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TESI DI DOTTORATO DI RICERCA

ANTIMICROBIAL PEPTIDES FOR THE PREVENTION OF BIOMEDICAL DEVICE-RELATED INFECTIONS

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Abstract

Among health-care associated infections (HCAIs), foreign-body related infections (FBRIs) represent a clinically relevant challenge. The increasing use of implanted medical devices poses the need for materials effective in preventing such type of infections. Indeed, among the causative pathogens, multidrug resistant (MDR) strains, often forming biofilms on implant surfaces, are frequent.

Antimicrobial peptides (AMPs) are naturally occurring molecules of the innate immunity that are receiving increasing attention as potential novel anti-infective agents. This is due to their potent and broad-spectrum antimicrobial activity also including MDR strains and biofilm, their scarce propensity to select resistant mutants, and their immunomodulatory properties.

In the present thesis, the potential of five cationic α-helical AMPs as possible candidates for the manufacturing of anti-infective biomaterials was explored. The peptides under study included two mammalian cathelicidins, i.e., BMAP27 and BMAP28 (26 and 27 amino acid residues, respectively), their (1-18) N-terminal fragments (18 residues), and the *in silico* designed P19(9/G7) (19 residues). The *in vitro* antimicrobial and antibiofilm activity against *S. aureus* and *S. epidermidis* reference strains and efficacy against 24 isolates from orthopaedic infections including *Staphylococcus*, *Streptococcus* and *Enterococcus* spp were tested. The effects of these peptides on the viability and differentiation of osteoblast cells have also been assessed. The three shorter peptides, which displayed good antimicrobial activity and were not toxic towards eukaryotic cells, underwent further characterization in conditions mimicking the orthopaedic pathophysiological environment, i.e. in the presence of serum, hyaluronic acid and synovial fluid. Under these conditions, the peptides demonstrated overall good antimicrobial efficacy and were safe for host cells at microbicidal concentrations. They were however differently affected by the presence of human serum. The peptide BMAP27(1-18) that best retained activity in most conditions was biotinylated at the N-terminus and immobilized onto streptavidin coated resin beads. The immobilized peptide proved active against *S. aureus* and *S. epidermidis* reference strains and safe to eukaryotic cells. As a preliminary approach to elucidate the mechanism of action of the immobilized peptide, a labelled analogue of BMAP27(1-18), obtained by substitution of its four

phenylalanine residues with cyano-phenylalanines, was immobilized onto the resin beads through the N- or, alternatively, through the C-terminus. Interestingly, the antimicrobial activity was retained in both cases, though to a slightly different extent.

These results encourage further studies for the development of BMAP27(1-18) as a possible peptide candidate for the functionalization of a clinically-usable biomaterial, for the prevention of biomedical-device related infections.

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Abbreviations

ALP:	alkaline phosphatase
AMP:	antimicrobial peptide
BHI:	brain heart infusion
CD:	circular dichroism
CFU:	colony forming unit
DMEM:	Dulbecco’s modified Eagle medium
FBRI:	foreign body reated infection
HA:	hyaluronic acid
HBSS:	Hank’s balanced salt soluton
HCAI:	health care associated infection
HS:	human serum
LDH:	lactic dehydrogenase
LPS:	lipopolysaccharide
LTA:	lipoteichoic acid
MBC:	minimum bactericidal concentration
MDR:	multidrug resistant
MH:	Mueller Hinton
MIC:	minimum inhibitory concentration
MRSE:	Methicillin resistant *Staphylococcus epidermidis*
NO:	nitric oxide
OM:	osteogenic medium
PBS:	phosphate buffered saline
PJI:	prosthetic joint infection
RPMI:	Roswell Park Memorial Institute
SF:	synovial fluid
TCA:	trichloroacetic acid

1. Introduction

1.1 Infections associated with medical devices

During care delivery, health-care associated infections (HCAIs) represent the most frequent complication. These are infections that occur in patients in any setting where they receive care (i.e. in health care facilities, in hospitals or at their own home) that were not in act or were in a latent stage at the time of admission and can become evident after discharge. HCAIs entail a relevant economic burden both for health system and patients' families, owing to prolonged hospital stays, long-term disability, increased antimicrobial resistance of pathogens, aside from being very dangerous, since they can even lead to death.

A significant contribute to HCAIs is given by foreign body-related infections (FBRIs). In fact, although the use of implanted medical devices is increasing in all fields of medicine, they are associated with a significant risk of bacterial and fungal infections. Contamination mostly occurs during implantation, with the contamination by a very small number of microorganisms derived mostly from the skin and mucous membranes of the patient or the hands of clinical staff, where they reside normally in as part of cutaneous microflora. Once introduced in the body, these pathogens often increase their morbidity, causing severe complications and eventually leading to death. The causative organisms of these infections are Gram-positive and –negative bacteria and fungi, with *Staphylococcus epidermidis* and other coagulase-negative staphylococci (CoNS) being the most encountered pathogens, together with *Staphylococcus aureus* [1].

