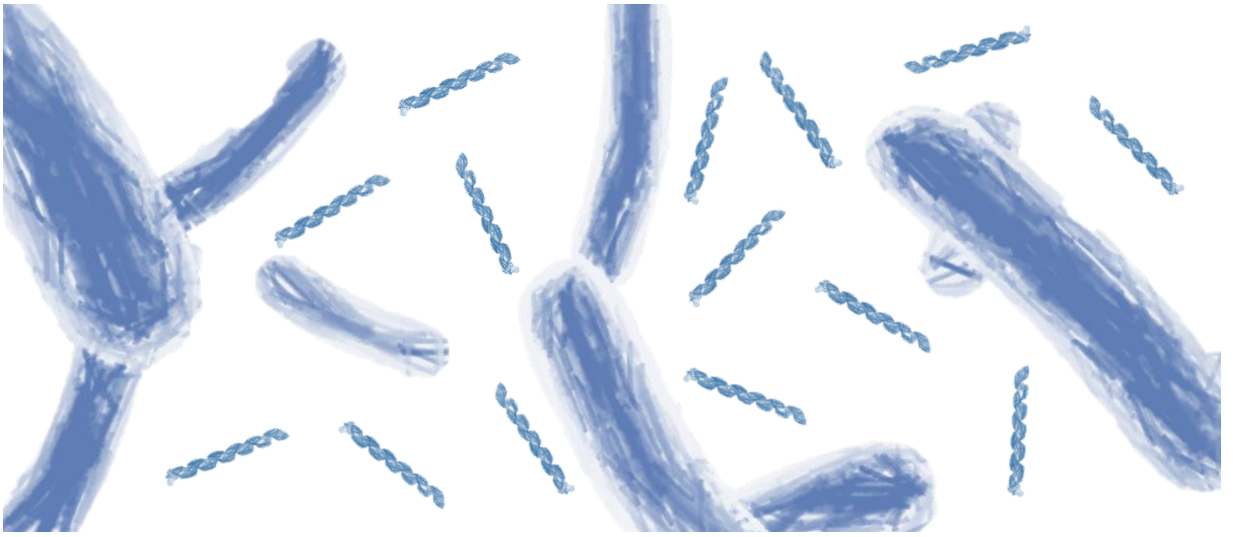

Exploring novel approaches to tackle *Mycobacterium avium*

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Abstract

The incidence of infections by nontuberculous mycobacteria (NTM) has been increasing and the most prominent NTM infections are caused by species of the *Mycobacterium avium* complex (MAC). *M. avium* is often considered an opportunistic pathogen, causing disease in individuals with either a history of chronic lung disease or immunosuppression. After infection, this mycobacterium is phagocytosed by the host's macrophages. However, it is able to escape the acidic environment of the phagolysosomes through a blockade of the phagosome-lysosome fusion, allowing for uptake of nutrients and consequent growth. With an intrinsic resistance to antibiotics, treatment of a MAC infection requires a multi-drug regimen with a three-times-weekly administration, lasting until the patient is culture-negative on therapy for one year. It is therefore imperative to find new antimicrobial agents as an alternative to antibiotics.

Antimicrobial peptides (AMP) have taken the role of being the most promising substitute for antibiotics over the last few decades. Found in every living organism, they not only display direct antimicrobial efficacy against viruses, fungi and bacteria but also display anti-inflammatory, anti-cancer and wound healing properties.

Therefore, this work aimed to test several AMP against *M. avium*. Moreover, using previous knowledge on AMP with good activity against *M. avium,* we aimed to understand what are the key structural and physiochemical properties determining the antimycobacterial effect of a peptide. Taking advantage of *in* silico predictors of AMP's antimicrobial activity and toxicity, several peptides were designed and one was chosen for synthesis and for testing against *M. avium in vitro.*

The obtained results indicate that the tested peptides with optimal activity against several Gram-positive and Gram-negative bacteria were ineffective against both axenically growing and intracellular *M. avium.* GOALA1, the *in silico* designed peptide, that was based on an AMP with described activity against *M. avium*, LFcin17-30, had a very low potency against several strains of axenically growing *M. avium* and also displayed no antimycobacterial activity against internalized *M. avium*. However, the peptide was effective against one strain of axenically growing *M. avium*, which was also more susceptible to clarithromycin, an antibiotic often administered in the treatment of a MAC infection. In the future, we pretend to proceed with these studies, trying to understand what are the features that make a good antimycobacterial peptide, combining both *in silico* and *in vitro* experiments.

Keywords: antimicrobial peptides, peptide synthesis, *Mycobacterium*

Resumo

A incidência de infeções por micobactérias não-tuberculosas tem aumentado, sendo que a infeção mais proeminente é causada por espécies do complexo *Mycobacterium avium* (MAC). *M. avium* é um patogénio oportunista, causando doença em indivíduos com doenças pulmonares crónicas e/ou imunossuprimidos. Após infeção, esta micobactéria é fagocitada pelos macrófagos do hospedeiro. No entanto, é capaz de escapar ao ambiente acídico do fagolisossoma através do bloqueio da fusão fagossoma-lisossoma, permitindo assim a captação de nutrientes e consequente crescimento. Com uma resistência intrínseca a antibióticos, o tratamento de uma infeção por MAC é complexo e com uma baixa taxa de sucesso, tendo como base a administração de vários antibióticos durante meses, até que a micobactéria não seja detetada no paciente durante um ano. Posto isto, é urgente encontrar novos agentes antimicrobianos que se apresentem como uma alternativa aos antibióticos convencionais.

Nas últimas décadas, os péptidos antimicrobianos (PAM) têm surgido como o mais promissor substituto dos antibióticos. Presentes em praticamente todos os organismos vivos, têm atividade antimicrobiana contra vírus, fungos e bactérias, como também propriedades anti-inflamatórias, anti-cancerígenas e regenerativas.

Como tal, este trabalho teve como objetivo testar vários PAM contra *M. avium*. Além disso, usando dados obtidos previamente de PAM com boa atividade contra *M. avium*, tentamos perceber quais são as propriedades estruturais e físico-químicas que determinam o efeito antimicobacteriano de um péptido. Recorrendo a ferramentas *in silico* que preveem a atividade antimicrobiana e toxicidade de PAM, três péptidos foram projetados, um foi sintetizado e testado contra *M. avium in vitro*.

Os resultados obtidos mostram que, péptidos com boa atividade contra bactérias Gram-positiva e Gram-negativa, não produziram qualquer efeito contra *M. avium* a crescer em culturas axénicas ou dentro de macrófagos. GOALA1, o péptido projetado *in silico* que foi baseado num péptido ativo contra *M. avium*, LFcin17-30, demonstrou baixa atividade antimicrobiana contra várias estirpes de *M. avium* a crescer axenicamente. Além disso, o péptido também demonstrou não possuir atividade contra *M. avium* a crescer intracelularmente. No entanto, o péptido foi ativo contra uma estirpe de *M. avium*, que também foi a mais suscetível à ação da claritromicina, um antibiótico administrado no tratamento de uma infeção por MAC. No futuro, pretendemos aprofundar estes estudos, tentando perceber quais são as propriedades que compõem um péptido antimicobacteriano, combinando as ferramentas *in silico* com experiências *in vitro*.

Palavras-chave: péptidos antimicrobianos, síntese peptídica, *Mycobacterium*

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List of abbreviations

NTM – Nontuberculous mycobacteria

MAC – *Mycobacterium avium* complex

SmT – Smooth transparent

SmOp – Smooth opaque

AIDS – Acquired Immunodeficiency Syndrome

SSTI – Skin and soft tissue infections

MDR – Multidrug resistant

MFS – Major facilitator superfamily

AMP – Antimicrobial peptides

HDP – Host defence peptides

ESKAPE group – *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter spp*

MIC – Minimum inhibitory concentration

IL-8 – Interleukin-8

LPS – Lipopolysaccharide

TNF-α – Tumor necrosis factor alfa

IL-6 – Interleukin-6

MHC – Major histocompatibility complex

SPPS – Solid-phase peptide synthesis

Fmoc – 1-(9H-fluoren-9-yl)-,ethoxycarbonyl

DMF – Dimethylformamide

DCM – Dichloromethane

eq – Equivalents

TFA – Trifluoroacetic acid

HPLC – High-performance liquid chromatography

ACN – Acetonitrile

ADC – Albumin-dextrose-catalase

PBS – Phosphate-buffered Saline

BMM – Bone marrow-derived macrophages

HBSS – Hank's Balanced Salt Solution

DMEM – Dulbecco's Modified Eagle's Medium

LCCM – L929 cells-conditioned medium

CFU – Colony forming units

OADC – Oleic acid-albumin-dextrose-catalase

IC_{50} – Concentration that inhibits by 50% the cellular viability

Introduction

Mycobacterium

Before the 20th century, infectious diseases constituted the main causes of death in humans.[1] A plethora of infectious diseases like leprosy, tuberculosis, smallpox, and cholera have all threatened the survival of entire populations throughout the past centuries. However, infectious diseases are not a matter of the past. While tuberculosis is still one of the top ten causes of death worldwide, other infections have seen a rapid uprise in prevalence over the last years, mainly due to increasing levels of bacterial resistance to antibiotics. Not only tuberculosis but also some other highly prevalent infectious diseases are caused by a genus of Actinobacteria, the *Mycobacterium*.[2]

Characteristics

Mycobacterium spp are acid-fast bacilli. These aerobic non-spore-forming bacteria[3] are characterized by their unique and complex cell wall. Its peptidoglycan is composed of N-glycolylmuramic instead of N-acetylmuramic acid, which is linked via a phosphodiester bridge to arabinogalactan, that, in turn, connects to mycolic acids **(Figure 1)**. A high content of lipids that can constitute up to 60% of the cell-wall weight makes it particularly unique. These lipids mainly consist of unique long-chain fatty acids that range between 60 and 90 carbons, the mycolic acids.[4,5]

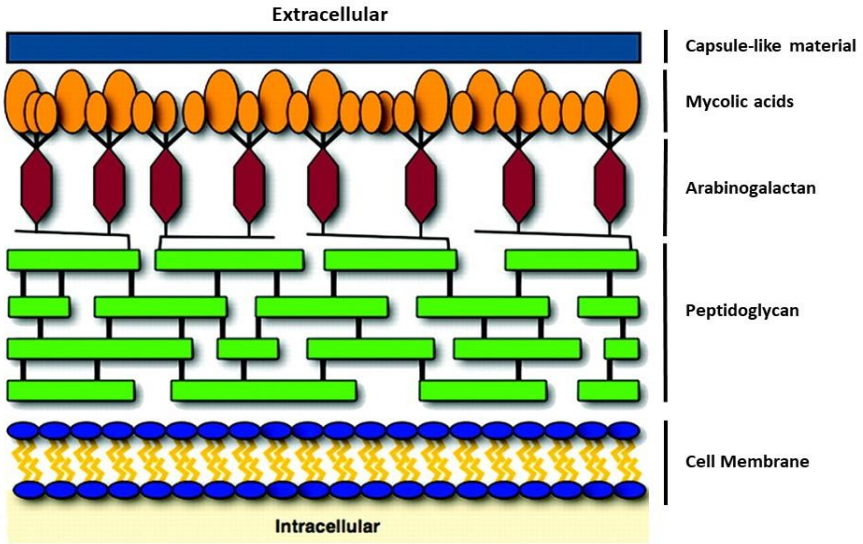

Figure 2. Mycobacterial cell wall. Schematic representation of the components of the mycobacterial cell wall and its organisation. Adapted from Hett *et al.* [6]

Coexisting in this lipidic environment provided by the mycolic acids are some other free lipids, cell-wall proteins, phthiocerol-containing lipids, lipomannan and lipoarabinomannan, with the latter being particularly important due to its wide spectrum

of immunomodulatory functions through binding to Toll-like receptors and physical insertion into membranes.[7]

The *Mycobacterium* genus includes several species capable of causing a variety of critical diseases, such as *M. tuberculosis*, responsible for tuberculosis, *M. leprae*, responsible for leprosy, and nontuberculous mycobacteria (NTM), responsible for various infections. NTM comprises mycobacteria species that do not belong to the *M. tuberculosis* complex and are also not *M. leprae*.[8]

NTMs are classified according to their growth rate in culture, which means they can be classified either as rapidly growing bacteria, like *M. abscessus*, which is able to form colonies in a solid medium within seven days or fewer, or as slowly growing bacteria, like *M. intracellulare* and *M. avium*, which are both enclosed under the same complex, *M. avium* complex (MAC), and require more than seven days to form mature colonies.[9] MAC colonies have more than one morphology, meaning that MAC strains have an isogenic variation. The most frequently seen in clinical isolates is the smooth transparent (SmT). However, environmental MAC strains can exhibit opaque variants, both smooth and rough (SmOp and ROp, respectively). A MAC colony that exhibits a SmT morphology is not only associated with increased virulence, in both chicken and mice[10], but also with higher resistance to antibiotics.[11]

Epidemiology

NTM cause various infections, with pulmonary infections being the most prevalent.[12] Despite not being as lethal as infections caused by *M. tuberculosis* or *M. leprae*, NTM infections have been increasing worldwide over the last three decades, even surpassing the rate of incidence of tuberculosis in some areas of the world.[13,14] This rapid increase in infections caused by NTM can be explained by an aging population, an increased prevalence of chronic lung disease, an augment of immunosuppressing diseases and treatments, and better detection techniques for the diagnosis of these infections. For instance, in Portugal, more specifically, at Hospital de São João in Porto, the number of NTM infections is increasing gradually each year, ranging from 52 isolates in 2008 to 137 isolates in 2012. Interestingly, the majority of the isolates found (58%) belong to MAC[15], in agreement not only with other studies conducted in Portugal[16] but also with global epidemiological studies.[17–19] Establishing the prevalence of this disease is hard because in most regions this is a disease that is not mandatorily reported to the authorities. Nonetheless, MAC is usually the most common NTM species isolated globally, ranging between 34% to 61% depending on the continent[17] and up to 55% in Portugal[20] **(Table 1)**.

Table 3. *M. avium* prevalence. Incidence of *M. avium* compared to other NTM across the different continents and Portugal, specifically.[17,20]

Continents	NTM	
	MAC	Others
Europe	34%	66%
Portugal	55%	45%
Asia	34%	66%
Africa	49%	51%
North America	51%	49%
South America	34%	66%
Oceania	61%	39%

Infections by M. avium

M. avium is often considered an opportunistic pathogen given its ability to cause disease in individuals with either a history of chronic lung disease or immunosuppression.[21] The important role that immunodeficiency plays in a MAC infection was first discovered at the beginning of the Acquired Immunodeficiency Syndrome (AIDS) pandemic, where a noticeable decrease of $CD4^+$ cells, <50 per mm^3, started to be associated with dissemination of NTM infections, more specifically, by MAC.[22]

There are four types of MAC infections: pulmonary, disseminated, MAC-associated lymphadenitis[23] and skin and soft tissue infections (SSTI). Despite the latter being more commonly associated to rapidly growing mycobacteria, all mycobacteria are capable to cause SSTI, including MAC.[24] Pulmonary infections are the most common disease caused by MAC, affecting mainly elderly women or people who have other lung-debilitating diseases, such as cystic fibrosis or chronic obstructive pulmonary disease. Disseminated MAC infections, as the name suggests, are infections that have spread throughout the body. A depleted immune system is required for the infection to disseminate; therefore, this type of infection is typically diagnosed in people with advanced AIDS. MAC-associated lymphadenitis is usually seen in young children that have a normal immune system and causes the swelling of the lymph nodes.[23] SSTI comprise a two-step process. Initially, colonization of a low number of bacteria happens in various regions of the skin, and as their number increases, bacterial invasion succeeds, followed by inflammation driven by the host response to the bacterial invasion.[25]

Despite some evidence of NTM being transmitted from person to person, namely *M. abscessus* among patients with cystic fibrosis,[26] there are no reports among patients with

pulmonary MAC. *M. avium* is commonly found in soil and water bodies, like rivers, lakes, and streams.[27] Moreover, given its lipidic cell wall, and thus, its hydrophobic nature, there is an innate enhancement of aerosolization potential, which translates into *M. avium* being most commonly found in showerheads. Therefore, inhalation of aerosolized NTM is characterized as one of the most common routes of NTM infection.[28]

Infection by MAC initiates either through the respiratory tract or through the gastrointestinal route,[29] the former being more common due to aerosols inhalation. Afterwards, the process of phagocytosis commences through the interaction of the mycobacteria with phagocytic receptors such as complement, mannose and type A receptors. In hosts with mycobacteria-specific antibodies, phagocytosis occurs via Fcγ receptors.[29] These receptors aggregate and initiate signalling pathways, through regulation of the actin cytoskeleton, that produce membrane protrusions for the pathogen to be involved. The mycobacteria are now enclosed in a vesicle that originated from the plasma membrane – the phagosome.[30] Usually, a phagosome undergoing maturation is characterized by progressive acidification and fusion with lysosomes, consequently forming the phagolysosome, structure characterized by its acidic composition and proteolytic enzymes that degrade invasive pathogens[31] **(Figure 2)**. However, *M. avium* disrupts the actin microfilament cytoskeleton, preventing both induction and activity of nitric oxide synthase[32] and delaying the movement of early endosomes, preventing the phagosome-lysosome fusion and inhibiting the acidification of the phagosome vacuole.[29] This protective mechanism prevents exposure towards a toxic low pH and lysosomal enzymes. Nonetheless, *M. avium* was shown to be capable of growing well in an acidified phagolysosome-like compartment (vacuoles containing *Coxiella burnetii*), displaying very robust characteristics that allow for survival in very aggressive conditions.[33] Moreover, the inability of *M. avium*-containing phagosomes to mature into a phagolysosome allows for the uptake of iron, required for its survival, since this nutrient localizes in cell membrane-derived vesicles (endosomes) with which the phagosomes can interact but the phagolysosomes not. Therefore, depriving the mycobacteria of its nutrients is essential to restrict its growth. Through chemokines signalling, such as IFNγ, the phagosome-endosome interaction is halted, and macrophages exert complete bacteriostasis over *M. avium*.[29]

Additionally, macrophages may also exert their antibacterial activity through mechanisms involving oxidative damage using hydrogen peroxide, nitric oxide and superoxide.[8] However, *M. avium* growing inside macrophages is resistant to the antimycobacterial effects of nitric oxide. As it happens, the effects of *M. avium* infection seem to be augmented due to a suppression of the immune system by the nitric oxide.[34] This is possibly explained by the fact that *M. avium* is a facultative intracellular pathogen

more commonly found disseminated in the environment, making it more adapted to aggressive conditions, providing for a possible explanation as to why *M. avium* is so resistant to antibiotics and disinfectants.

