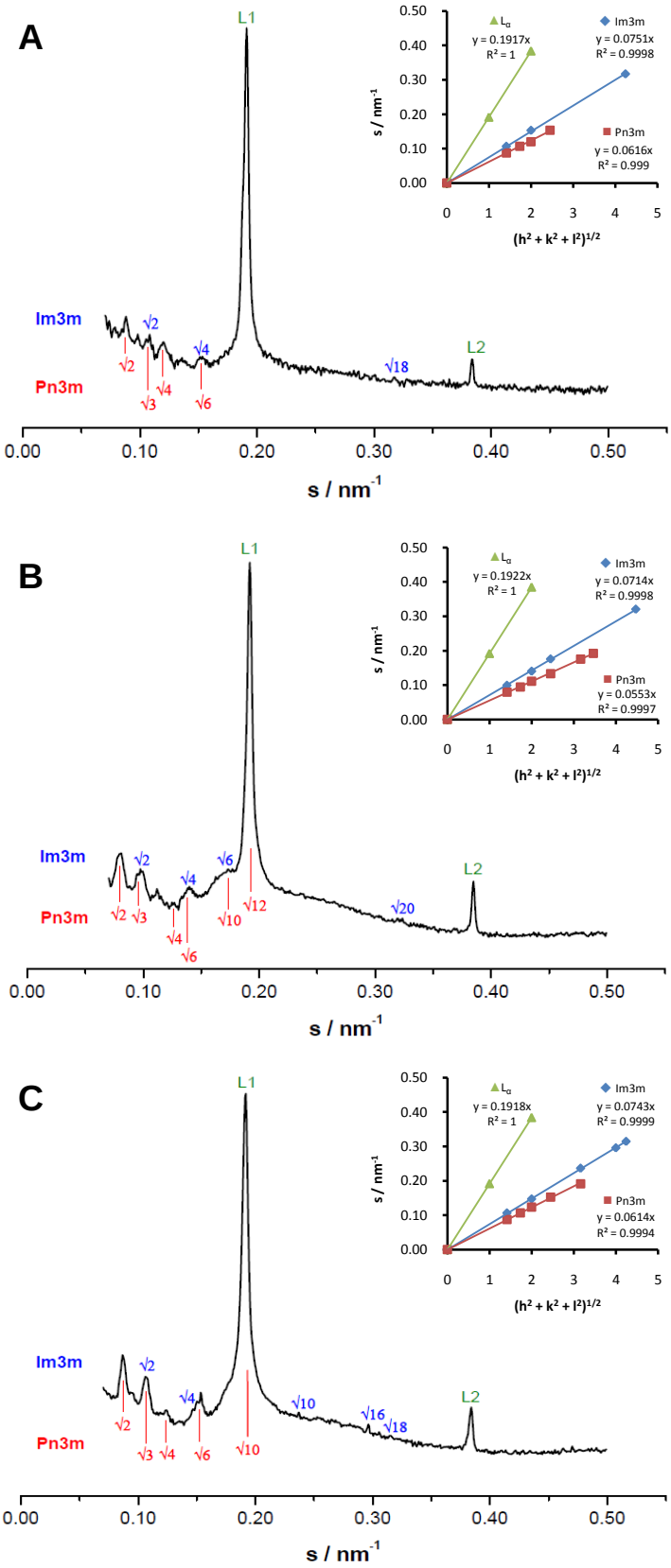

Figure 52 – Cubic phases (Im3m and Pn3m) in POPE membranes with lactoferrin-derived peptides. SAXD patterns for peptide/lipid mixtures at 50 ºC on cooling. **A)** LFcin17-30 at PL=1:8, **B)** LFampin265-284 at PL=1:8 and **C)** LFChimera at PL=1:18. Insert: A plot of s = f($\sqrt{h^2+k^2+l^2}$) for all observed reflections is shown.

PART IV

DISCUSSION & CONCLUSIONS

DISCUSSION & CONCLUSIONS

In this work we used microbiological and biophysical techniques to study the effects of cecropin A-melittin derived and lactoferrin-derived peptides, on different pathogens and on model membranes.

The **antimicrobial activity of cecropin A-melittin derivatives** was assessed **against *Mycobacterium avium*** in axenic cultures (bacteria growing in liquid medium) and in cultures of macrophages, the cell inside which mycobacteria usually grow. CAM, K6 and K7 directly inhibit *M. avium* growth above 50 μM but even at 200 μM the growth inhibition did not reach 50%. This lack of significant effect suggests that these peptides are not able to crossover the highly impermeable and complex mycobacterial cell wall. In addition, these peptides are toxic towards macrophages at much lower concentrations, the cytotoxicity of CAM and K6 at 20 μM overpassing 50%. For concentrations at which the toxicity does not reach 10%, no effect on the viability of *M. avium* growing inside macrophages was seen.