Pathogenicity of microorganisms that cause FBRIs is mainly due to their capacity to adhere to materials and develop in a thick, multi-layered biofilm. Biofilm is defined as an "aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface" [2]. This EPS is composed mainly by extracellular DNA, proteins, and polysaccharides produced by the microorganisms themselves. Biofilm formation on devices allows microorganisms to persist as reservoirs that can readily spread into patients. Microorganisms grown in a biofilm acquire new features with respect to their planktonic counterparts (i.e. single-cells floating in a liquid medium); in particular, they result largely more resistant to antimicrobial agents. Moreover, the formation of a biofilm permits pathogens to overcome innate immune defences, resulting in long-term persistence of these infections [3]. Different factors drives microorganism growth in biofilm, i.e. specific or unspecific attachment on surfaces, nutritional signals, or sometimes exposure to sub-inhibitory concentrations of antibiotics [4]. To grow as a biofilm, cells undergo a differential regulation of various genes, with a phenotypic shift in behaviour [5].

The first step for biofilm formation is the attachment of microbial cells to the surface, and it depends on the surface characteristics of both the implant and the microorganism. These factors are mainly physicochemical forces between the naked material and pathogen's surface proteins, but involve also specific recognition between receptors, present on the microorganism, and plasma and connective tissue proteins that rapidly coat medical devices once implanted [6]. After the adhesion of the first cells, a second step of multiplication and accumulation occurs. To permit the formation of multi-layered cell clusters, intercellular adhesion is needed, achieved by the expression of specific polysaccharides and proteins on the surface of the colonizing cells [7]. *Quorum sensing* (QS, i.e. intercellular signal) is a regulatory system of gene expression according to cell-population density. The signal molecules released lead to an alteration in gene expression, resulting into regulation of diverse physiological activities, i.e. symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation. QS consents bacteria to coordinate the behaviour of the entire community [8]. Therefore, microorganisms embedded in biofilms are often scarcely susceptible to the immune system and far more resistant to antibiotics and other antimicrobials as compared to their planktonic counterparts, resulting in difficult eradication of these communities from medical implants. Once biofilm is formed, a phase of dispersal occurs, in which cells detach from the biofilm, disseminating and leading to colonisation of new sites within the host and eventually leading to systemic infections [9] (Fig.1.1).

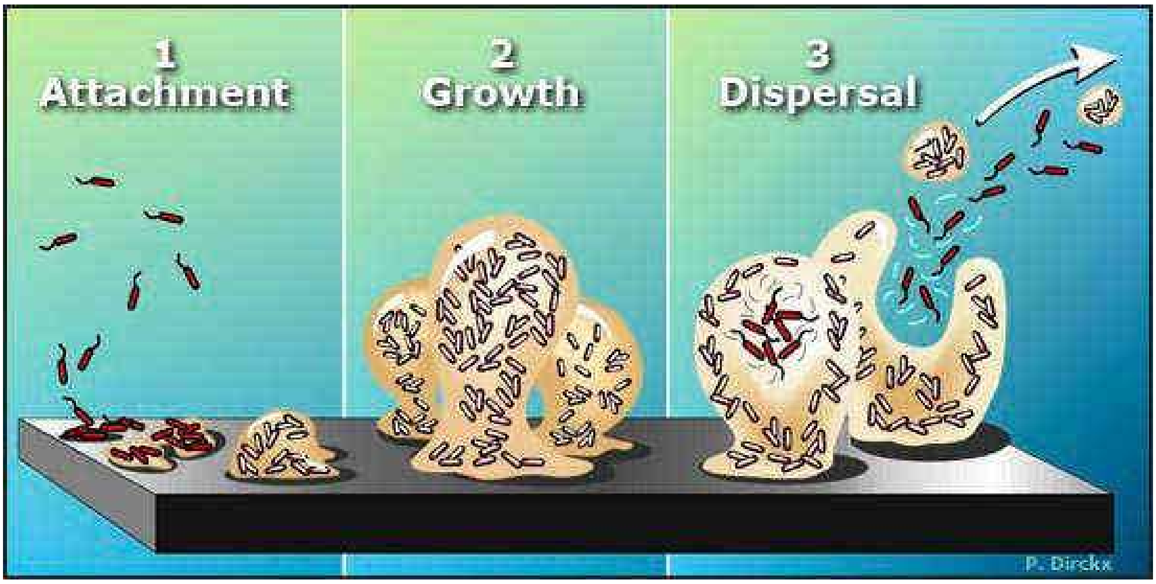

Figure 1.1. Biofilm life cycle. The attachment of planktonic microorganisms occurs rapidly, followed by the production of extracellular polymeric substances (EPS) and surface colonization (1). Within hours, biofilm develops in a complex, three-dimensional structure, under the influence of different environmental factors (2). Single cells or clumps can detach from the original biofilm, colonizing other surfaces (3) (modified from BIOFILMS: The Hypertextbook)