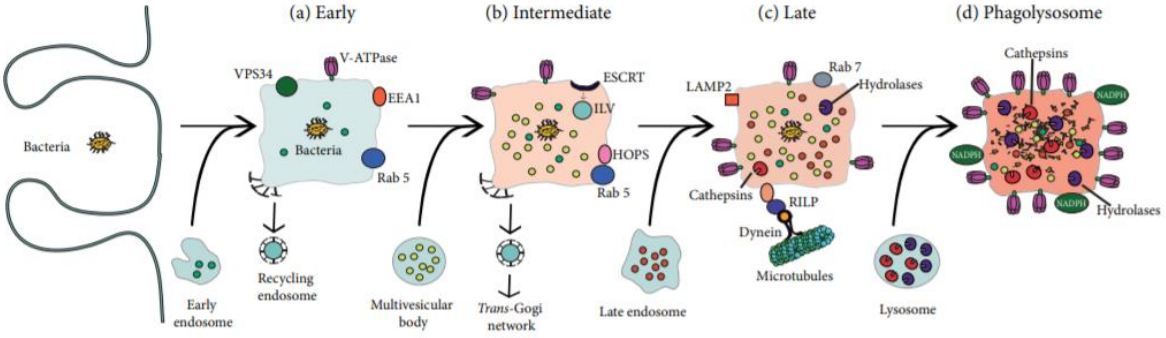

Figure 2. The process of phagosome maturation. Schematic representation of the various steps involved in a normal phagosome maturation inside the macrophage after phagocytosis of an invasive pathogen. *M. avium* halters the process of fusion between the late phagosome and lysosome, inhibiting its acidification while also disrupting the actin microfilament cytoskeleton. Adapted from Rosales *et al.* [30]

Drug resistance

Antibiotics

When bacteria cause disease, the usually prescribed treatment is antibiotics. Antibiotics are organic compounds that display antimicrobial activity. Their action resides in either killing bacteria or preventing their growth and are classified as bactericidal or bacteriostatic, respectively. There are various types of antibiotics, but they can be differently grouped depending on their target. **(Figure 3)**

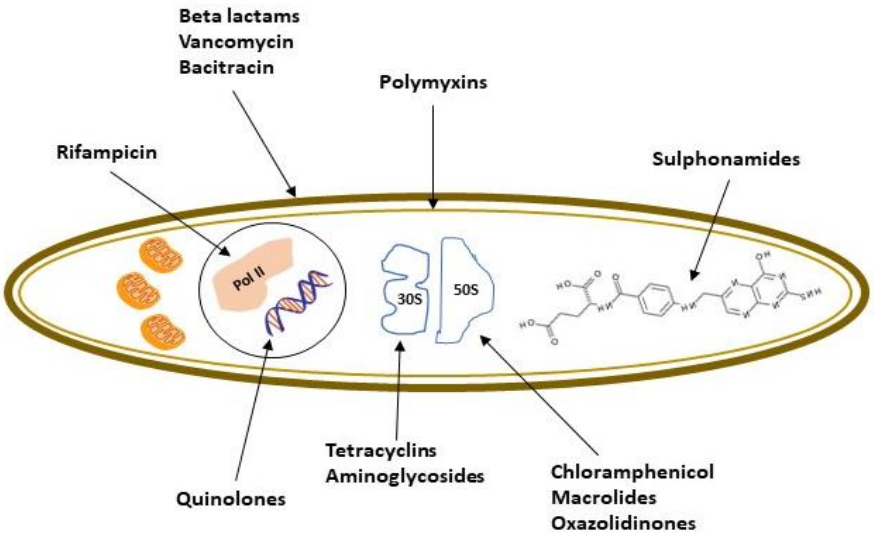

Figure 3. Antibiotics target sites. Schematic representation of the various groups of antibiotics and their respective target sites. Beta lactams, vancomycin and bacitracin act on the cell wall through interruption of its synthesis, polymyxins disrupt both outer and inner bacterial membranes after binding to LPS in gram-negative bacteria. Sulphonamides act as competitive antagonists against para-aminobenzoic, inhibiting the synthesis of dihydrofolic acid, a precursor of acid folic. Quinolones exert their antibacterial activity through inhibition of gyrase enzyme, responsible for nicking the double stranded DNA during replication. Rifampicin by binding RNA polymerase and consequently inhibiting it. Tetracyclines and aminoglycosides are both inhibitors of the 30S ribosomal subunit and therefore act as inhibitors of protein synthesis altogether. Chloramphenicol, macrolides and oxazolidinones act by inhibiting protein synthesis, however, they do it through inhibition of the 50S ribosomal subunit.[35–39]

The treatment of NTM infections demands the administration of various antibiotics, at the same time, for the patient to be cured. More specifically, MAC pulmonary infections require treatments based on 3 antibiotics administered on a three-times-weekly regimen, lasting until the patient is culture-negative on therapy for one year. The antibiotics usually administrated on this regimen are rifampicin (600 mg), clarithromycin (1000 mg) or azithromycin (500 mg) and ethambutol (25 mg/kg).[40]

Both clarithromycin and azithromycin are macrolides, which are very potent antibiotics given their inhibition of protein synthesis through the 50S ribosomal subunit. Rifampicin

is an antibiotic that, as previously mentioned, is highly effective against mycobacteria and acts through inhibition of RNA polymerase II. Ethambutol, however, is not classified into any of the prementioned groups of antibiotics. Administration of ethambutol is critical towards the success of the treatment.[41] It has a bacteriostatic activity, meaning that it is not capable of killing any bacteria on its own, it only inhibits their growth.[42] Its mechanism of action revolves around arabinogalactan and lipoarabinomannan, components specifically synthesized in mycobacteria, inhibiting their synthesis and, consequently, impairing the biosynthesis of the mycobacterial cell wall.[43] Therefore, ethambutol facilitates the uptake of the remaining antibiotics.

Intrinsic Resistance

Mycobacteria are intrinsically resistant to antibiotics. This resistance can be partially explained by the fact that NTM and *M. avium,* specifically, reside in soil and water, which means that they were driven over the years to develop various resistance mechanisms, to ensure their survival in these environments.[44]

A correlation between drug susceptibility and a SmT type colony has been previously described, where SmT colonies are more resistant to antibiotics than their opaque counterparts.[11] However, other studies demonstrate that there is an additional morphotypic switch. The so-called red and white colonies of MAC can be observed when *M. avium* is grown on a solid medium that contains the lipoprotein stain Congo red. Red colonies can change their morphotypic appearance to transform into white colonies. Independently operating from the opaque-transparent switch, this red-to-white morphotypic appearance switch is accompanied by an exacerbation of the resistance to antibiotics displayed by MAC colonies.[45]

The characteristic thick, hydrophobic cell wall that characterizes mycobacteria **(Figure 1)** is 10 to 20 times less permeable in NTM like *M. chelonae* when compared to *M. tuberculosis*.[44] This can be attributed to the environment where each mycobacterium exist. While *M. tuberculosis* is an intracellular pathogen that primarily infects macrophages,[46] this mycobacteria can only survive inside a cell and therefore does not have the resistance mechanisms to subsist in more hostile environments like the ones inhabited by NTM such as *M. chelonae* and *M. avium*. In fact, the mycobacterial cell wall acts as a permeation barrier for the entrance of antibiotics and other therapeutic agents. This is easily supported by the fact that the efficacy of an antibiotic is increased not only when the cell wall structure is affected by the occurrence of mutations but also when the culture is treated with Tween, a common detergent.[4]

The diffusion mechanism of these molecules is, naturally, dependent of its solubility. There are two available pathways: the hydrophilic and the lipophilic pathway. One could

expect that the characteristic low fluidity of the mycolic acids that compose the mycobacterial cell wall would slow down the import of compounds through the lipophilic pathway, however, lipophilic solutes should, naturally, be able to transverse any lipophilic membrane. The hydrophilic pathway is carried out by small cell wall proteins (59 kDa), porins, that have a channel diameter of only an estimated 2 nm. This pathway is very limiting as only compounds small enough can transverse these porins. Moreover, the number of porins that remain open and the channel length determine the transport velocity. Most importantly, when compared to the enterobacterial porins of *Escherichia coli*, which are an abundant component of its cell wall, these small proteins constitute a minor percentage of the cell wall composition. Furthermore, they also have a lower permeability than an equal weight of an *E. coli* porin.[47] These results can explain the low permeability of *Mycobacterium* to hydrophilic compounds, and, consequently, its natural resistance to antibiotics and other therapeutic agents.

Nonetheless, trespassing the mycobacterial cell wall is not the only barrier that drugs must surpass to kill or inhibit mycobacterial growth. Given their ability to pump outside of the cell any xenobiotic that is detected, efflux pumps are also a mechanism of intrinsic resistance displayed by NTM. While some are responsible for the extrusion of a very narrow range of antibiotics, many are non-specific and are responsible for the multidrug resistance (MDR) portrayed by mycobacteria. Efflux pumps can be divided into five different categories: major facilitator superfamily (MFS), ATP-binding cassette superfamily, small multidrug resistance family, resistance-nodulation-cell division superfamily and the multi antimicrobial extrusion protein family.[48] More specifically, the MFS family is found in various species of the *Mycobacterium* genus and when overexpressed, they confer pertinent levels of MDR to many mycobacteria.[49] This was corroborated by Machado *et al.* in 2015, where it was demonstrated that using an MFS efflux pump inhibitor boosted the antibiotic effect portrayed by macrolides.[50]

However, when antibiotics enter and successfully remain inside the cell, mycobacteria share resistance mechanisms with many other bacteria, such as polymorphisms of the antibiotics target gene or the induction of specific genes that modify the binding site of a certain antibiotic.[44] Lastly, mycobacteria are capable of directly modifying antibiotics that have entered the cell, either through enzymatic degradation or enzymatic modifications, such as the addition of chemical groups to the antibacterial compound, rendering them inactive or at least making them a less active and effective metabolite than its parent antibiotic.[49]

Nonetheless, this resistance is highly exacerbated by humans' antibiotics misuse and overuse not only in countries where there is no regulation but also in countries where antibiotics are regulated. Furthermore, over and incorrect prescriptions also contribute

to this problem. Adjemian *et al.* demonstrated that in the United States, out of 744 patients diagnosed with pulmonary NTM caused by MAC, only 13% of the antibiotic regimens prescribed by their physicians followed the 2007 ATS/IDSA guidelines. While 57% did not even include a macrolide (e.g azithromycin and clarithromycin), other patients (16%) were treated with a macrolide monotherapy. Other regimens were also noted, such as some alarming two-drug combination like macrolides with rifampin (13%) or macrolides alone with a fluoroquinolone (1%). The latter regimen constitutes a more dangerous problem since fluoroquinolone has no supporting evidence for its use in the treatment of a MAC infection and exacerbates the development of macrolide-resistant strains when this type of antibiotic is the only one used in the treatment of MAC-infections that has a correlation between *in vitro* susceptibility and clinical response.[51]

Overall, it may be said that resistance mechanisms are not only rapidly emerging but also spreading at a very fast rate, which is turning common infectious diseases that once were easily treatable into incurable deadly diseases.

Antimicrobial peptides

Heading into a “post-antibiotic era”, research towards finding a new antibacterial agent has become imperative. Since their discovery in the early 1980s,[52] antimicrobial peptides (AMP) have taken the role of being the crowning achievement of this research.

AMP are found in every living organism, from bacteria to animals and plants, and display direct antimicrobial efficacy against viruses, fungi, and bacteria. Not only that, but they also display anti-inflammatory, anti-cancer and wound healing properties.[53] Being part of the foundations of the immune system, AMP are essential in the combat of a given organism against various attacking pathogens, being present, usually, in the first line of defence.[54] Given its multifaceted biological properties **(Figure 4)**, AMP have been coined as host defence peptides (HDP).

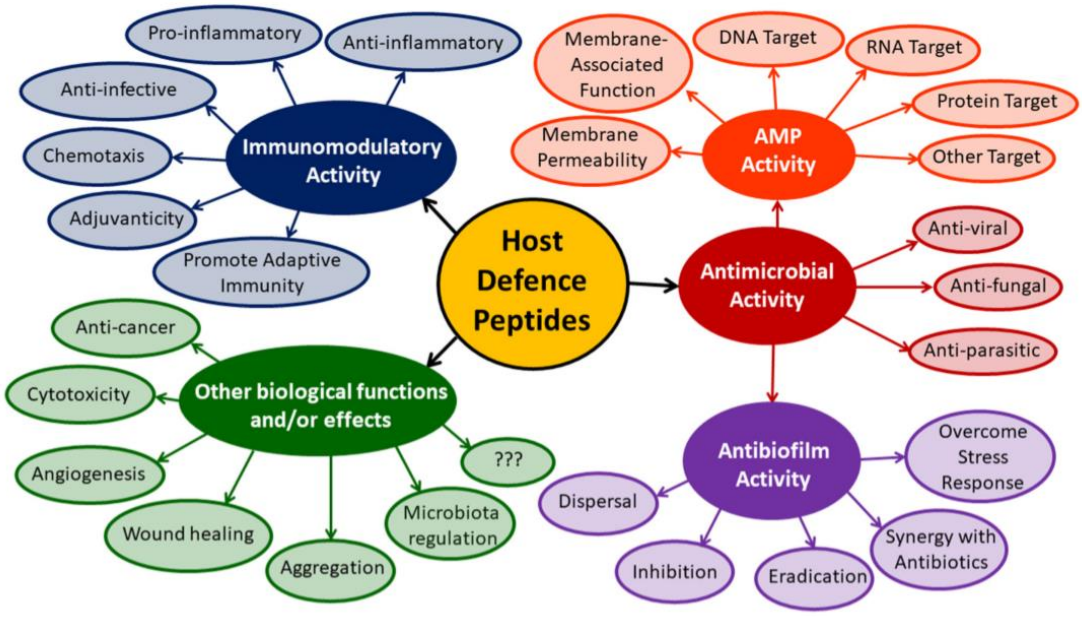

Figure 4. The many activities portrayed by HDP. Schematic representation of the various immunomodulatory, antimicrobial and antibiofilm activities as well as other biological functions. Adapted from Haney *et al.* [53]

Structural Properties

There are 20 amino acids that compose all naturally occurring peptides and proteins **(Figure 5)**. Each amino acid has an amino group, a carboxyl group, and a side chain. The side chain varies from amino acid to amino acid, and it is responsible for the specificity in terms of polarity, charge, and different interactions with other side chains of these peptide’s “building blocks”.

Because of this specificity, a change in a single amino acid of a peptide or protein can not only exacerbate, halter, or inhibit its function entirely, but also promote or impede interactions with other molecules as well as modify its structure. This is the case in sickle

cell anaemia, the most severe form of sickle cell disease. A single amino acid mutation in the β-globin subunit of the haemoglobin protein is responsible for the erythrocyte's deformation into a "sickle cell". The substitution of glutamic acid for valine at position 6 of the protein occurs and if it affects both β-globin chains, sickle cell anaemia follows.[55]

The primary structure of a given peptide is formed by the linkage of an amino acid α-carboxyl group to the α-amino group of another amino acid. This linkage happens through an amide bond, often called the peptide bond, which is kinetically stable. The primary structure is, therefore, the order in which the different amino acids are linked from the N-terminus to the C-terminus, being specific for each peptide or protein. Peptides have their structural properties like charge, hydrophobicity, polarity, and secondary/tertiary structure heavily influenced by their primary structure.

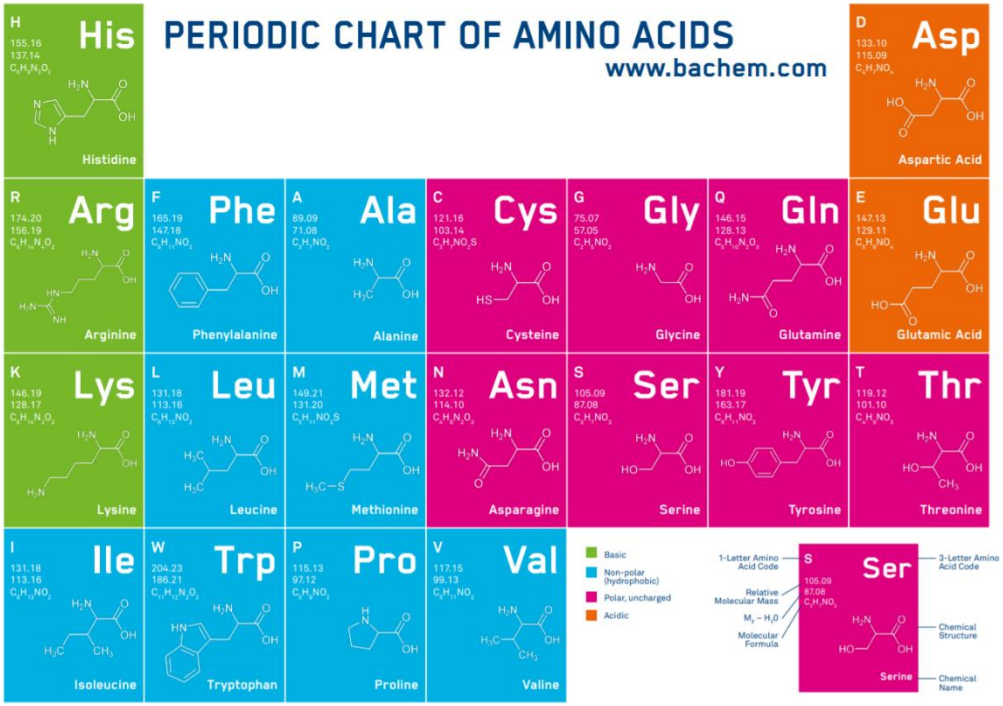

Figure 5. Periodic chart of amino acids. Schematic representation of the various naturally synthesized amino acids and their chemical properties. Adapted from www.bachem.com.