All these results discourage the use of these particular peptides as therapeutic agents against mycobacterial infections, as their activity was very low and they cannot be used on macrophages, the host cell of these bacteria. Nevertheless the results are interesting in some points of view. For instance, recent studies showed that the hemolytic activity of the trimethylated peptides K6 and K7 was very low (Fernandez-Reyes, M. *et al.* 2010), which was seen as a promising characteristic in terms of therapeutic utility. However, our results showing that the peptides were toxic towards macrophages, indicate that although erythrocytes and macrophages are both mammalian cells, there must be crucial differences in their cellular membranes. This suggests that the use of erythrocytes only as target cells for determination of AMP toxicity may be misleading. The differential effects of cecropin A-mellitin peptides on different cell types could be more clarified with biophysical studies using model membranes mimicking these different cells. Until now the most common model membrane of choice for mammalian cells is solely composed by phospatidylcholine (PC) the main lipid of the cellular membrane of erythrocytes. The lipid composition of the cytoplasmic membrane of macrophages must be very different from the membrane of erythrocytes, containing other phospholipids and possibly changing the relative amounts, and this could explain the differences in behavior that we encountered. However the knowledge about the lipid composition of the macrophage cytoplasmic membrane is poor and so no model membranes more specific for these cells are known up to now. The use of lipidomics, for instance, could be useful to try to uncover this composition, and be subsequently used to make model membranes, leading to a deeper

knowledge on how these peptides, and others, interact with these types of cells, and in that way refine the applicability of the biophysical techniques as screening methods for the antimicrobial activity of the AMPs. We hope to use this in the future.

We have also investigated the **effects of several lactoferrin-derived peptides on** two intramacrophagic pathogens, ***Leishmania* and *Mycobacterium avium***.

In this study, we found that Lactoferricin (LFcin17-30), lactoferrampin (LFampin265-284) and LFChimera were active against *L. donovani* promastigotes, and LFChimera is of special interest due to its high activity both against *L. donovani* promastigotes and *L. pifanoi* amastigotes. Our results indicate that the leishmanicidal mechanism of these AMPs consists on targeting the plasma membrane, leading to its permeabilization that culminates into bioenergetic collapse of the parasites due to loss of the ionic gradient and leakage of ATP, and also leakage of the cytoplasmic content, ending in parasitic death. The mechanism of action of LFcin17-30 was also very interesting and peculiar, as it appears to induce cellular aggregation. The fact that this peptide was able to reach intracellular amastigotes when they were inside its host cell strongly suggests that it will also be able to exert its leishmanicidal effect inside macrophages. However, the effect of this peptide on the parasitic load inside macrophages was not determined. Future experiments will be performed to address this question.

The activity of LFChimera against *L. donovani* promastigotes was much higher than the sum of its separated components, revealing that having the two peptides, LFcin17-30 and LFampin265-284, bond to each other is important for the antimicrobial activity. To try to understand what are the special features in LFChimera that make this peptide so active, a set of surrogates that varied some of the characteristics of this peptide, such as charge, order of the constituent peptides (LFcin17-30 and LFampin265-284), and the bond between them, were also tested. The activity against *Leishmania* parasites of all the peptides was equal or higher than the parental peptide. Two of these peptides, Di-LFcin and especially LFampin-LFcin, were found to have higher activity than LFChimera, against both forms of the parasite, promastigotes and amastigotes. This is in contrast with the LFChimera, which was less active in amastigotes as compared to promastigotes. These results suggest that the charge (higher in Di-LFcin in comparison to LFChimera) is important for the activity of AMPs and that the bond between the peptides appears to play an important role too, as in the most potent surrogate (LFampin-LFcin) instead of having the peptides linked by an additional lysine in a special linkage through the side chain (like in LFChimera), they are linked trough a normal peptide bond. So, the attempt to reach structure-activity relationships is not yet fully accomplished, as the experiments so far

were limited to the small number of surrogates available, and so, further studies will be done with different surrogates to obtain deeper information about structure-activity relationships. Nevertheless we should stress that at least two very promising peptides were found, with high antimicrobial activity that makes them good candidates to fight leishmaniasis. Further studies should be done in order to understand what are the antimicrobial mechanisms of these peptides. Also of importance, their toxicity towards mammalian cells, such as macrophages and erythrocytes, should be carefully evaluated.