Once formed, biofilms are very difficult to eradicate. Often antimicrobial therapy is not sufficient and removal and possibly replacement of the device is needed. So, it is evident that prevention of surface contamination of implanted medical devices is fundamental for a good outcome. Good clinical practices, standardisation of aseptic care, antibiotic prophylaxis and hygienic measures of medical structures, workers and patients are surely pivotal, but not always sufficient [1, 10].

Among FBRIs, prosthetic joint infections (PJIs) are particularly dreaded. Indeed, although orthopaedic surgery is nowadays common and operations are mostly successful, and despite these infections have drastically decreased in the past decades, they still pose strong concern, in particular in developing countries, for their high morbidity and economic burden. The formation of biofilm on a prosthesis forces to the use of high doses of antibiotics and, if the therapy doesn't work, there could be the need of revision surgery, with a consequent prolonged rehabilitation and hospitalization. Moreover, revision surgeries have a high cost and the chances of a new infection are increased [11]. Table 1.1 summarizes the principal organisms causative of prosthetic joint infections [12].

Common causes of prosthetic-knee and prosthetic-hip infection
Gram-positive cocci (approximately 65%)
Coagulase-negative staphylococci
Staphylococcus aureus
Streptococcus species
Enterococcus species
Aerobic gram-negative bacilli (approximately 6%)
Enterobacteriaceae
Pseudomonas aeruginosa
Anaerobes (approximately 4%)
Propionibacterium species
Peptostreptococcus species
Finegoldia magna
Polymicrobial (approximately 20%)
Culture-negative (approximately 7%)
Fungi (approximately 1%)

Table 1.1. Causative organisms of prosthetic joint infections (modified from Del Pozo et al., 2009)

1.2 Anti-infective biomaterials

With the progress of medicine and material sciences, new strategies to create anti-infective biomaterials resistant to pathogens' colonisation have been explored. These biomaterials can be also used to prevent, treat and reduce infections, by delivering of bioactive substances [13]. Obviously, biomaterials aimed to eradicate pre-existing infections should have a high bactericidal potential. In the years, many approaches were explored to obtain such anti-infective biomaterials [14] (Fig. 1.2).