AMP have a short primary structure, up to 50-60 amino acids and are commonly unstructured in an aqueous solution. However, when in environments where either biological or mimetic membranes are present, they usually fold into an amphipathic conformation. An amphipathic conformation requires the presence of cationic and hydrophobic amino acids being separated into well-defined polar and non-polar regions. Concomitantly, AMP are typically cationic, with an overall charge of +2 to +9, and display a high concentration of non-polar residues (30% to 50%).[56,57]

There is a close correlation between charge and antibacterial activity of an AMP. While there are some reports of anionic AMP,[58] the majority of these peptides are characterized

by a global cationic charge. This property is thought to be crucial for an AMP's activity because a higher concentration of peptide accumulates at the membrane's surface when the AMP is cationic, given that the primary interaction of the peptide with the membrane is mostly electrostatic and that the membranous environments of AMP targets, such as bacteria and cancer cells, are anionic.[59] Nonetheless, peptides' charge is commonly associated with a key aspect, closely related to its toxicity – selectivity. Indeed, the viability of an AMP can be greatly impaired if its charge has surpassed a certain value, due to increased haemolytic activities.[60,61] There are structural differences between mammalian and bacterial membranes. In mammals, acidic phospholipids are usually located in the inner leaflets of the membrane while the outer leaflets are mainly composed of zwitterionic lipids. However, in bacteria not only are the outer leaflets more negatively charged due to a higher presence of acidic phospholipids, but their cell walls also contain several anionic molecules. This allows AMP to establish electrostatic interactions with more ease towards bacteria when compared to mammalian cells.[62]

Similarly, a high concentration of hydrophobic residues greatly contributes to a peptide's antimicrobial activity due to the peptide-membrane interaction. The hydrophobic residues not only allow the peptide to adopt an amphipathic structure in conjugation with cationic/polar residues but also controls the extent in which a greater portion of the peptide is able to partition into the membranous layer.[59] Much like the peptide's charge, an optimal concentration of non-polar amino acids is required for the peptide to have a low haemolytic activity without compromising its antimicrobial efficacy.[63]

A folded AMP typically adopts four different secondary structures to insert themselves into biological membranes: α-helical, β-sheet, extended and looped structures.[52] While α-helical and β-sheet are the most common and regular secondary structures, looped structures are an irregular class composed of diverse structures that serve the purpose of connecting, through turns, random-coils and strands, other secondary structures. Similarly, extended structures are also not regularly structured and are characterized by a high proportion of specific amino acids like arginine, tryptophan and proline.[64]

One could argue that an AMP needs to adopt a certain structure to exert its biological function. However, a study in 2007 demonstrated that aurein 2.2 and 2.3, natural cationic AMP with the same length, net charge and an amidated C-terminus, adopt an α-helical structure in membrane-mimetic environments. However, an analogue of aurein 2.3 with a carboxylated C-terminus does not possess any antimicrobial activity, despite adopting the same structure as its parental AMP,[65] raising the idea that part of an AMP activity cannot be solely attributed to one conformation that is more active than others. In fact, due to their small length, AMP have fabled flexibility, allowing them to have conformational plasticity. This ensures that AMP exert antimicrobial activity against a

wide range of microorganisms, either by killing them directly, modulating the immune response of the host, or both.[53] To understand better how AMP are able to exert activity, various molecular models have been used.[66]

Mechanisms of action – Membranous interactions

AMP interact with the membrane to permeabilize it, either killing the bacteria or facilitating the entrance of the AMP itself. This can be done by either receptor-mediated or non-receptor-mediated interactions.

The receptor-mediated mechanism is generally present in peptides produced by bacteria and requires a specific domain capable of recognizing and binding molecules present in the bacterial membrane, such as the lipid II molecule. This pathway is rarer than its counterpart.[66] Nisin is an antimicrobial peptide produced by Gram-positive bacteria (i.e, *Lactococcus* and *Streptococcus* species) that has been acting as a food preservative for the last several decades. However, reports have recently shown the efficacy of nisin against antibiotic resistance staphylococci, allowing for the possibility of using this AMP to treat SSTI — commonly caused by *Staphylococcus aureus*.[67]

The non-receptor-mediated is the pathway preferred by most vertebrate and invertebrate AMP and it is the one that, as previously mentioned, has electrostatic interactions as the driving force between the anionic membrane and the cationic peptide. These interactions, alongside the hydrophobic ones that occur simultaneously, allow for an accumulation of peptide on the membranous surface of the bacteria.[66] Various models try to explain the subsequent events after the peptide's adsorption onto the membrane, and many may occur simultaneously or in a sequential manner. However, there is a concentration dependant threshold that needs to be surpassed to set said events in motion. This concentration dependant threshold is highly variable and given the peptide's conformational plasticity, it is possible that its structure influences that threshold and consequently affects the mechanism "chosen" by a certain AMP. The peptide to lipid ratio, peptide concentration, the kinetics of all interacting molecules in the environment around both the peptide and the membrane and even the bacterial membrane composition are also factors capable of playing a key role that influences the determination of the threshold.[59] Of the many models that have been studied, the pore-formation and carpet-like mechanisms have been extensively studied and mostly used to describe AMP' mechanisms of action.

The pore-formation mechanisms involve 2 different pathways for the peptide to insert itself into the membrane: the "barrel-stave" and the "toroidal-pore" models. In the "barrel-stave" model **(Figure 6)**, a peptidical reorientation ensues so that the AMP perpendicularly insert themselves, promoting interactions like those seen in protein-ion

channels, spanning the membrane. The hydrophobic residues interact with the membrane lipids, while the hydrophilic residues constitute the lumen of the channels formed. It is, therefore, imperative for the AMP to adopt an amphipathic structure. However, only two known secondary structures partake in this mechanism, α-helical and β-sheet. If the peptide adopts an α-helical structure, a minimum length of twenty-two amino acids is required and if it adopts a β-sheet structure, a minimum of eight residues is mandatory.[59,66]

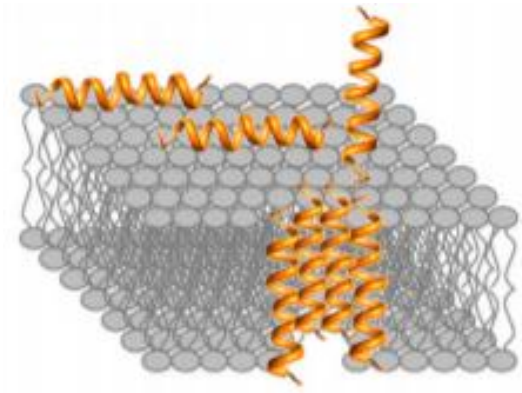

Figure 6. The "barrel-stave" model. Schematic representation of the "barrel-stave" mechanism, a pore-formation model that promotes the formation of a pore in the bacterial membrane, disrupting and leading to cell death. Adapted from Kumar *et al*.[66]

Much like the latter model, the "toroidal-pore", also called "wormhole" model, involves a perpendicular insertion of the peptide into the membrane **(Figure 7)**. However, lateral peptide-peptide interactions are not present. The pores are, instead, formed partially by both the peptides and the phospholipids' heads, meaning that the peptides, contrary to the "barrel-stave" model, still interact with the lipids' polar heads. In this model, AMP induce a local curvature in the lipid bilayer. The major difference from this to the previous "barrel-stave" model is that in the latter, the hydrophobic and hydrophilic disposition of the lipids is maintained after the AMP insertion, however, in the "toroidal-pore" model that very same disposition is disarranged. The positive curvature strain, alongside the disarrangement of the hydrophobic/hydrophilic disposition, destabilizes the integrity of the bacterial membrane, disrupting it and forming pores that allow for the leakage of cell components, effectively killing it. It is important to know that this model is much more transient than the "barrel-stave" mechanism since it is not as stable.[59,64,66]

Not all mechanisms of action involve forming pores in the membrane. Among the mechanisms that act through non-specific membrane interaction, the most studied pathway is the "carpet-like" model. In this model, after being initially attracted through electrostatic forces as well, AMP start to effectively cover the membrane in a parallel manner, much like a "carpet". There is no formation of any other structure in the

membrane and this “carpet-like” region starts to cause a change in the membrane energetics and fluidity, destabilizing it, causing a loss in its integrity. Consequently, a “detergent-like” effect follows, where micelles are formed.[59,66]

Evidence suggests that the “carpet-like” mechanism is a pre-requisite step necessary for the “toroidal-pore” model to take place since the “toroidal-pore” requires the AMP to arrange themselves parallelly before reorienting themselves to insert perpendicularly into the membrane. This consolidated model is called the “Shai-Matsuzaki-Huang” model **(Figure 7)**.[66,68]

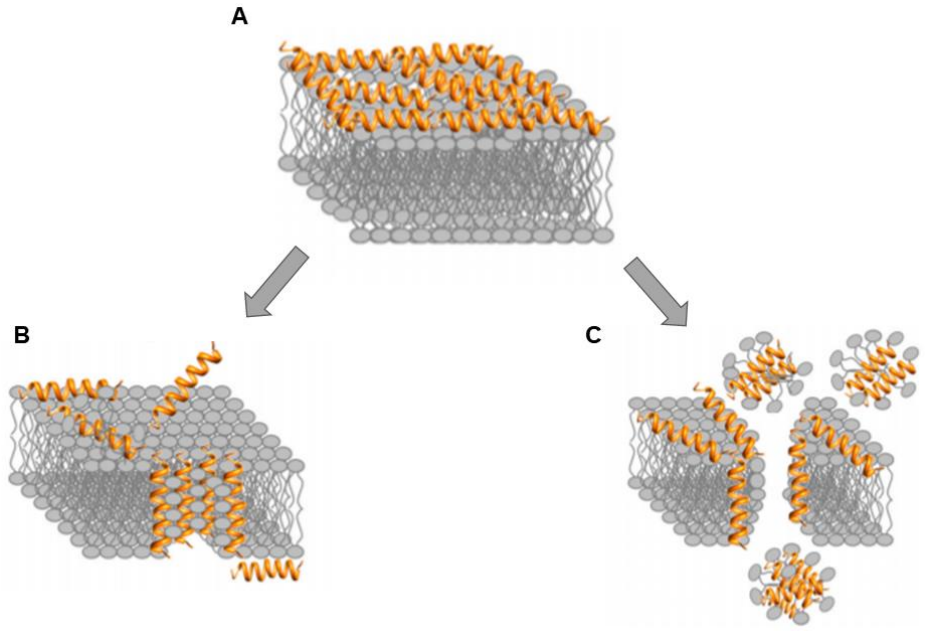

Figure 7. The “Shai-Matsuzaki-Huang” model. Schematic representation of the “Shai-Matsuzaki-Huang” model, a unified mechanism that comprises the “carpet-like” mechanism as a predecessor to both the “toroidal-pore” and “detergent-like” mechanisms. **A)** The “carpet-like” mechanism. The peptides parallelly align themselves with the membrane and start causing changes in its energetics and fluidity. **B)** The “toroidal-pore” mechanism. The peptides insert themselves perpendicularly into the membrane, but still, maintain interactions with the lipids’ polar heads. The pores formed promote leakage of cell components and lead to its death. **C)** The “detergent-like” model. The formation of micelles leads to disruption of the cell membrane and with consequent cell death. Adapted from Kumar *et al*.[66]

It is important to note that these mechanisms are not the only ones capable of killing bacteria. They have, however, been extensively studied and can be classified as structurally defined mechanisms. However, the majority require the peptide not only to adopt an α-helical structure but also to be long enough to span the membrane. AMP dispose of a whole plethora of mechanisms capable of provoking cell lysis or at least permeabilize its membrane, besides the ones mentioned above. Electroporation is one of those mechanisms, where a high concentration of peptide on the outer leaflet of the membrane allows its potential to go beyond a certain threshold, making it permeable for a short period, allowing the peptide to enter the cell.[64] “Interfacial activity” and “sinking raft”, are some of the other mechanisms that allow for small, unstructured AMP, unable

to span the bacterial membrane, to exert their antimicrobial function.[69] Moreover, many AMP are capable of binding to intracellular targets after translocating the membrane, displaying a varied arsenal of mechanisms. Localizing in the cytoplasm, AMP can inhibit not only DNA, RNA and protein synthesis, but also cytosolic enzymatic activity, proving once more the advantageous manoeuvrability that peptides offer when compared to antibiotics.[52,64]

Mechanisms of action – Non-membranous interactions

As previously stated, AMP have many properties besides being just antibacterial. While many share wound healing properties while being antibacterial,[70] others present activity against cancer cells.[71] However, many peptides are now being described and immunomodulatory peptides. These are AMP that do not exert antibacterial activity solely through disruption of the membrane. These immunomodulatory peptides are capable of exerting antibacterial activity through recruitment and regulation of other molecules important in the context of an infection. Given this broad spectrum of activities, AMP can also be designated as HDP.[53]

HDP are a component of the innate immune system since they are produced by the same cells that compose the first line of defence against invasive microorganisms and can act both in an autocrine and paracrine manner.[72] As a direct result, they are capable of various immunomodulatory activities, such as but not limited to, enhancement of phagocytosis, induction of autophagy and activation of signalling pathways such as extracellular signal-regulated kinases 1/2 and p38 mitogen-activated protein kinases. The most common and primary way that peptides modulate our immune system is through chemotaxis. Human neutrophil peptides and a cathelicidin antimicrobial peptide, LL-37, which have their direct antimicrobial activity dampened in the presence of ionic compositions similar to those of the human organism,[52] are all capable of recruiting different key players of the innate immune response such as monocytes, neutrophils, immature dendritic cells and eosinophils to the site of infection. Moreover, these peptides are also capable of stimulating chemokines such as interleukin 8 (IL-8), albeit in low concentrations, in both immune cells and structural cells. They are also known to up-regulate the expression of chemokine receptors. It is therefore evident that HDP play a crucial role in the immune cells' recruitment to the site of infection.[73] LL-37 is also known for inhibiting lipopolysaccharide (LPS)-induced secretion of tumor necrosis factor-α (TNF-α) and, similarly, human lactoferricin decreases the expression of LPS-induced interleukin-6 (IL-6), highlighting anti-inflammatory properties, reducing the risk of sepsis development.[74,75] HDP can also play a key role against the invasion of microorganisms by suppressing the apoptosis of neutrophils through the inhibition of caspase-3.[73]

Neutrophils play a key role in the immediate innate response of our immune system as they can phagocytize the invading microorganisms. Prolonging their lifespan, is, therefore, rather important during an immune response **(Figure 8).**

Additionally, HDP affect the adaptive immune system, a response that relies on memory and specificity to defend reoccurring infections through stimulation of B and T-lymphocytes. While a humoral response is led by B-lymphocytes, T-lymphocytes are in charge of the cellular response. This pathway involves the recognition of antigenic peptides, presented by major histocompatibility complexes (MHC). There are two types of MHC. While MHC-I bind peptides from proteins expressed by self-cells and consequently derived from the proteasome, MHC-II bind peptides expressed extracellularly or from self-cells degraded in the endosomal pathway.[76] Following bacterial phagocytosis, MHC-II molecules residing in lysosomes are presented with bacterial peptide fragments, product of the many proteases' action inside the lysosome. Moreover, autophagosomes, fusing with lysosomes, provide endogenous antigenic peptides to the MHC-II. MHC-II loaded with antigenic peptides are then transported to the cell surface in endolysosomal tubules, where they present the peptides to $CD4^+$ T cells of the immune system.[77] Once activated, these cells can secrete cytokines that regulate the immune response, leading the immune system to an enhanced response with each subsequent infection (**Figure 8**), highlighting the importance of peptides and how they are able to modulate the innate immune system response in a pathogen invasion.

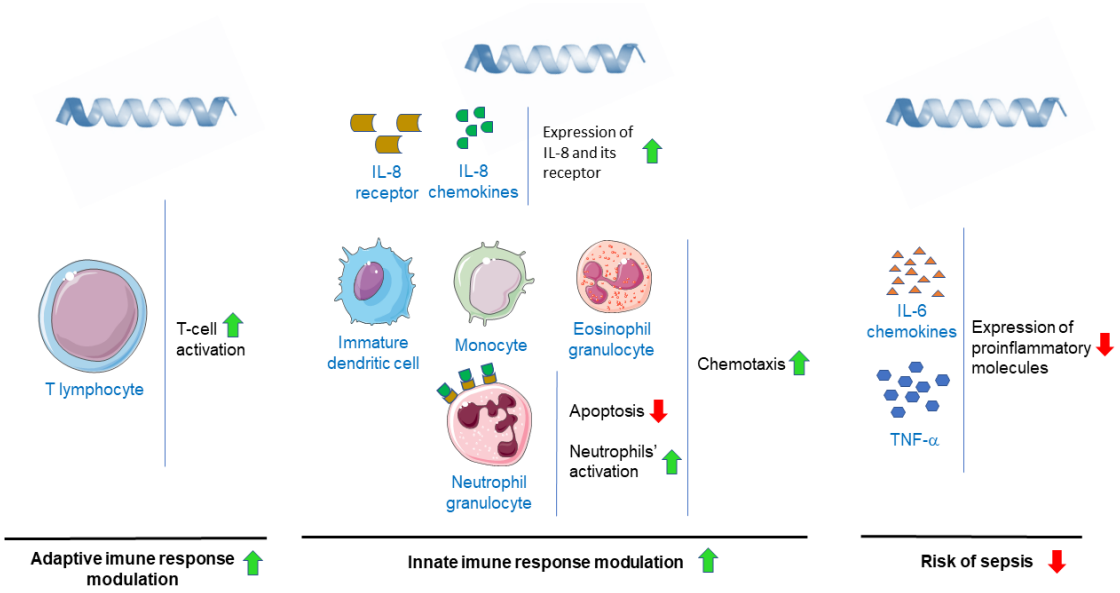

Figure 8. Immunomodulatory properties of HDP. Schematic representation of the various immunomodulatory properties that an HDP possesses. The modulation of the adaptive immune response comes from an interaction of MHC-II with antigenic peptides, inducing the activation of T-lymphocytes. Through upregulation of IL-8 and IL-8 receptors expression on the surface of neutrophils, HDP portray a higher activation of these important phagocytes, while, at the same time, reducing their apoptosis and increasing the chemotaxis of various cells of the innate immune response to the site of infection. It also reduces the risk of sepsis through downregulation of proinflammatory molecules such as IL-6 and TNF-α. Image constructed based on Mahlapuu *et al.*[57]

Ub2

Ub2 is a ubiquitin-derived peptide first identified by Kieffer *et al.* in 2003. It corresponds to the C-terminal sequence of the ubiquitin protein, comprising its last twelve amino acids – STLHLVLRLRGG.[78] With a reported optimal activity against mycobacteria, Ub2 possesses a minimum inhibitory concentration (MIC) of 5 μM against *M. tuberculosis* acting through membranous interactions.[79]