Lactoferrin-derived peptides had variable impact on the growth of *M. avium*. Lactoferrampin had no effect against *M. avium* either in axenic cultures or growing inside macrophages. Lactoferricins (human, hLFcin1-11, and bovine, LFcin17-30, peptide) significantly inhibited the growth of *M. avium* axenically at and above 25 μM. Although they did not inhibit the growth of *M. avium* inside macrophages up to 40 μM, they showed a synergistic effect with ethambutol in this model. These results suggest that the peptide is increasing the access of the antibiotic to the mycobacteria that are growing inside the phagosome of the macrophage. This synergistic effect of lactoferricin with conventional antibiotics was already shown for several other pathogens (Gifford, J.L. *et al.* 2005; Jenssen, H. & Hancock, R.E. 2009; Leon-Sicairos, N. *et al.* 2006; Oo, T.Z. *et al.* 2010; Sanchez-Gomez, S. *et al.* 2011). It is thought that this peptide disturbs the membrane, in this case the cytoplasmic membrane of the macrophage and/ or the phagosome membrane, probably by forming transient pores increasing the permeability of the membrane not only to the peptide itself but also to compounds that are close to these pores, such as the antibiotic. The combined efficacy could also be due to a more localized effect on the mycobacteria cell wall. The peptide could induce dissipation of the proton-motive force resulting in decreased activity of ATP-dependent multidrug efflux pumps, allowing in that way the entrance of the antibiotic (Gifford, J.L. *et al.* 2005). Nevertheless, whatever the mechanisms are, the combination of lactoferricin with antibiotics like ethambutol, for the treatment of mycobacterial infections, as for others, has many advantages such as the decrease in the antibiotic dosages and in the duration of the treatment, and more important the decreased ability of the bacteria to acquire resistance to both AMPs and antibiotics.

LFChimera, contrary to the effect observed on *Leishmania* and on other pathogens (Bolscher, J.G.M. *et al.* 2009; Flores-Villasenor, H. *et al.* 2010; Leon-Sicairos, N. *et al.* 2009; Lopez-Soto, F. *et al.* 2010), did not inhibit the growth of *M. avium*. In fact the number of *M. avium* growing inside macrophages increased almost two-fold in the presence of LFChimera at 10 μM. This result was found to be dose-dependent and statistically significant as early as 4 days after infection, with no apparent impact on

macrophage viability. Indeed the macrophage viability after 7 days of infection and incubation with LFChimera was maintained at somewhat higher levels than in untreated macrophages. These unexpected results raise the question of the mechanisms underlying the increased growth of *M. avium*. We can try to propose some explanations. This peptide could be: i) inhibiting a mechanism that normally contributes for bacterial growth restriction (like production of reactive oxygen and/ or nitrogen species); ii) facilitating the access of the bacteria to some critical nutrient or growth factor; iii) interfering with lipid metabolism and iron availability; iv) interfering with the characteristics of the membranes of mycobacteria-containing phagosomes, since AMPs act on membranes, and so many more. Understanding the processes driving this effect could help to clarify the mechanisms by which macrophages actually control the intracellular growth of mycobacteria, leading to the discovery of possible new targets for antimicrobial agents.

Also somewhat conflicting are other results revealing that LFChimera was toxic towards peritoneal macrophages infected with *L. pifanoi* amastigotes. This difference in toxicity between mycobacteria-infected BMMØ and leishmania-infected-peritoneal macrophages could result from:

- the peptide-to-cell ratio. Although the concentrations of peptide was the same in both assays the amount of peritoneal cells was 5 times less than the amount of bone marrow derived macrophages (BMMØ) used in the mycobacterial infections, so the number of peptide molecules per cell is much higher in the case of peritoneal macrophages.
- the type of medium. The peritoneal macrophages were kept in HBSS buffer supplemented with D-glucose 10 mM, whereas BMMØ were cultured in a complete medium with 10% of fetal bovine serum that can potentially decrease the toxicity of the peptide.
- peritoneal macrophages and BMMØ are primary macrophages but the first ones are more differentiated, with less capacity to maintain viability in culture, potentially being more susceptible to the activity of the peptides.

So the comparison of results obtained with different techniques and different cells should be done very carefully and every variable should be considered before taking any definitive conclusion.

In an attempt to shed some light on the mechanism of action of these families of peptides, the **interaction between AMPs and simple model membranes** was characterized in this work by a biophysical technique, SAXD.