During an infection, the fusion of the phagosome with the lysosome facilitates the killing of the invading pathogen through both oxidative and non-oxidative mechanisms. Ubiquitin-derived peptides are the primary mediators of those non-oxidative mechanisms.[80] Alonso *et al.* reported that a lysosomal extract had antimycobacterial properties against *M. tuberculosis*. He later found that the ubiquitin itself, present in the lysosomal extract, did not have any antimycobacterial activity. However, as a consequence of the proteases' action present in the extract, its derived peptides, namely Ub2, had that type of activity.[79] The mechanism through which this peptide acts depends on the formation of a secondary structure involving a β-sheet, which is evident in the context of sodium dodecyl sulfate micelles and phospholipid vesicles. Ub2 inserts itself into the bacterial membranes, being capable of disrupting them to an extent where their internal contents are released.[81] A loss of membrane integrity exposes the bacteria to

adverse conditions in the lysosome, such as acidic pH and other bactericidal compounds while promoting cytoplasmic leakage over time, contributing to bacterial death. Being a short peptide, Ub2 is likely unable to span the membrane, so the mechanism through which it acts is most likely the micellar aggregate "detergent-like" model (**Figure 7C**). Nonetheless, Ub2 has also been shown to localize in the cytoplasm of the bacteria. Ub2 is also most efficient as an AMP when in a pH of 5.5-6, which is the same pH present in mycobacteria-containing phagosomes.[80]

"In-house" chimeric peptides

While membrane-lytic peptides can adopt various conformations and exert activity, the cationic and α-helical peptides represent the most abundant and well-characterized class of AMP.[82] Therefore, there has been a great interest in generating peptides with leucines and lysines, amino acids that increase the potential of forming α-helices (LK peptides).[83] Tryptophan residues are also important in AMP, allowing the peptide to interact with the membranous surface of the bacteria often increasing its activity through strong membrane-disruptive abilities.[84] Therefore, Kang *et al.* set out to synthesize *de novo* LK peptides with a single tryptophan at the critical amphipathic interface – between the hydrophilic ending site and the hydrophobic starting site of the helix. Out of twelve peptides synthesized, the most potent peptide had eleven residues, six leucines, four lysines and one tryptophan – KKLLKWLLKLL. Designated 3.1, this peptide was particularly potent *in vitro* against Gram-positive bacteria with a MIC of 1.6 μg/mL and between 3.1-25 μg/mL against Gram-negative bacteria.[85]

Drug resistance is of special concern in healthcare because patients are very prone to hospital-acquired infections (HAI), caused by MDR bacteria belonging to the "ESKAPE" group: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter* spp.[86] HAI not only hinders medical procedures but also the treatment of SSTI – worsening the severity of the skin wounds caused by SSTI. Some AMP have been linked with wound-healing properties in addition to their antibacterial activity. MSI-78 has even entered clinical trials in the treatment of skin injuries, namely diabetic foot ulcers.[70] Considering this, Gomes *et al.* hypothesized the formulation of a chimeric dual-action AMP that could not only treat the infection caused by the ESKAPE group but also treat the skin lesions through induction of collagen synthesis. Thus, the 3.1 AMP was coupled to pentapeptide-4 (PP4), a collagen-boosting peptide (amino acid sequence – KTTKS), whose palmitoylated form is widely used as a cosmeceutical known as "Matrixyl" (C16-PP4). Palmitoylated and non-palmitoylated hybrids were also synthesized.[87] While palmitoylated hybrids displayed higher levels of toxicity against human fibroblasts (HFF-1 cells), the non-

palmitoylated constructs were all active against both Gram-positive and Gram-negative bacteria with PP4-3.1 being more active than 3.1-PP4. Nonetheless, PP4-3.1 also proved to be more toxic. 3.1-PP4 was also able to maintain the collagen-boosting properties that C16-PP4 possessed in HFF-1 cells.[87]

PepH

PepH is an example of a peptide that can exert its activity through immunomodulatory activities. With an uprise of drug-resistant tuberculosis, Sharma *et al.* set out to discover new antimicrobial peptides based on a human α-defensin, human neutrophil peptide-1, given that its specific mode of action against mycobacteria had already been established.[88,89]

Using an *in silico* approach Sharma *et al.* analysed fifteen amino acid long peptide fragments of the defensin and chose one of those fragments based on overall charge and antimicrobial scores given by four different servers (AntiBP, AntiBP2, CAMP and AMPA). Out of sixteen fragments, PepH was chosen with an overall charge of +3 and the following sequence: RRYGTCIYQGRLWAF.[89]

PepH revealed antimycobacterial efficacy against *M. tuberculosis* H37Rv, having a MIC of 10 μg/mL. However, when tested against *M. tuberculosis* H37Rv growing inside human monocyte-derived macrophages, PepH revealed a higher antimycobacterial activity, with an increase in bacterial growth inhibition from 60% against mycobacteria growing *in vitro* to 91% against internalized mycobacteria at 5μg/mL .[89] Upon treatment with PepH macrophages had increased levels of interferon gamma and reactive nitrogen oxide species in their supernatants. Moreover, there was also a significant decrease in the pro-inflammatory cytokines TNF-α, IL-6 and monocyte chemoattractant protein-1, highlighting possible immunomodulatory properties.[89]

AMP challenges and future applications

Being coined as the perfect replacement to antibiotics ever since their discovery, crossing the bridge that separates AMP from being a therapeutical promising molecule to actually being commonly used in the clinic had been a winding path. Despite more than three thousand AMP being reported and well-characterized, only seven have been approved by the FDA, while many peptides fail before clinical trials.[90]

The primary reason is the susceptibility to proteolytic enzymatic activities. Pepsin, trypsin and chymotrypsin are all barriers that AMP need to surpass when administered orally since these enzymes operate throughout the digestive tract. If administered intravenously, the difficulties encountered will be no different, as there are many proteases present in the blood.[91] Moreover, an intravenous administration translates into

a shorter peptidic half-life due to hepatic and renal clearance.[57] Due to the presence of those proteases, many consider topical applications the most successful administration route for AMP.[91] Nonetheless, there are some strategies applied to prevent proteolytic degradation. Acetylation, acylation and formylation are three common modifications applied to the peptidic N-terminus.[91,92] On the other side of the peptide, amidation is the most common chemical modification applied on the C-terminus. Peptide cyclization is also a possibility, however, the typical conformational plasticity of an AMP would be lost. Another popular strategy is based around the incorporation of non-natural amino acids, such as N-alkylated, α,α-disubstituted glycines, β-substituted α-amino acids and β-amino acids or proline analogues, with the most common being the incorporation of D-amino acids instead of the naturally occurring L-amino acids.[92] The D-enantiomer retains the antimicrobial efficacy since the interactions with the bacterial membrane are not dependant on specific receptors.[91] In fact, in some cases, trading the L-amino acids for its isomeric counterpart increases its antimicrobial efficacy, as reported by Silva *et al.* in a study against *M. avium* using a shorter version of bovine lactoferricin.[93] Moreover, peptide stability can also be achieved through conjugation to a polymer or through transient modifications using a prodrug approach. Structural modifications are also possible through reduction of peptide bonds, substituting the α-CH group of the backbone for an isoelectronic nitrogen atom, synthesizing *retro-inverso* peptides (the normal sequence is reversed) or by synthesizing peptoids, where the side chains are annexed to the nitrogen atom of the backbone instead of the α-carbon.[92] Moreover, drug delivery systems, using different types of vehicles such as nanoparticles, liposomes or different gel formulations, have also been a strategy employed to reduce proteolytic degradation.[94] Nanoparticles allow for the protection of peptides, controlled plasma levels, a prolonged and/or controlled release and reduction of administration frequency. Consequently, all these advantages translate into lower toxicity for the host.[95] In 2016, Silva *et al.* were able to encapsulate a peptide into a self-assembling hyaluronic acid nanogel in aqueous environments with a high efficiency (70%), allowing for the treatment of *M. tuberculosis* infection, with a significant reduction of the lungs' bacterial burden of infected mice.[96]

Another challenge that researchers focused on AMP need to surpass is the undesired peptide toxicity towards eukaryotic cells. The haemolytic activity that a peptide displays, is, as previously mentioned, closely bound to structural characteristics like charge and hydrophobicity. Generally, an increase in the peptide's antimicrobial efficacy is accompanied by increased haemolytic activity.[61,63] However, some strategies can be applied towards reducing a peptide's toxicity, with some of them occurring in strategies applied to augment a peptide's stability. Changing the primary structure can reduce the

toxicity of a given peptide, however, since there is a direct compromise between primary structure and antimicrobial activity, changing the amino acids composition might not be beneficial towards potency, so an equilibrium must be found. Much like stability-increasing methods, cyclizing the peptide or perform a *retro-inverso* synthesis can be done towards lowering toxicity, as well as insertion of modified amino acids, especially D- amino acids. N-acetylation and amidation of the peptidic C-terminus are chemical modifications done to lower a peptide's toxicity. Amino acids lipidation is a modification that not only lowers the toxicity of a peptide but also increases its hydrophobicity index. Lipids can be added directly to the amino acids through a linker. Lastly, the addition of polyethylene glycol molecules to the peptide has also been found to reduce a peptide's toxicity without compromising its antimicrobial efficacy.[97]

Considering the broad range of activities that HDP possess, continuous large-scale production is required, especially since the bioavailability of those naturally synthesized is rather low. However, producing HDP synthetically has proved to be a slow and costly process, impairing the medical industrialization that HDP may require one day. Consequently, biotechnological approaches have been developed to enhance the production of HDP. Resorting to the insertion of recombinant DNA into specific vectors for the expression of the desired HDP provides with less cost and time wasted, while at the same time allows for easier scale-up. However, there is a downside of AMP-mediated inhibition of growth by the host and since the vectors are usually bacteria and yeast, post-translational modifications are not allowed. Using transgenic expression in plants, especially higher plants, involves fewer production costs, fewer risks of contamination, faster scale-up, synthesis of proteins with the correct folding and post-translational modifications. Chloroplasts have also been explored as bioreactors for AMP synthesis since they are organelles known to have their own genome and consequently, their own genetic system. Using chloroplast engineering allows for higher AMP expression when compared to the nucleus transformation of a single plant cell because there are numerous chloroplasts per cell.[98,99]

Overall, the broad spectrum of properties that peptides possess make them one of the best possible replacements for a new "post-antibiotic" era. Taking into consideration the high intrinsic resistance that *M. avium* possess and the increasing incidence of MAC infections all around the world, various research groups are now trying to tackle these mycobacteria exploring this approach.

Objectives

The aims of this study were:

1. Assess the antimycobacterial activity of existing peptides with reported activity against other bacteria, namely Ub2, “in-house” chimeric peptides and PepH;
2. Use previous knowledge on peptides with activity against *M. avium* to understand what are the key structure-activity relationships determining the antimycobacterial effect of a peptide;
3. Predict the activity and toxicity of peptides *in silico*, designing the best possible peptide, synthesizing and testing it against *M. avium*.

Methods

Peptides

In this work, several antimicrobial peptides (**Table 2**) were tested against various strains of not only *M. avium* but also *M. tuberculosis*. Ub2, PepH and GOALA1 were synthesized by me in Paula Gomes' lab at REQUIMTE/FCUP, while the remaining peptides were synthesized by others in the same group.

Table 2. Proper synthesized peptides used in this study.

Peptide	Sequence	Molecular weight (g/mol)
Ub2	STLHLVLRLRGG-OH	1320,8
PepH	RRYGTCIYQGRLWAF-NH2	1889
3.1-PP4	KKLLKWLLKLLKTTKS-NH2	1940,54
PP4-3.1	KTTKSKKLLKWLLKLL-NH2	1940,54
3.1	KKLLKWLLKLL-NH2	1394,9
PP4	KTTKS-NH2	526,7
C16-PP4	Palmitoyl-KTTKS-NH2	801,1
GOALA1	ALAKRWWAWRKKRL-NH2	1867,13

Peptides' synthesis

The synthesis of a peptide can occur through three different pathways: solution-phase synthesis, solid-phase peptide synthesis (SPPS) or a combination of both. In this work, the peptides' synthesis was carried through SPPS.

In SPPS, the peptides are synthesized from the C-terminus to the N-terminus of the sequence while anchored to an insoluble polymer through its C-terminus. This solid support consists of small resin beads. Given the attachment of the sequence to this solid support, excessive reagents and undesired side reaction products can be easily washed. The assembly occurs through successive addition of amino acids containing protective groups in a repetitive manner, often called synthetic cycles. The protective groups can be categorized into two different types: temporary groups that allow for protection of the α-amino group of a given amino acid, and "permanent" protecting groups that block side-chains interactions. The introduction of both temporary and "permanent" protective

groups is called orthogonal protection and it is the best strategy to avoid an untimely removal of the protective groups, which could cause the formation of undesired by-products. This orthogonal protection allows for milder overall reaction conditions. A synthetic cycle includes the following steps: cleaving the α-amino protecting group, washing the cleavage reagent, coupling of the following protected amino acid and washing the excessive material.

Ub2 was assembled through manual SPPS with the appliance of an orthogonal 1-(9H-fluoren-9-yl)-methoxycarbonyl)/*tert*-butyl (Fmoc/tBu) chemistry protecting group/scheme, using a Fmoc-Glycine Wang resin (0,79 mmol/g, 100-200 mesh, NovaBiochem). This resin was pre-conditioned using dimethylformamide (DMF, Fischer Scientific) for 20 min and dichloromethane (DCM, Fischer Scientific) for another 20 min before the removal of the Fmoc protecting group using a solution of 20% piperidine (Sigma-Aldrich) in DMF for 20 min. This reaction exposed the reactive glycine amine group bound to the resin. This deprotected group was coupled with the C-terminal Fmoc-protected amino acid using 5 equivalents (eq) of the Fmoc-AA-OH (Bachem), 5 eq of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU, Novabiochem) and 10 eq of N,N-diisopropylethylamine (DIEA, VWR). This coupling step was carried out for 1 hour. The remaining amino acids were sequentially coupled to the resin in a similar fashion of deprotecting and coupling cycles.

On the other hand, PepH and GOALA1 were assembled through an automated SPPS Symphony X synthesizer (Gyros Protein Technologies). Much like Ub2, an orthogonal Fmoc/tBu scheme was applied using a Rink Amide LL resin (0,33 mmol/g for PepH, 0,36 mmol/g for GOALA1, 100-200 mesh, NovaBiochem). This resin was pre-conditioned in DMF for 10min. Removal of the Fmoc protecting group followed by treating the resin twice with a solution of 20% piperidine in DMF for 5min. The C-terminal Fmoc-protected amino acid was coupled to the deprotected group twice for 10 min using 10mM of the Fmoc-AA-OH, 10 mM of the *in situ* coupling reagent O-(6-chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HCTU, NovaBiochem) and 200 mM of N-methylmorpholine (NMM, Sigma-Aldrich) in DMF. The remaining amino acids were sequentially coupled to the resin in a similar fashion of deprotecting and coupling cycles.

Peptides' purification

Once fully assembled, the peptides were released from the resin through a 2-hour acidolysis using a cocktail solution containing 87,5% trifluoroacetic acid (TFA, Alfa Aesar), 5% phenol (Sigma-Aldrich), 5% deionized water and 2,5% triisopropylsilane (TIS, Alfa Aesar). The nature of this cocktail, especially the presence of phenol, is due to the existence of arginines and histidines in the peptides' sequence.

The crude peptides that were obtained had their composition analysed through liquid chromatography-mass spectrometry (LC-MS) and were then purified through reverse-phase chromatography, using a preparative high-performance liquid chromatography (HPLC), more specifically, an Hitachi-Merck LaPrep Sigma system (VWR, Radnor) that is equipped with a LP3104 UV detector, a LP1200 pump and a reverse-phase C18 column (250x25 mm ID and 5 µm pore size Merck). A gradient elution composed of 0.05% TFA in water as solvent A and acetonitrile (ACN, Fischer Scientific) as solvent B was applied in a manner of 5% to 20% of ACN for Ub2, 25% to 40% for PepH and 20% to 35% for GOALA1. All elutions were completed in 60 min with a flow rate of 15 mL/min. Peptide purity was analysed through an analytical HPLC with a Hitachi Merck LaChrom Elite system equipped with a diode array detector and a thermostated automated sampler. Provided with a reverse-phase C18 column (150x4,6 mm ID and 5 µm pore size, Merck), the analyses were performed using a gradient of 1-100% of solvent B (ACN) in solvent A (0,05% TFA in water) for 30 min using a flow rate of 1 mL/min while being detected at 220 nm. Pure peptide fractions were then freeze-dried to produce the peptide as a low density white solid and then stored at -20ºC, until further use.

In silico predictors

In an attempt to design a more active antimycobacterial peptide, *in silico* tools were used, namely an antimycobacterial activity predictor (AntiTBPred), based on machine learning models, that uses physicochemical properties of reported antimycobacterial peptides to predict activity.[100] Host toxicity, predicted by ToxinPred,[97] was combined with the previous results, and the GOALA1 sequence was chosen for synthesis.

Bacteria

Mycobacterium avium 2447 SmT strain was provided by Dr. Françoise Portaels (Institute of Tropical Medicine, Antwerp, Belgium) and isolated from the bone marrow of an AIDS patient.

M. avium 2-151 SmT and SmOp variants were isolated from an AIDS patient and kindly provided by Dr. John Belisle (Colorado State University, CO, United States).

M. avium 25291 SmT was isolated from chicken and acquired from the American Type Culture Collection (ATCC).

Axenic assays

To evaluate the AMP' antimycobacterial activity against the different strains of *M. avium*, bacteria were grown in liquid culture medium using increasing peptide concentrations in

a 96-well plate. The mycobacteria's viability was then assessed through resazurin reduction.

An axenic culture of *M. avium* was expanded in Middlebrook 7H9 medium (BD Difco™) containing 0,05% of Tween 80 (Sigma-Aldrich) and 10% of Albumin-Dextrose-Catalase (ADC) supplement. The culture was incubated at 37ºC and the bacterial growth was monitored daily, through measurement of its optical density (OD) at 600 nm. Upon reaching the exponential phase, the inoculum was diluted 1:100 in 7H9/10% ADC, having a final concentration of approximately 10^5 CFU/mL. In a 96-well flat-bottom plate, each peptide was diluted successively 1:2 in 7H9/10% ADC. Posteriorly, the previously prepared 1:100 culture was seeded into each well and incubated with the peptides, making a final volume of 200μL. Non-treated wells and blank wells containing only medium were also included. Each condition was tested in triplicates. Then, the plate was incubated at 37ºC for six days. A solution of 2,5 mM resazurin (Sigma-Aldrich) in phosphate-buffered Saline (PBS) 1x was prepared and 10% (v/v) was added to each well. Resazurin is a blue oxidation-reduction indicator that is non-fluorescent until reduced to the highly fluorescent pink indicator that is resorufin. This simple non-destructive assay can be used to measure cell viability, since only metabolically active cells can reduce resazurin to resorufin, meaning that the level of resazurin reduction can be used as a measure of cellular viability in each well. The fluorescence of resorufin was measured, after incubation for 24 hours at 37ºC, at λ_{ex} = 530 nm and λ_{em} = 590 nm in a Synergy™ Mx microplate reader using the software Gen5.

GraphPad Prism 8 (GraphPad Software, LLC) was then used to analyse the obtained results. The results were calculated as the percentage of the fluorescence obtained in treated wells relative to the corresponding non-treated wells and expressed as percentage of mycobacterial viability.

Bone marrow-derived macrophages

Macrophages were used to assess both the viability and antimicrobial activity of the peptides. Bone marrow-derived macrophages (BMM) were obtained from both BALB/c and C57BL/6 strains of mice bred at the i3S animal facility.

The animals were euthanized by CO_2 inhalation. Posteriorly, the femurs and tibias were removed, and bone marrow cells were flushed with 10 mL of Hank's Balanced Salt Solution (HBSS, Gibco). The obtained cell suspension was centrifuged for 10 min at 259 G, 4ºC, and re-suspended in 4 mL of Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10 mM glutamine, 10 mM of 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid (HEPES) buffer, and 10% Fetal Bovine Serum (FBS, Biowest). They were then transferred to a cell culture dish containing 12 mL of complete

DMEM and 1,5 mL of L929 cell-conditioned medium (LCCM) as a source of Macrophage Colony Stimulating Factor (M-CSF). The cells were incubated overnight at 37ºC in a 7% CO_2 atmosphere to remove fibroblasts.

The non-adherent cells were collected, and the petri dish was washed three times with cold HBSS. The resulting cell suspension was centrifuged at 259 G, 4ºC for 10 minutes, resuspended with complete DMEM and had its cells counted with a Neubauer chamber. The concentration was then adjusted to $4x10^5$ cells/mL with complete DMEM plus 10% LCCM, and the cell suspension was plated on either 24-well plates (1mL per well) or 96-well plates (200 μL per well). The former was used to determine a given compound's antimicrobial activity while the latter was used to ascertain the compounds' toxicity against mammal cells.

The plates were then incubated at 37ºC in a 7% CO_2 atmosphere and, after four days, 10% of LCCM (v/v) was added to each well. On the seventh day after incubation, the culture medium was renewed.

10 days after the plate's incubation, the cells were fully differentiated into macrophages. The culture medium was removed and a bacterial suspension of *M. avium* 2447 SmT at $5x10^6$ CFU/mL previously prepared in complete DMEM was added to the adherent cells (200 μL per well). The plates were incubated at 37ºC in a 7% CO_2 atmosphere for four hours. Afterwards, the cells were washed four times with warm HBSS to remove extracellular bacteria and re-incubated with 1 mL of new complete DMEM/10% LCCM with or without different concentrations of each compound. Each condition was tested in triplicates.

The intracellular growth of *M. avium* 2447 SmT was evaluated by Colony Forming Units (CFU) assay, 5 days after infection, in the 24-well plates. The macrophages were lysed with 0,1% saponin (final concentration). The resulting bacterial suspension was then serially diluted in water containing 0,05% of Tween-80 and plated in Middlebrook 7H10 agar medium (BD Difco™) supplemented with 10% of oleic acid-albumin-dextrose-catalase (OADC). After a 7-day incubation at 37ºC, the number of colonies was counted and compared with the number of colonies present at time zero. Similarly, the intracellular growth of *M. tuberculosis* H37Rv was evaluated by CFU assay 3 days after infection, in 24-well plates. However, the resulting bacterial suspension after macrophage lysis and serial dilutions was plated in Middlebrook 7H11 agar medium (BD Difco™) supplemented with OADC. After a 30-day incubation at 37ºC, the number of colonies was counted and compared with the number of colonies present at time zero.

Macrophages cultured in 96-well plates and treated with increasing concentrations of the peptides tested herein had their viability determined by resazurin reduction. Four days after infection and treatment, a solution of 1,25 mM resazurin in PBS 1x was prepared

and 10% (v/v) was added to each well. After a 24-hour incubation at 37ºC, the fluorescence of resofurin was measured at λ_{ex} = 530 nm and λ_{em} = 590 nm in a Synergy™ Mx microplate reader using the software Gen5.

GraphPad Prism 8 was then used to analyse both the data obtained by resazurin reduction and by CFU count.

Results

Peptide synthesis

All synthesized peptides (Ub2, PepH and GOALA1) (**Table 2**) were successfully obtained in high purity degrees (≥ 98%) and presenting ESI-MS data (**Annex Figures**) in agreement with the expected molecular weights, as shown in the following **Table 3**:

Table 3. Synthesized peptides and respective analytical data. Data obtained by HPLC with a gradient elution of 1-100% ACN in water (0.05% TFA), for 30 minutes, at a flow rate of 1 mL/min (purity and retention time – R_t) and ESI-IT MS (detected m/z).

Peptide	Molecular weight (g/mol)	Purity (%)	Rt (minutes)	Detected m/z
Ub2	1320,80	98	10,8	$[P+H]^{1+}$ = 1322.53; $[P+2H]^{2+}$ = 662.27; $[P+3H]^{3+}$ = 441.67.
PepH	1889,00	98	12,3	$[P+H]^{1+}$ = 1889.87; $[P+2H]^{2+}$ = 945.60; $[P+2H+TFA]^{2+}$ = 1002.60 $[P+3H]^{3+}$ = 630.93.
GOALA1	1867,13	100	10,5	$[P+H]^{1+}$ = 1869.20; $[P+2H]^{2+}$ = 935.80; $[P+4H+2TFA]^{2+}$ = 1048.73; $[P+2H+TFA]^{2+}$ = 992.27; $[P+3H]^{3+}$ = 624.20; $[P+4H]^{4+}$= 468.47.

"In-house" chimeric peptides

A series of different AMP – 3.1, PP4, C16-PP4, 3.1-PP4 and PP4-3.1 – were previously synthesized in Paula Gomes' lab and tested against bacteria of the ESKAPE group, revealing interesting effects.[87] To test the activity of these peptides against bacteria of the *M. avium* species, we started by performing axenic assays with the 2447 SmT strain. Increasing concentrations of the peptides were incubated with *M. avium* 2447 SmT for seven days and mycobacterial viability was assessed by resazurin reduction.

The parental peptides 3.1 and PP4 behaved differently in terms of efficacy against *M. avium* 2447 SmT. The former displayed activity between 25 μM - 100 μM and inhibited 50% of bacterial growth at the highest concentrations tested (**Figure 9A**), presenting an IC_{50} of 76,3 μM (**Table 7**). The latter displayed no antimycobacterial activity (**Figure 9B**), much like its commercial form, C16-PP4 (**Figure 9E**).

The chimeric peptides 3.1-PP4 and PP4-3.1 displayed some activity against *M. avium* 2447 SmT in the same concentration range as the parental peptide 3.1. However, since they were incapable of inhibiting at least 50% of bacterial growth at any of the concentrations tested, these peptides are deemed ineffective against *M. avium* 2447 SmT (**Figure 9C and 9D**).

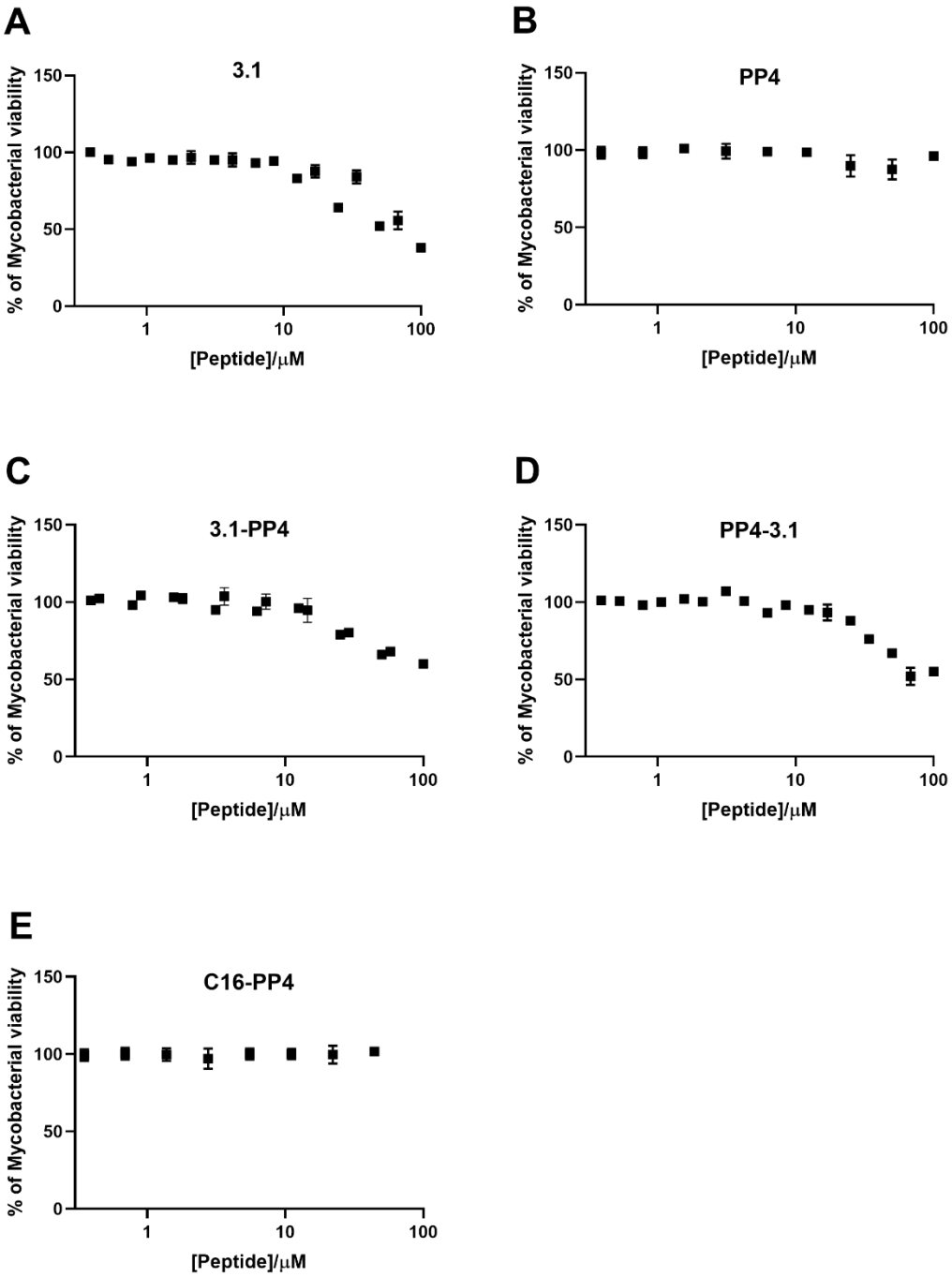

Figure 9. Antimycobacterial activity of the chimeric peptides. *M. avium* 2447 SmT was incubated with increasing peptide concentrations in a 1:2 manner, for 7 days at 37ºC. A) 3.1; B) PP4; C) 3.1-PP4; D) PP4-3.1; E) C16-PP4. The bacteria viability was measured by resazurin reduction. The graphs show the averages ± standard deviations of both four (A, B, C and D) and three (E) individual experiments, presented as percentages of viable mycobacteria relative to the non-treated mycobacteria.

Since, *M. avium* is an intracellular pathogen, residing mainly on macrophages upon infection, we proceeded to evaluate the activity of 3.1, PP4-3.1 and 3.1-PP4 on *M. avium* 2447 SmT growing inside BMM.

Firstly, the toxicity of these peptides was evaluated against the host cells, measured by resazurin reduction, to assess which peptide concentration range could be used in the BMM assay without affecting the macrophage's viability.

3.1, 3.1-PP4 and PP4-3.1 displayed toxicity towards the macrophages at high concentrations. While 3.1 and 3.1-PP4 had no toxicity until 10 μM (**Figure 10A and 10C**), PP4-3.1 proved to be the most toxic peptide out of the three (**Figure 10D**), having an IC_{50} of 8,4 μM (**Table 7**). 3.1 and 3.1-PP4 had IC_{50} of 15,4 μM and 20,0 μM, respectively (**Table 7**), meaning that 3.1-PP4 was the least toxic peptide out of the three that were tested.

Despite not having any activity against *M. avium* 2447 SmT in axenic assays, and, therefore, not being tested against BMM, the toxicity of PP4 and C16-PP4 was also tested to infer if any of these peptides were harmful towards the macrophages. No toxicity towards BMM was shown, up to 100 μM (**Figure 10B and 10E**).

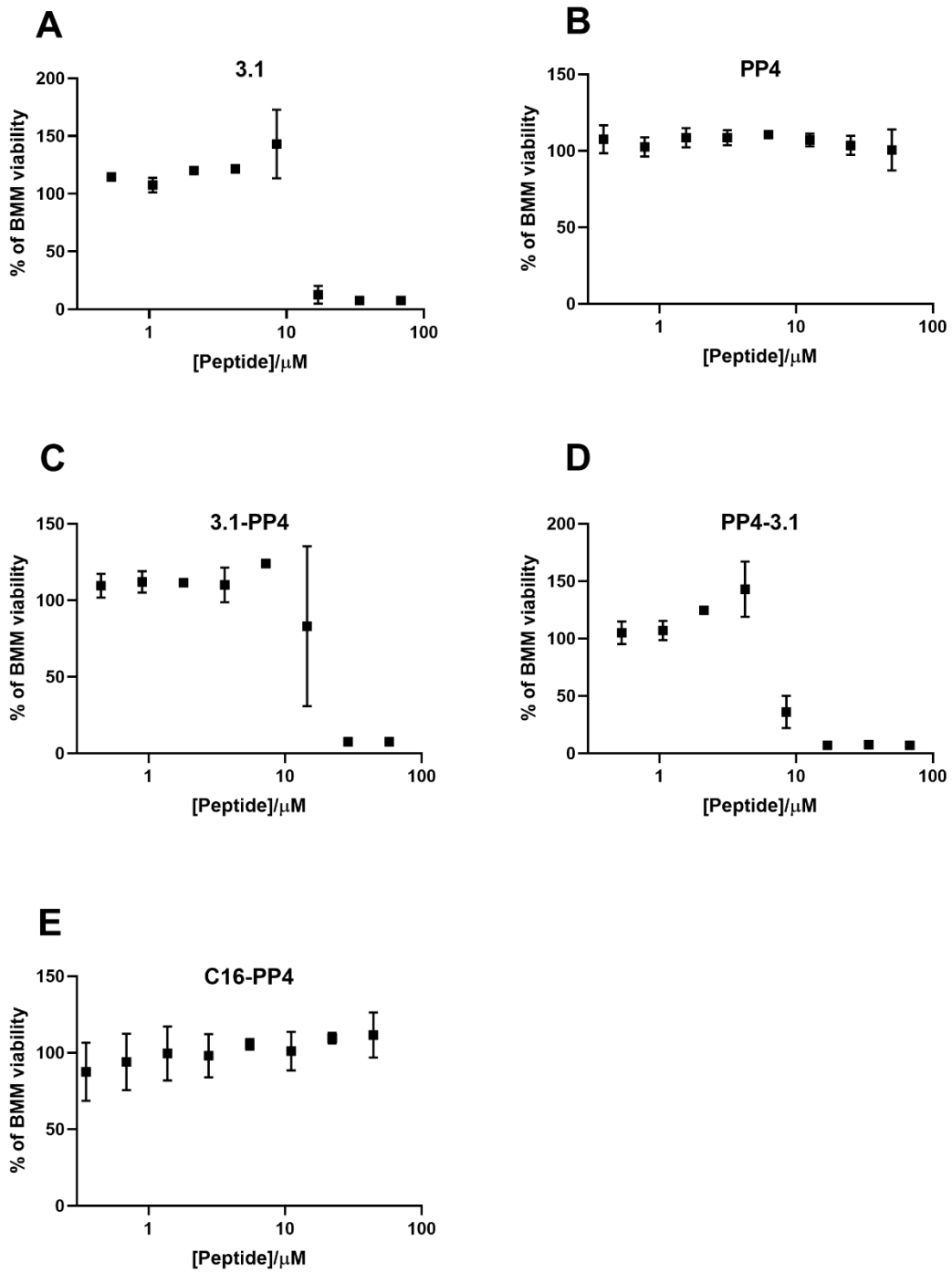

Figure 10. Toxicity of the chimeric peptides. BMM were incubated with increasing peptide concentrations in a 1:2 manner, for 7 days at 37ºC. A) 3.1; B) PP4; C) 3.1-PP4; D) PP4-3.1; E) C16-PP4. The bacteria viability was measured by resazurin reduction. The graphs show the averages ± standard deviations of two individual experiments, presented as percentages of viable macrophages relative to the non-treated macrophages.

We then moved towards testing the peptides against intracellular mycobacteria. The intracellular growth of *M. avium* 2447 SmT phagocytosed by BMM was evaluated by CFU assay, five days after infection. As depicted in **Figure 11**, none of the peptides tested, 3.1, 3.1-PP4 and PP4-3.1, displayed antimicrobial activity against *M. avium* 2447 SmT growing inside mouse macrophages.

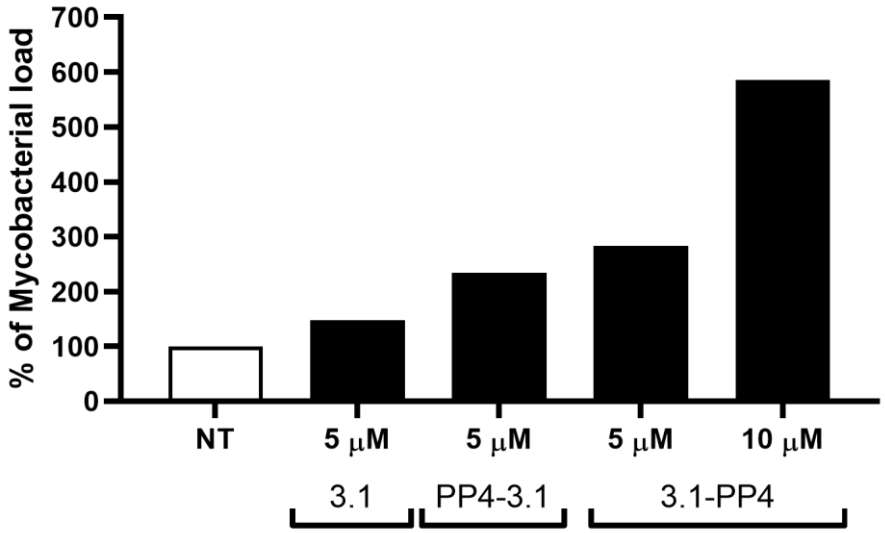

Figure 11. Antimycobacterial activity of 3.1-PP4, PP4-3.1 and 3.1 against internalized *M. avium*. BMM were infected with 1x10^6 CFU of *M. avium* 2447 SmT, and treated with 5 μM 3.1, 5 μM of PP4-3.1 and 5 and 10 μM of 3.1-PP4. Intracellular bacterial growth was evaluated by CFU assay 5 days after infection and treatment. The graphs show the average of one individual experiment, presented as percentages of mycobacterial colonies relative to the number of non-treated mycobacteria colonies.