The choice of the lipid composition of the model membranes is important when we want to use biophysical results to help interpret the biological action. As such, we have used mainly model membranes of phosphatidylethanolamine (PE), as it is the most abundant phospholipid of the bacterial membranes. Studies were also performed with lipid mixtures composed of PE and phosphatidylglicerol (PG), as PG is also one of the major phospholipids in the bacteria cell membrane, being one of the main responsible for its characteristic negative charge. Nevertheless, these results are still being analyzed. Studying the phase behavior of the model membranes in the absence and presence of AMPs can provide information about their mechanism of action. By SAXD we can retrieve this structural information by studying the lamellar/non-lamellar phase transitions and the changes induced by the presence of the peptide. In the last years growing evidence is showing that AMPs are able to induce or favor the formation of non-lamellar phases, namelly three dimensional cubic phases, as part of their mechanism of action (Bastos, M. *et al.* 2011; Haney, E.F. *et al.* 2010; Zweytick, D. *et al.* 2008). The formation of such cubic phases, could indicate that the peptides act by promoting an extensive curvature of the bilayer, leading eventually to disruption of the membrane and leakage of the cell content and thereby cell death. Also the existence of cubic phase could indicate that the peptide is inducing cubic domains in the membranes compatible with the formation of toroidal pores, a proposed mechanism for the action of AMPs.

All the peptides analyzed induce the formation of bicontinuous cubic phases in POPE liposomes (Pn3m and Im3m) on the cooling process. However each family of peptides affects differently the transition temperatures from lamellar (L_α) to hexagonal phase (H_{II}), in the heating process. In the cecropin A-melittin family all peptides induce changes in the hexagonal phase, either by the existence of two hexagonal phases superimposed or, in the case of the trimethylated peptides (K6 and K7), by the early appearance of the hexagonal phase (10ºC bellow the temperature observed for the pure lipid system), whereas in the case of lactoferrin-derived peptides no changes were observed in this transition ($L_\alpha \rightarrow H_{II}$).

The main conclusion at this point is thus that the interaction strongly depends on the peptides, and even in the same peptide family quite different patterns of interaction can be observed (*e.g.* trimethylated peptides, K6 and K7). It should be noted that the behavior also depends on lipid composition – in a recent study (Bastos, M. *et al.* 2011) we found that lactoferrampin (LFampin265-284) induces the formation of a micellar cubic phase (Pm3n) in model membranes of PC/PG, whereas in the case of PE membranes the same peptide induces the formation of bicontinuous cubic phases. So the interaction between AMPs and lipid membranes is not as unspecific as it was thought some years ago, the

details of the interaction go beyond the plain existence of charge at the surface of the bilayer. Although this is work still in progress, we can clearly say at this point that the use of biophysical techniques can help to define the importance of a lipid composition in the overall mechanism of action of antimicrobial peptides and predict their antimicrobial activity.

To follow this work, **further experiments** with lactoferrin-derived peptides are warranted.

The potential of LFcin17-30, as well as lactoferricin variants (to be designed *de novo*) for synergism with other antibiotics, such as rifampicin and clarithromycin, will be assessed against *M. avium* growing inside macrophages.

The mechanism by which LFChimera promotes the growth of *M. avium* inside macrophages will be assessed by:

- infecting macrophages with different intracellular pathogens in order to determine if the effect observed for *M. avium* is exclusive for this bacteria;
- activating macrophages with LPS and IFN-γ, for example, and evaluate the effect of LFChimera on the levels of macrophage antimicrobial molecules (*e.g.* NO);
- infecting phox- and iNOS-deficient macrophages with *M. avium* and treating with LFChimera to ask whether the growth-promoting effect of the peptide depends in any way on oxygen and nitrogen reactive species;
- characterizing LFChimera's distribution inside non-infected and infected macrophages and the ability of the peptide to reach *M. avium* containing phagosomes by confocal microscopy using fluorescence-labeled LFChimera;
- determining the impact of LFChimera on the degree of maturation of *M. avium*-containing phagosomes by confocal microscopy, using appropriate fluorescent probes and molecular markers;
- assessing the integrity of phagosomal and other macrophage membranes when treated with LFChimera by electron microscopy;
- determining the effect of LFChimera surrogates on *M. avium* growing inside macrophages, trying to determine which features of LFChimera are important for the mechanism of promoting the intracellular growth of *M. avium*.

The interaction of these peptides with simple model membranes prepared with different combinations of PC, PG, PE, LPS, phosphatidylserine and cardiolipin, will be assess by Differential Scanning Calorimetry (DSC) and by X-ray diffraction (SAXD and WAXD). More complex preparations of model membranes with extracts of bacteria and extracts of

macrophage membranes will also be used, trying to show distinctive patterns characteristic of each peptide/ cell interaction.

As a final note we would like to stress that although no immediate or straightforward correlation is apparent between the microbiological and biophysical results, it has been the aim of the groups of Salomé Gomes and Margarida Bastos to perform these studies in parallel, as we have been getting input from the results in one area to lead to changes in procedure or way of “reasoning” in the other. The biophysical insight can help understanding some features of microbiological action, and conversely the later results direct the way that future biophysical experiments are designed.

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