PepH

Following the relatively negative results obtained with the first series of peptides, we next searched for an AMP with reported activity against mycobacteria. We found PepH, previously described as having antimycobacterial activity against *M. tuberculosis*[101]. We first tested PepH against *M. avium* 2447 SmT in an axenic assay. Despite having reported activity against *M. tuberculosis*, results presented here show that it did not affect *M. avium* 2447 SmT (**Figure 12A**).

Since PepH was more active against intracellular *M. tuberculosis* than *M. tuberculosis* growing axenically[101] it was decided to test the peptide against *M. avium* 2447 SmT growing inside macrophages. First, we tested its toxicity against BMM and found no toxicity up to 100 μM (**Figure 12B**). Regarding the activity against intracellular *M. avium*, we found that despite having no activity in axenic assays, PepH caused growth inhibition of 31,5% at 5 μM and of 45% at 25 μM (**Figure 12C**).

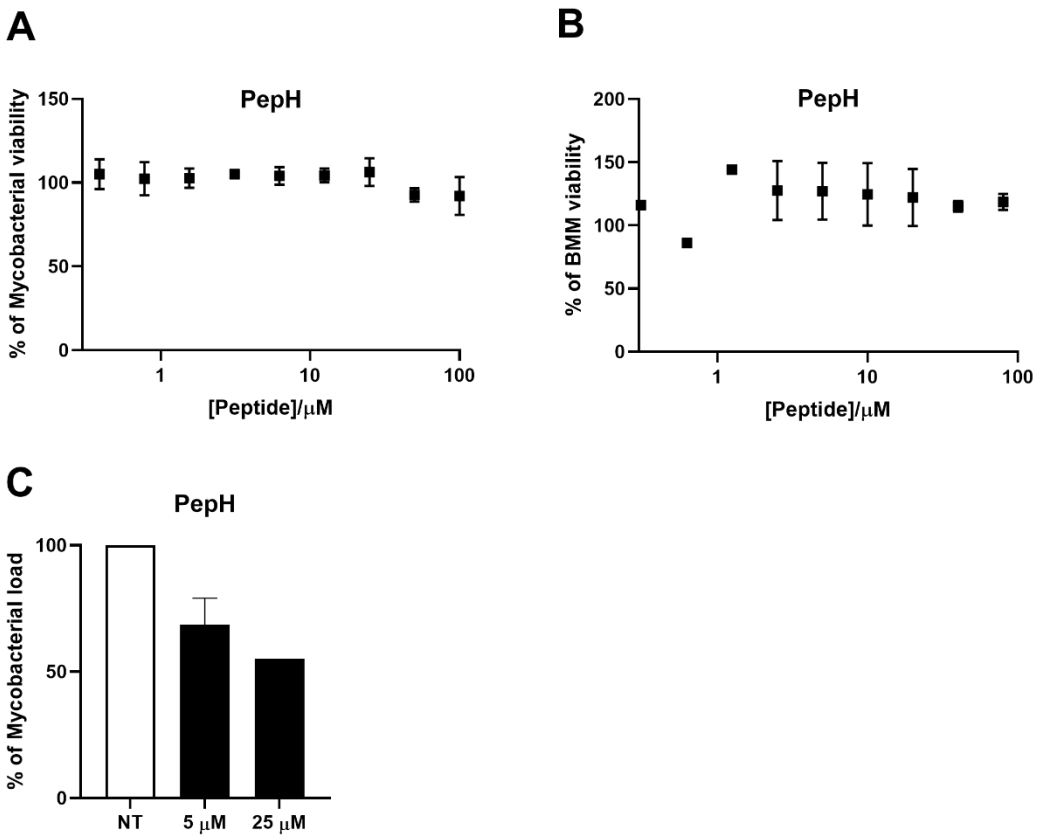

Figure 12. Antimycobacterial activity and toxicity of PepH. Both *M. avium* 2447 SmT (A) and BMM (B) were incubated with increasing peptide concentrations in a 1:2 manner, for 7 days at 37ºC. The bacterial and macrophage viability was measured by resazurin reduction. The graphs show the averages ± standard deviations of three (A) and two (B) individual experiments, presented as percentages of viable mycobacteria or macrophages relative to the non-treated cells. (C) BMM were infected with 1x10^6 CFU of *M. avium* 2447 SmT and treated with 5 μM and 25 μM of PepH. Intracellular bacterial growth was evaluated by CFU assay 5 days after infection and treatment. The graphs show the average + standard deviations of one (25 μM) and two (5 μM) individual experiments, presented as percentages of mycobacterial colonies relative to the number of non-treated mycobacteria colonies.

GOALA1 design

Previous work in our group reported a lactoferricin peptide with antimycobacterial efficacy against *M. avium* 2447 SmT strain, with an IC_{50} of 14,2 μM and an IC_{90} of 18,9 μM – LFcin17-30.[93] The low potency of all peptides tested up to this point, with 3.1 being the most potent, and the optimal efficacy of LFcin17-30 raised a question – why does LFcin17-30 possess such a good activity and 3.1 does not when it has been reported as such a good AMP against both Gram-positive and Gram-negative bacteria?[85]

Previous studies demonstrated that good antimycobacterial peptides typically possess some common characteristics. However, these are characteristics shared amongst most AMP, namely an overall positive charge allowing for greater interactions with bacterial anionic membranes, an amphipathic nature allowing greater conformational plasticity, and the presence of cysteines (C) that allow the formation of disulfide bridges, stabilizing the peptide.[102] However, a study by Usmani *et al.* allowed to identify two key characteristics commonly found in antimycobacterial peptides – specific peptidic composition and where certain amino acids localize in the peptidic sequence.[100]

In terms of peptidic composition, it was noticed that there is a common predominance of lysine (K), arginine (R), leucine (L) and tryptophan (W). However, the localization of these amino acids in the sequence seems to affect the potency of a given antimycobacterial peptide, more specifically, positions in both terminals of the peptide. There is a tendency for R to be inserted in positions one and four of the N-terminal and L in positions two, three and five. As for the C-terminal, L was more commonly situated in positions one, four and five, while R in two, and W in position three. With this information, we decided to compare LFcin17-30 with 3.1, considering these characteristics (**Table 4**).

Table 4. Head-to-head between LFcin17-30 and 3.1. Comparison between the amino acid sequence of the two peptides (peptidic sequence below their ID).

	3.1 KKLLKWLLKLL	LFcin17-30 FKCRRWQWRMKKLG
More basic		✓
More amphipathic		✓
More L	✓	
More R		✓
More W		✓
Presence of C		✓
N-terminal positions	L in position three	R in position four
C-terminal positions	L in position one, four and five	

As we can see in **Table 4**, LFcin17-30 shares many more characteristics with peptides that present greater antimycobacterial efficacy than 3.1 does, which is in agreement with their activities against *M. avium* 2447 SmT. Usmani *et al.* not only identified these characteristics but also built a predictor based on them, which allowed to design and predict the antimycobacterial activity of a peptide – AntiTbPred.[100] **Table 5** demonstrates that according to that very same predictor, LFcin17-30 is a better antimycobacterial peptide than 3.1, not only proving to be more amphipathic but also more basic, with a higher antimycobacterial score overall.

Table 5. Comparison between LFcin17-30 and 3.1. Key characteristics that the two peptides presented, calculated with the AntiTbPred predictor.[100]

Peptide	Score	Amphipathicity	Charge
LFcin17-30	1,68	1,40	6
3.1	1,64	1,33	4

Since this predictor could design a theoretically optimal antimycobacterial peptide, we decided to introduce mutations in LFcin17-30 to increase its score as an antimycobacterial peptide. These mutations are displayed in **Table 6.**

Table 6. Mutations applied to LFcin17-30 to increase its AntiTbScore. All mutations and their consequences in terms of score, steric hindrance, amphipathicity and charge of the peptide. All values are taken from the AntiTbPred predictor.[100]

Name	Peptidic sequence [a]	Score	Amphipathicity	Charge
LFcin17-30	FKCRRW**Q**WRMKKLG	1,68	1,40	6
	↓ a			
	FKCRRWAWR**M**KKL**G**	2,06	1,31	6
	↓ b			
	AKR**R**WAWRKK**L**L	2,04	1,53	6
	↓ c			
GO1	AKRWWAWRKKRL	2,25	1,53	6
	↓ d			
GOALA1	A**LA**KRWWAWRKK**R**L	2,40	1,31	6
	↓ e			
GOALA2	ALAKRWWAWRKKLL	2,52	1,14	5

[a] Amino acids in red were deleted from the sequence; In green were added; In bold were substituted.

Mutation "*a*" saw a substitution of a glutamine for an alanine. In *b* various mutations occur. Firstly, we have a substitution of the phenylalanine for an alanine and of the glycine for a leucine. Afterwards, we have the removal of the methionine and cysteine. Mutation *c* resulted in a substitution of one of the arginines in the N-terminal for a tryptophan. Additionally, a leucine in position two of the C-terminal was substituted by an arginine. Then, in mutation *d*, an alanine and a leucine were added, so that the peptide could have a leucine in the position two of the N-terminal. Lastly, in *e* the arginine previously added in *c* was substituted again by a leucine.

Having found three sequences that had a good compromise between AntiTbScore, amphipathicity and charge, we decided to evaluate *in silico* if these peptides would be toxic. For this purpose, we used a predictor based on the same model that AntiTbPred was, using a main dataset of 1805 toxic peptides.[103] If the peptide had a negative predicted score than it would be deemed by the predictor as non-toxic. **Table 7** demonstrates the prediction given by ToxinPred for the peptides.

Table 7. Toxicity prediction of the peptide chosen based on its AntiTbScore. All values are taken from the ToxinPred predictor.[103]

Name	Peptide	Score	Amphipathicity	Charge
GO1	AKRWWAWRKKRL	-0,91	1,53	6
GOALA1	ALAKRWWAWRKKRL	-0,87	1,31	6
GOALA2	ALAKRWWAWRKKLL	-0,92	1,14	5

We thus decided to firstly synthesize the peptide with the **ALAKRWWAWRKKRL** sequence, because it allowed a better compromise between antimycobacterial activity score, amphipathicity, charge and toxicity score – the peptide was named GOALA1. The peptide with the sequence **AKRWWAWRKKRL** was named GO1 and the peptide with the sequence **ALAKRWWAWRKKLL** was named GOALA2.

GOALA1 activity assessment

To assess the antimycobacterial activity of GOALA1, axenic assays were performed not only against *M. avium* 2447 SmT but also other strains of *M. avium* – 25291 SmT, 2-151 SmT and 2-151 SmOp – each with its different susceptibility and virulence, allowing to retrieve its antimycobacterial efficacy *in vitro.*

However, we firstly decided to assess the potency of the antibiotic clarithromycin against these strains. The IC_{50} values of clarithromycin against *M. avium* are already well established and assessing its potency in this project serves the purpose of controlling the susceptibility of the different strains used.

As expected, clarithromycin is highly potent as an antibiotic against all four strains of *M. avium* (**Figure 13**) and the IC_{50} of this antibiotic against all four strains is < 1 μM (**Table 8**). Nonetheless, the antimycobacterial potency is higher against the 2447 SmT and 2-151 SmOp strain (**Figure 13A and 13D**), with the latter having the lowest IC_{50} of all strains – 0,12 μM (**Table 8**).

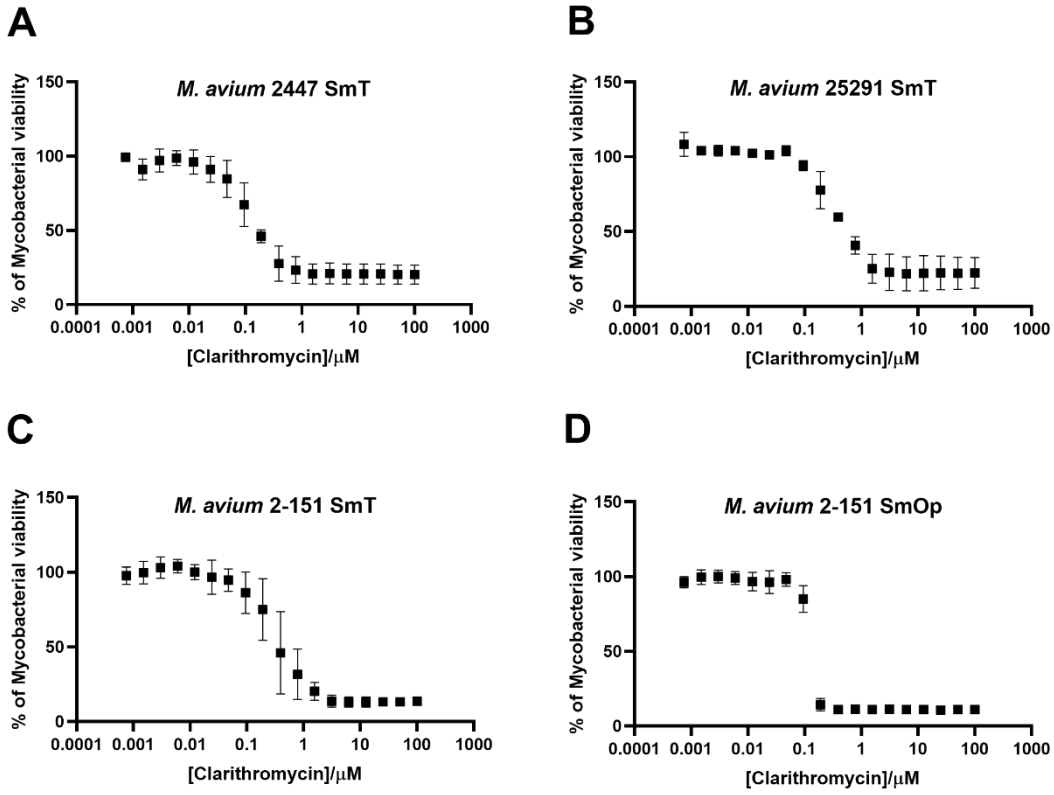

Figure 13. Antimycobacterial activity of clarithromycin. All *M. avium* strains were incubated with increasing antibiotic concentrations in a 1:2 manner, for 7 days at 37ºC. A) *M. avium* 2447 SmT; B) *M. avium* 25291 SmT; C) *M. avium* 2-151 SmT; D) *M. avium* 2-151 Op. The bacteria viability was measured by resazurin reduction. The graphs show the averages ± standard deviations of three individual experiments, presented as percentages of viable mycobacteria relative to the non-treated mycobacteria.

We then tested GOALA1 against the 4 strains of *M. avium*. GOALA1 did not inhibit more than 50% of the *M. avium* 2447 SmT growth in any of the concentrations tested (**Figure 14A**). *M. avium* strains 25291 SmT and 2-151 SmT were equally not susceptible to GOALA1 (**Figure 14B and 14C**) with inhibition of around 50% only at the highest concentrations. However, the peptide was more effective against the 2-151 SmOp strain, presenting activity at higher concentrations (above 25 μM) (**Figure 14D**). Despite not being optimal, GOALA1 presented an IC_{50} of 26 μM, the highest of all peptides assessed in this project (**Table 8**).

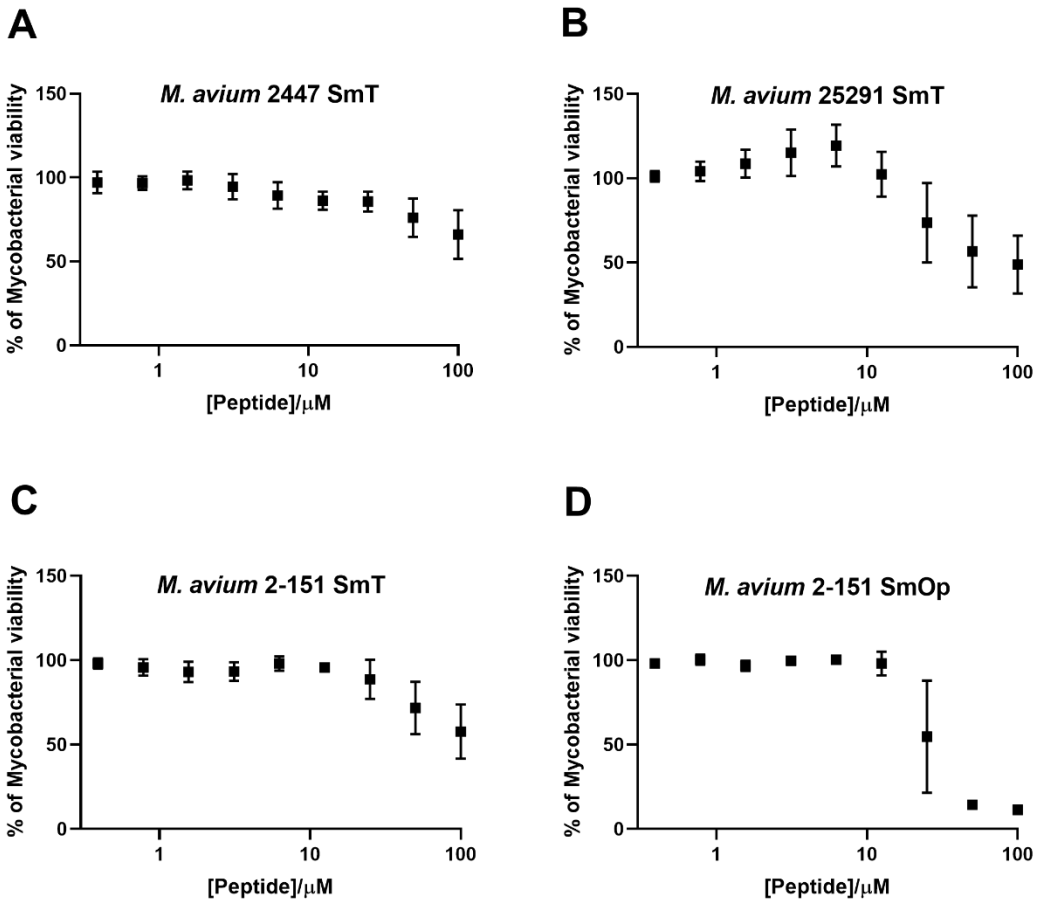

Figure 14. Antimycobacterial activity of GOALA1. All *M. avium* strains were incubated with increasing peptide concentrations in a 1:2 manner, for 7 days at 37ºC. A) *M. avium* 2447 SmT; B) *M. avium* 25291 SmT; C) *M. avium* 2-151 SmT; D) *M. avium* 2-151 Op. The bacteria viability was measured by resazurin reduction. The graphs show the averages ± standard deviations of both six (A) and three (B, C and D) individual experiments, presented as percentages of viable mycobacteria relative to the non-treated mycobacteria.

We also tested GOALA1 against *M. avium* inside BMM. Contrary to PepH, GOALA1 did not display any antimycobacterial efficacy against *M. avium* 2447 SmT growing inside BMM, at any of the concentrations tested (**Figure 15**).

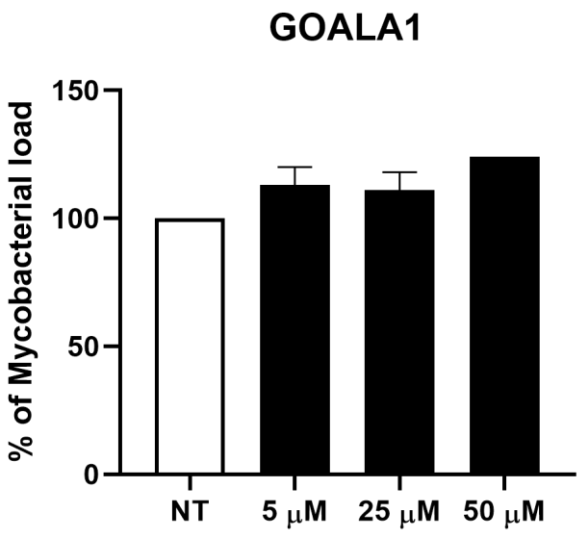

Figure 15. Antimycobacterial activity of GOALA1 against internalized *M. avium*. BMM infected with *M. avium* 2447 SmT was treated with 5, 25 and 50 μM of GOALA1. Intracellular growth was evaluated by CFU assay 5 days after infection and treatment. The graphs show the averages + standard deviations of one (50 μM) and two (5 and 25μM) individual experiments, presented as percentages of mycobacterial colonies relative to the number of non-treated mycobacteria colonies.

Since many peptides that compose the database used to predict the activity of GOALA1 were based on AMP that were tested against *M. tuberculosis*, we decided to assess the activity of this peptide against it. Concurrently, PepH was also tested, despite already being previously tested by Sharma *et al.*[101] To that purpose, the intracellular growth of *M. tuberculosis* phagocytosed by BMM was evaluated by CFU assay three days after infection. As depicted in **Figure 16**, GOALA1 can be deemed as an ineffective peptide against *M. tuberculosis* since it demonstrated no antimycobacterial activity against the bacteria at 5 μM. Even at 25 μM, the bacteria inside macrophages treated with the peptide were capable of growing almost as much as the bacteria growing inside the non-treated macrophages. On the other hand, PepH, as expected, was active against *M. tuberculosis*. However, in this assay, the peptide showed to be less efficient than Sharma *et al.* had assessed, with the bacteria incubated with the peptide being capable of displaying a 30% inhibition of growth at 25 μM and 46% at 5 μM.

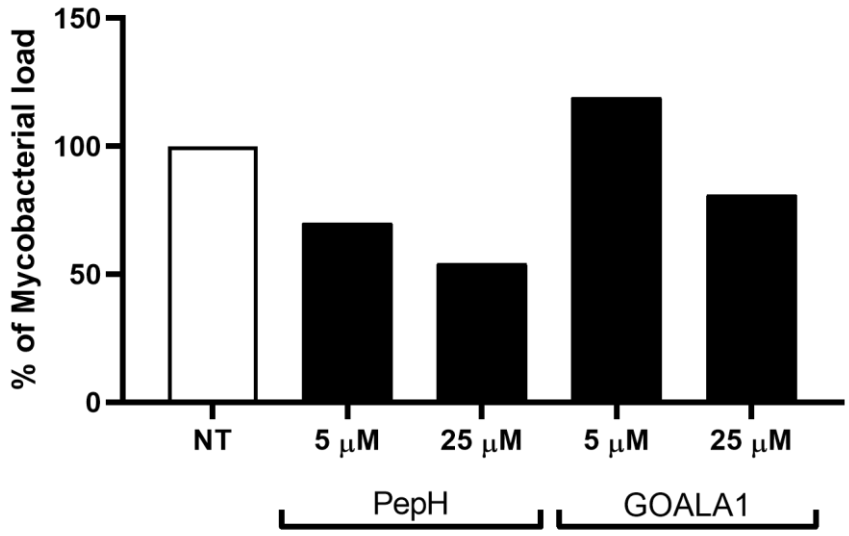

Figure 16. Antimycobacterial activity of PepH and GOALA1 against internalized *M. tuberculosis*. BMM infected with *M. tuberculosis* H37Rv was treated with 5 and 25 μM of PepH or GOALA1. Intracellular growth was evaluated by CFU assay 3 days after infection and treatment. The graphs show the averages of one individual experiment, presented as percentages of mycobacterial colonies relative to the number of non-treated mycobacteria colonies.

IC_{50} of all compounds

Table 8 summarizes the activity of all compounds tested in this project against the four different strains of *M. avium* and toxicity towards macrophages. For the cases where there are no values of IC_{50} presented, the inhibitory effect of the compound was not enough to inhibit 50% of bacterial growth or macrophage viability until 100 μM.

Table 8. IC_{50} of compounds. Summarized results of all IC_{50} (compound's concentration that inhibits by 50% either the macrophage's or the mycobacteria's viability) in μM.

	IC_{50}				
	BMM	*M. avium*			
		2447 SmT	2-151 SmT	2-151 SmOp	25291 SmT
3.1	15,4	76,3	nd	nd	nd
3.1-PP4	20,0	>100	nd	nd	nd
PP4-3.1	8,4	>100	nd	nd	nd
Clarithromycin	nd	0,16	0,38	0,12	0,51
GOALA1	nd	>100	>100	26,0	>100

nd – not determined; >100 – IC_{50} not determined in the range of concentrations tested

Discussion

Conjugating an increasing resistance towards antibiotics displayed by bacteria throughout the years with a harsh regimen of antibiotic administration, this project aimed to find and design new possible therapeutics to help in the treatment of mycobacterial infections, namely those by *Mycobacterium avium* complex.

The first step towards this objective relied on testing the Ub2 peptide, which already had been proven effective against *M. tuberculosis*.[79] However, despite a successful manual synthesis (**Annex Figures 1 and 2**), we failed to test this peptide due to solubility problems. Noticing that the peptide would form a precipitate when added to 7H9 supplemented with ADC, we found out through chromatography analysis of both the supernatant and the precipitate that the peptide would conjugate itself with the albumin present in ADC, making it impossible to test. Since Ub2 was synthesized with a free C-terminal, It would be interesting to synthesize Ub2 with an amide C-terminal to infer if that is the reason why it is conjugating with albumin.

The chimeric peptides followed. As expected, the results displayed by both 3.1 and PP4 against *M. avium* 2447 SmT growing axenically, were very different. Both PP4 and its commercial palmitoylated form, C16-PP4, displayed no activity against *M. avium* (**Figures 9B and 9E**). Being capable of inducing collagen synthesis, this peptide was not supposed to have antibacterial activity. On the other hand, 3.1, with reported high activity against Gram-positive and Gram-negative bacteria,[85] displayed activity against *M. avium* as well (**Figure 9A**), albeit presenting a high IC_{50} of 76,3 μM (**Table 8**). Both chimeric peptides also displayed activity against *M. avium 2447* SmT. However, contrary to what Gomes *et al*. had assessed against other bacteria,[87] both 3.1-PP4 and PP4-3.1 were less active than 3.1 against this strain of *M. avium* (**Figures 9C and 9D**), with both chimeras incapable of inhibiting 50% of the mycobacterial growth. Nonetheless, much like the results reported by Gomes *et al.*[87] PP4-3.1 proved to be slightly more effective than 3.1-PP4, evidencing once more the importance of peptide orientation in peptidic conjugates.[87,104–106]

Selectivity is one of the most important factors for this new generation of antibacterial compounds. This means that an ideal compound would exert microbicidal activity against the pathogen of interest without compromising the integrity of the host cell. In this case, since we are working with *M. avium*, the peptides tested need to have a high antimycobacterial activity without being toxic towards macrophages, since phagocytosis of the bacteria is a required step in *M. avium* infection. PP4-3.1 seems to be the most toxic peptide towards BMM (**Figure 10D**), with an IC_{50} of 8,4 μM (**Table 8**), which is in accordance with the findings reported earlier where 3.1-PP4 was the least toxic peptide of the two chimeric forms.[87] However, contrary to what has been reported, 3.1 was more toxic than 3.1-PP4 (**Figures 10A and 10C**)(**Table 8**). This difference in toxicity is due to

the type of cells used in both toxicity assays. While bone marrow-derived macrophages were used in this study, Gomes *et al.* used a human cell line of fibroblasts, HFF-1 cells. Overall, the toxicities reported in this study for 3.1, 3.1-PP4 and PP4-3.1 were lower than those reported by Gomes *et al.*,[87] and PP4 and C16-PP4 displayed no toxicity in either study (**Figures 10B and 10E**).

Having presented activity against axenically growing *M. avium* 2447 SmT, 3.1, 3.1-PP4 and PP4-3.1 were tested against *M. avium* 2447 SmT previously internalized by bone marrow-derived macrophages. Results appear to indicate that neither of the peptides tested displayed any kind of activity (**Figure 11**). However, only one experiment was performed, meaning that the inefficiency of the peptides can not be concluded with certainty. A possible justification for this lack of activity relies on the inability of the peptide to cross the cytoplasmic membrane of the macrophage and/or the phagosome membrane. This inability can be explained based on the structural differences observed between mammalian and bacterial membranes, more specifically, the localization of the acidic phospholipids. In bacterial membranes, the outer leaflet is more negatively charged due to a higher presence of acidic phospholipids, while in mammalian cells, these are usually located in the inner leaflet. Therefore, it might be harder for the 3.1, 3.1-PP4 and PP4-3.1 to establish electrostatic interactions with the mammalian membranes of the BMM.[62] In addition to the lack of antimicrobial activity, abnormal bacterial growth was also observed in the presence of 10 μM of 3.1-PP4 (**Figure 11**). This possibly reflects an extracellular growth of the bacteria due to macrophage death, since, at 10 μM, 3.1-PP4 exhibited some toxicity towards the host cells (**Figure 10C**). Extracellular bacteria can grow at a faster rate than the ones internalized. Nonetheless, to confirm this hypothesis, a CFU assay of the macrophages' supernatants separated from the cells' lysate is required.

With a very high antimycobacterial activity against *M. tuberculosis,*[101] PepH was a promising candidate against *M. avium*. To assess that, the peptide was tested against axenically growing *M. avium* 2447 SmT. As depicted in **Figure 12A**, the peptide displayed no activity. Taking into consideration that *M. avium* can survive not only in harsh environments such as soil and water bodies but also intracellularly, it is assumed that *M. avium* is more resistant to antibacterial compounds than *M. tuberculosis*, which is an obligatory parasite, meaning that it needs the host to survive, infecting primarily macrophages. This assumed higher resistance can be linked to a cell wall harder to penetrate, which can help explain why PepH was able to exert antimycobacterial activity against *M. tuberculosis* but not against *M. avium* growing axenically. Like PP4 and C16-PP4 (**Figures 10B and 10E**), PepH displayed no toxicity towards BMM (**Figure 12B**). Against internalized *M. avium 2447* SmT, PepH displayed an apparent concentration-

dependent inhibition of growth (**Figure 12C**). With inhibition of 31,5% at 5 μM and 45% at 25 μM, PepH seemingly displayed higher activity against intracellular bacteria than axenically grown bacteria. However, this can only be confirmed once more experiments are performed, given that the activities presented for the peptide are the results of not more than two assays. This might indicate that PepH is exerting its activity through immunomodulatory pathways inside the macrophage, much like those displayed by the peptide against *M. tuberculosis*.[101] Nonetheless, further assays are required to prove this theory, such as the peptide localization, measurements of cytokine levels in the cultures' supernatants, and modulation of cellular processes (e.g. autophagy).

In what concerns the GOALA1 peptide, its amino acid sequence resulted from a series of mutations applied to the sequence of a lactoferricin peptide with previously described activity against *M. avium* – LFcin17-30 (IC_{50} of 14,2 μM against *M. avium* 2447 SmT).[93] These mutations were based on a study conducted by Usmani *et al.* where key characteristics of antimycobacterial peptides were identified allowing to build a predictor capable of designing and predicting the activity of antimycobacterial peptides – AntiTbPred.[100] This predictor analyses the amino acid sequence of a peptide and scores based on its predicted antimycobacterial activity. The higher the score, the higher presence of antimicrobial regions and characteristics common to antimycobacterial peptides. The mutations applied were done so that the AntiTbPred score given to LFcin17-30 could increase, meaning that theoretically, GOALA1 with a score of 2,40 was more potent against mycobacteria than LFcin17-30, which only had a score of 1,68. It is important to note that any peptide with a score equal or higher to 1, was deemed antimycobacterial. **Table 6** demonstrates the mutations that were applied. Mutation "*a*" was suggested by the predictor and resulted in the substitution of glutamine for an alanine. Alanine's side chain is, contrary to glutamine's, nonpolar, which facilitates nonpolar interactions that can occur in the presence of tryptophan, a key amino acid in antimycobacterial peptides, allowing for a better separation of polar and non-polar regions. In *b* various mutations occur. Firstly, we have a substitution of the phenylalanine for an alanine. Alanine is a much smaller amino acid which results in a lower steric hindrance, possibly allowing for greater interactions between the peptide and the membrane. The substitution of glycine for a leucine allowed for the insertion of leucine in position 1 of the C-terminal. Then, we have the removal of methionine and cysteine. The suggested removal of cysteine is contradictory since the presence of cysteines in antimycobacterial peptides is a common feature, and the score decreases; however, the amphipathicity of the peptide increases, hence the reasoning of these deletions. Mutation *c* saw a substitution of one of the arginines in the N-terminal for a tryptophan. This substitution heavily increased the peptide's score given the addition of a tryptophan,

despite giving up on arginine in position four of the N-terminal, a common feature in antimycobacterial peptides. However, concurrently with an augment in the peptide's score, it also meant a loss of amphipathicity and charge, two very important features in the activity of any AMP. Therefore, the substitution of leucine for arginine in position 2 of the C-terminal was done so that we could still maintain a charge of +6. Additionally, arginine is a commonly found amino acid in that position of an antimycobacterial peptide. This sequence was named GO1. Afterwards, in mutation *d*, alanine and leucine were added, so that the peptide could have a leucine in the position two of the N-terminal. This was the sequence for GOALA1. For GOALA2, in mutation *e,* the substitution of the arginine in position two of the C-terminal for a leucine was suggested by the predictor. Despite increasing the peptide's score, the additional replacement of arginine for the non-polar amino acid leucine not only decreased the peptide's overall charge but also decreased its amphipathicity.

The assessment of GOALA1's activity was done against four different strains of *M. avium*: 2447 SmT, 2-151 SmT, 25291 SmT and 2-151 SmOp. Concurrently, these bacteria were also incubated with clarithromycin, a macrolide antibiotic that displayed an IC_{50} < 1 μM for all strains (**Table 8**)(**Figure 13**). In 2012, Inger *et al.* presented data of both pharmacokinetics and pharmacodynamics of all major drugs usually prescribed to patients diagnosed with a MAC infection, assessing that *M. avium* complex was highly susceptible to clarithromycin, with a MIC equal or lower than 4 μg/mL (5,35 μM).[107] Our IC_{50} values for the four different strains tested in this study (**Table 8**) are all in agreement with what Inger *et al.* reported, meaning that the bacterial strains tested were all susceptible to clarithromycin.

GOALA1 was not nearly as effective as clarithromycin was. While it presented activity at higher concentrations, it was incapable of inhibiting 50% of bacterial growth in three of four strains tested (**Figures 14A, 14B and 14C**). The only strain where GOALA1 could moderately exert its activity was against *M.* avium 2-151 SmOp (**Figure 14D**), presenting an IC_{50} of 26,0 μM (**Table 8**). This means that *M. avium* 2-151 SmOp was the most susceptible strain against both clarithromycin and GOALA1. In fact, the smooth opaque variant of MAC colonies is linked with a higher susceptibility to antimicrobial compounds while the most resistant is the transparent variant. This higher susceptibility was proven with the use of long-chain fatty acids with a strong hydrophobicity. To exert their antibacterial activity, fatty acids penetrate cell surface structures, including the outer membrane and cell wall. Therefore, Saito *et al.* tested the susceptibility of SmOp, SmT and rough variants of *M. avium* to these fatty acids. The SmT variants of the colonies tested were the least susceptible to the fatty acids' toxicity, being more resistant to both saturated and unsaturated fatty acids than the SmOp variants were.[108] This can be

explained at the bacterial cell wall level. Preliminary scanning and transmission electron microscopy revealed that the smooth transparent variants not only possessed a structured outer layer of polysaccharide, absent in the smooth opaque variant but its cell wall was also thicker. This outer layer possibly impairs the entrance of AMP into the bacteria, rendering them ineffective.[109]

Having assessed the antimycobacterial activity of GOALA1 against various strains of axenically growing *M. avium*, we proceeded to test the peptide's activity against *M. avium* 2447 SmT internalized by BMM. GOALA1 did not display any activity against *M. avium* at any of the concentrations tested (**Figure 15**). Given the lack of activity of GOALA1 against internalized *M. avium,* we decided to assess if the peptide was able to exert antimycobacterial activity against *M. tuberculosis*. The reason for this peptide to be tested against *M. tuberculosis* resides in the nature of the predictor that helped design its sequence. AntiTbPred was based on an extensive database of peptides with reported activity against various species of mycobacteria – AntiTbPdb.[110] While this predictor has peptides with reported activity against *M. ranae, M. smegmatis, M. vaccae, M. abscessus, M. avium, M. bovis and M. intracellulare*, the most predominant species represented is *M. tuberculosis*, with 56% of all peptides inserted into the database having reported activities against this species.[110]

Alongside GOALA1, PepH was also tested against *M. tuberculosis* H37Rv, despite having reported activities against that strain already.[89] However, our results indicate that this peptide had much lower activity than previously reported. Nonetheless, the BMM assays performed against *M.* avium and *M.* tuberculosis were insufficient to safely say that the peptide does not possess meaningful activity against these mycobacteria. These results only indicate tendencies towards lack of activity. The difference observed in the activity of PepH can be explained by the nature of the macrophages used. While Sharma *et al.* used monocyte-derived human macrophages,[89] we used bone marrow-derived macrophages from mice. Despite being very moderate, GOALA1 seemed capable of exerting some activity at 25 μM against *M. tuberculosis* (**Figure 16**). This tenuous difference observed between the GOALA1's activity against the two species of mycobacteria can be explained by the nature of the database in which the predictor is based, as stated above. Taking into consideration that the most predominant species represented in the databank is *M. tuberculosis*, the sequence built for GOALA1 may be optimized for an antimycobacterial peptide against *M. tuberculosis* and not *M. avium*. Another possible explanation for this difference in activity relies on the already mentioned nature of both species. Being an obligatory intracellular pathogen, *M. tuberculosis* is thought to be naturally less resistant to antimicrobial compounds than *M. avium,* a bacterium capable of surviving in harsh conditions.

In terms of toxicity, due to the current pandemic situation, we could not test if the peptide was indeed non-toxic as ToxinPred predicted (**Table 7**)**.** However, during the BMM assays, observation through use of an optical microscope revealed that the peptide appeared to have no toxic effect on the macrophages, at the tested concentrations.

Overall, GOALA1 seemed to be ineffective against both *M. avium* and *M. tuberculosis.* Its antimycobacterial activity was nowhere near the activity reported for LFcin17-30,[93] when, according to the predictor that helped design it, it was supposed to be even more potent. As stated by Usmani *et al.* the inaccuracy of the predictor can be explained by the non-availability of negative data. A lack of experimentally verified non-antitubercular peptides hinders the capabilities of AntiTbPred to be as accurate as possible. To overcome this problem, more negative results, such as this one, need to be stored in the repository so that this *in silico* method can be improved.[100] Nonetheless, as of now, the predictor failed to design a peptide that experimentally matches the theoretical potency that has been attributed.

Altogether, these results show that despite possessing great potential, *in silico* predictors require extensive experimental data behind them of both positive and negative results to increase prediction accuracy. Nonetheless, some results are interesting. GOALA1 proved to be effective against the 2-151 SmOp and revealed some mild differences between *M. avium* and *M. tuberculosis* in terms of susceptibility. Moreover, the chimeric peptides helped realize how more resistant mycobacteria are to antimicrobial compounds when compared to Gram-negative and Gram-positive bacteria since the chimeric peptides revealed to be less active than the parental peptide 3.1 against *M. avium* 2447 SmT. It would be interesting to infer if PepH exerts its activity through immunomodulatory pathways, as well as assessing which characteristics, such as charge and amphipathicity are more important in an antimycobacterial peptide. This could be done through point mutations in peptides with an amino acid sequence much similar to that of LFcin17-30 than the one GOALA1 had. Having such a distinct sequence from the original peptide, it is hard to infer what has antimycobacterial value in that sequence, which consequently makes it harder to understand what can be changed to increase its efficacy. Nonetheless, the GOALA1 amino acid sequence can also be mutated to that same effect, especially when considering that it is a synthetic peptide built solely with the idea of being active against mycobacteria. In fact, both peptides GO1 and GOALA2 were supposed to be synthesized during this project to infer if a higher amphipathicity value while maintaining the same charge meant a higher antimycobacterial activity despite being predicted to be less potent than GOALA1 (GO1 peptide) or if the peptide would be more potent than GOALA1 as predicted by AntiTbPred while being less amphipathic and having less charge (GOALA2 peptide). This would

allow for a better understanding of what properties are more important in an antimycobacterial peptide. However, given the current pandemic situation, I was not able to synthesize both GO1 and GOALA2. Moreover, resolving the secondary structure of GOALA1 would be very helpful towards a better understanding of why there is a lack of activity against SmT strains. Taking into consideration its amino acid sequence and that alanine, leucine and arginine are three of the amino acids with the most propensity towards the formation of α-helices,[111] a next possible step would be to resort to circular dichroism to infer on the potential of the peptide to form an α-helix.

Being coined as the next “go-to” antibacterial agent for the last decades, antimicrobial or host defence peptides are one of the many solutions currently in development to overcome the emerging danger that antibiotic-resistant bacteria represent. However, these have been failing in translating from research labs to clinical use helping to combat the incidence of infectious diseases that affect millions of people per year. Yet, its high plethora of applications and mechanisms is still one major advantage that peptides offer when compared to other solutions. Our understanding of the mechanisms through which peptides act has never been so great and research needs to continue so that we can understand how peptides work, fully unlocking their potential.

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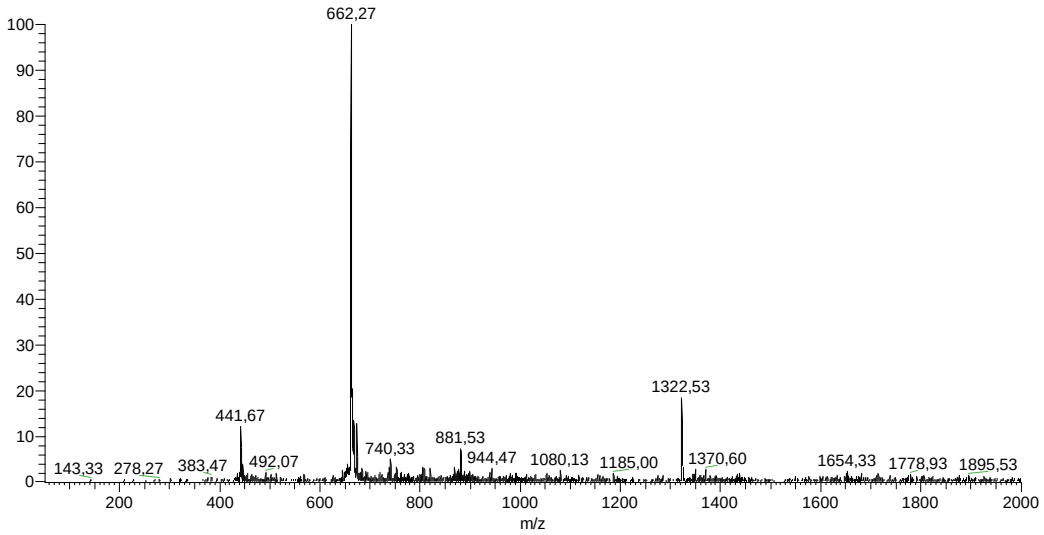
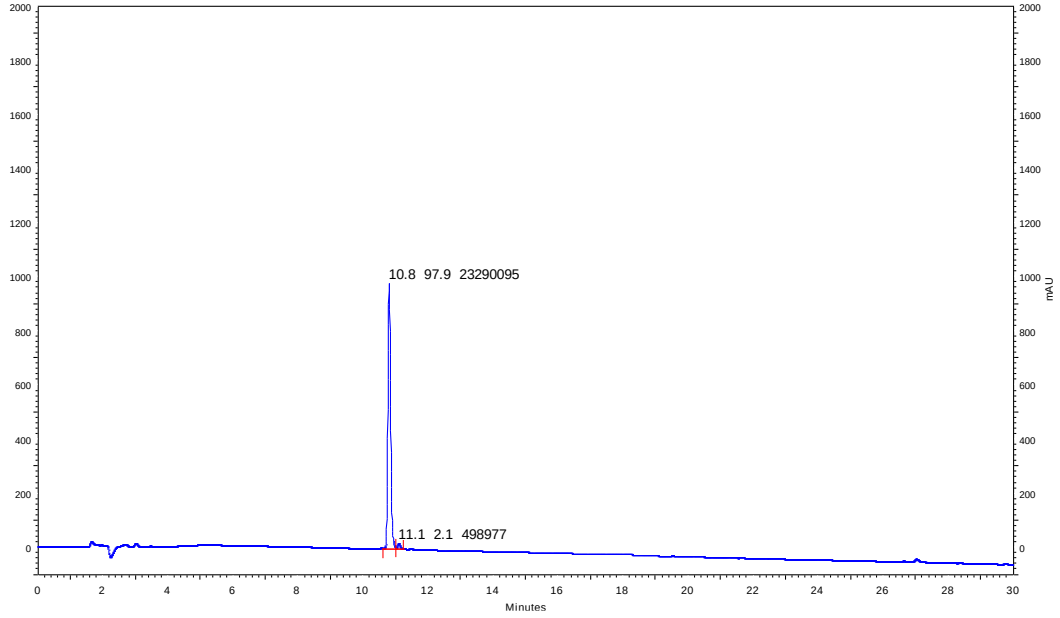
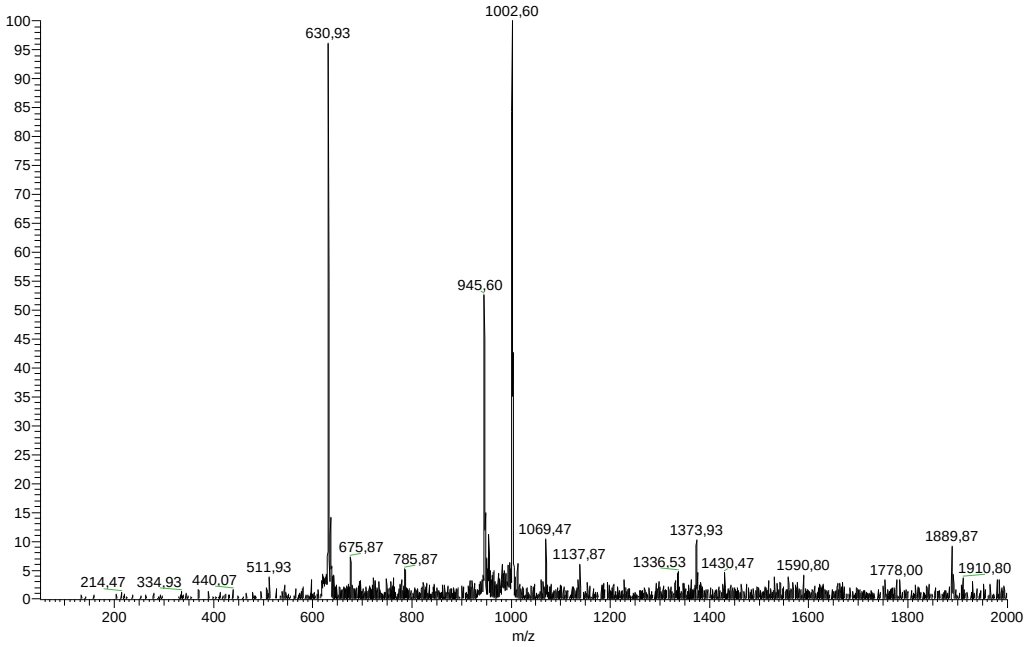
Annex



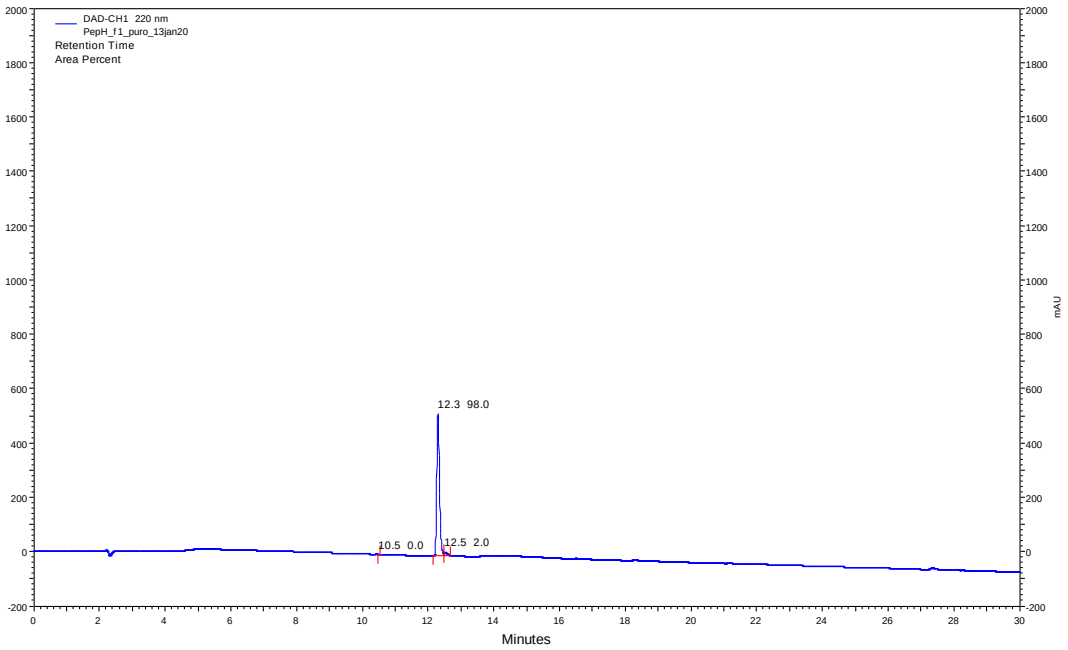
Annex Figure 1. ESI-IT mass spectrum (positive mode) of Ub2. $[P+H]^{1+}$ = 1322.53; $[P+2H]^{2+}$ = 662.27; $[P+3H]^{3+}$ = 441.67.



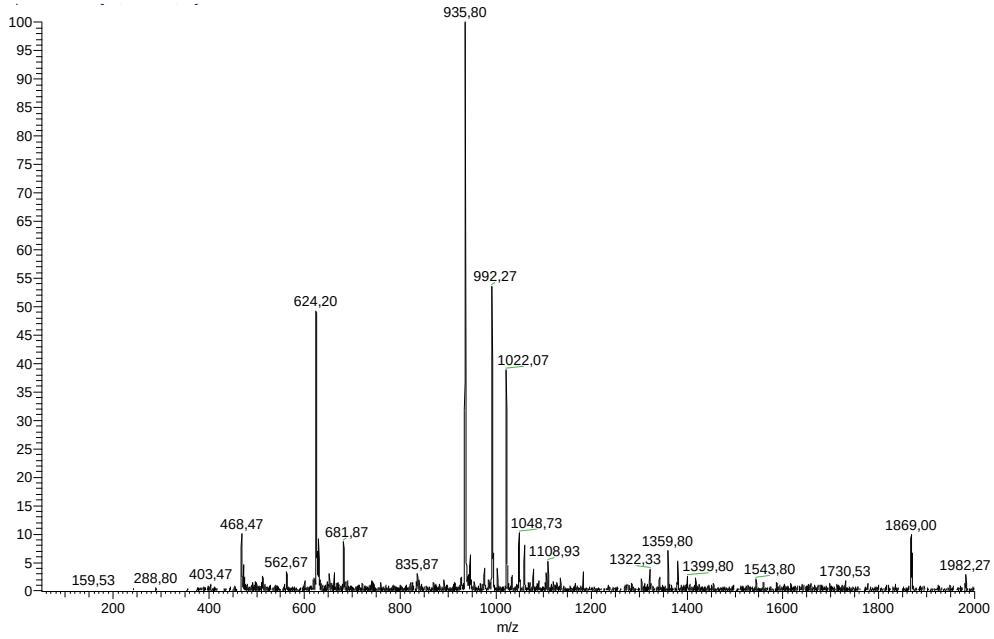
Annex Figure 2. HPLC chromatogram of Ub2. The chromatogram revealed that Ub2 had a purity of 97,9% and its retention time was 10,8 minutes.



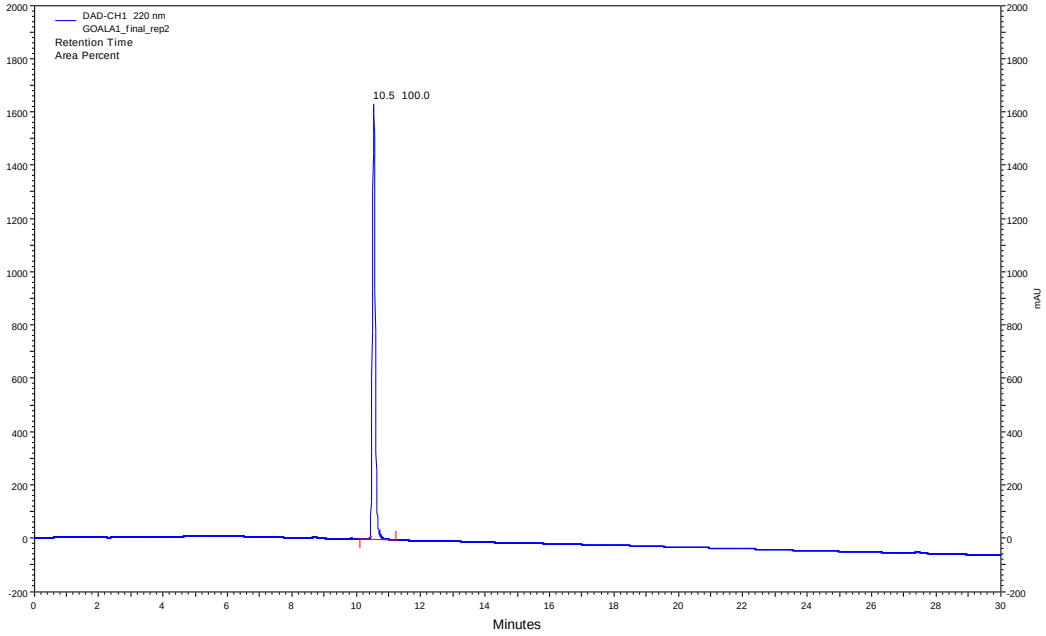
Annex Figure 3. ESI-IT mass spectrum (positive mode) of PepH. $[P+H]^{1+}$ = 1889.87; $[P+2H]^{2+}$ = 945.60; $[P+2H+TFA]^{2+}$ = 1002.60 $[P+3H]^{3+}$ = 630.93.



Annex Figure 4. HPLC chromatogram of PepH. The chromatogram revealed that PepH had a purity of 98,0% and its retention time was 12,3 minutes.



Annex Figure 5. ESI-IT mass spectrum (positive mode) of GOALA1. $[P+H]^{1+}$ = 1869.00; $[P+2H]^{2+}$ = 935.80; $[P+2H+TFA]^{2+}$ = 992.27; $[P+3H]^{3+}$ = 624.20.



Annex Figure 6. HPLC chromatogram of GOALA1. The chromatogram revealed that GOALA1 had a purity of 100,0% and its retention time was 10,5 minutes.