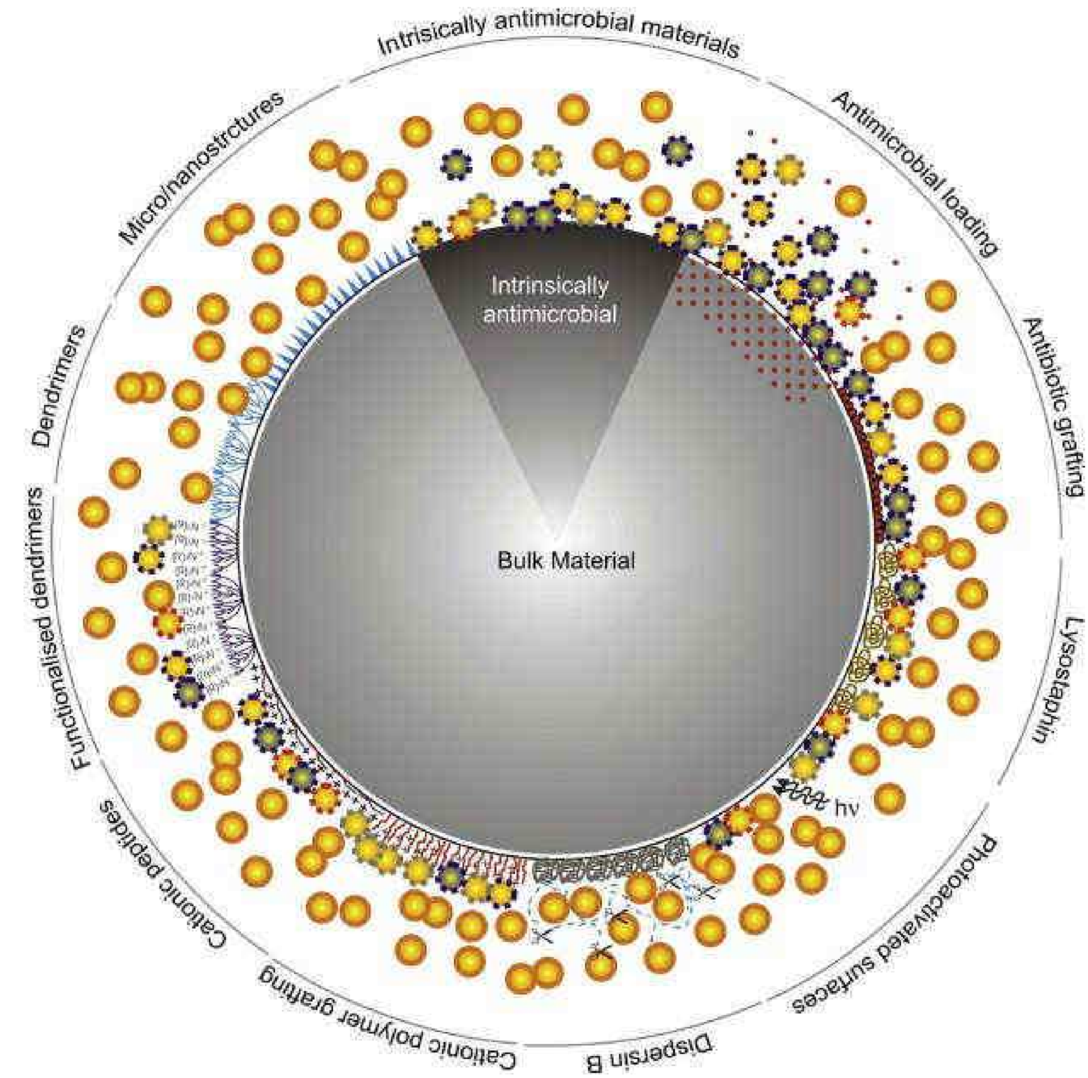

Figure 1.2. Summary of biomaterials and surfaces developed for the prevention of implant-associated infections (modified from Campoccia et al., 2013)

Due to the amplitude of applications they have to be used for, the development of these biomaterials follows different strategies, each trying to target different stages of microbial colonisation (Fig. 1.3).

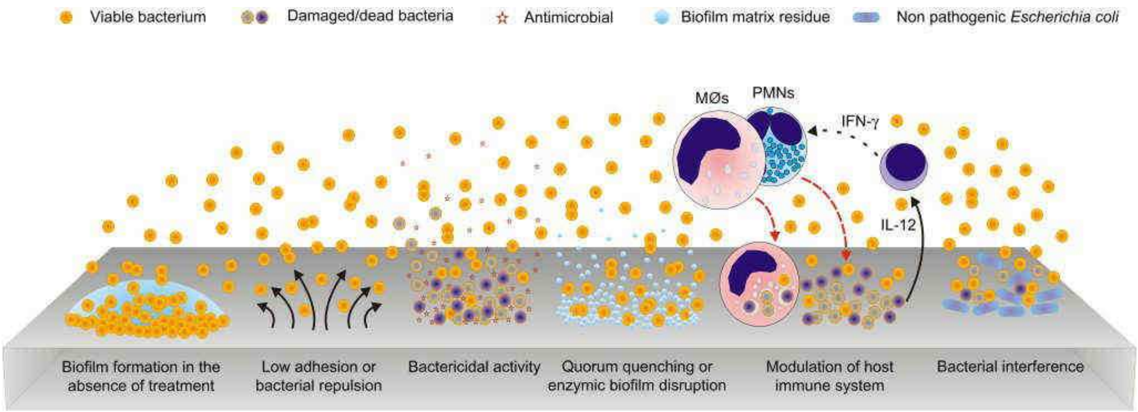

Figure 1.3. Overview on the approaches used for the prevention of the attachment of microorganisms on implant surfaces. (modified from Campoccia et al., 2013)

The first obvious target is the attachment of microorganisms to the surface. As previously mentioned, attachment can occur by passive adsorption through physicochemical interactions, which is a mechanism common to all microbial species, or by adhesion of molecules present on microorganisms' surface which are species-specific, i.e. adhesins. While the contamination occurring through mechanical transfer in dry state could be avoided simply by adequate sterility measures and procedures, the situation changes when infection derives by the contact with infected fluids, and if it is a proteinaceous fluid or not. In the case of fluids not containing proteins, electrostatic forces, hydrophobic and Van der Waals interactions or hydrogen bonding drive the interaction between groups on both bacterial surface and biomaterial [15]. These type of interactions could be reduced by the modification of surface properties of the bulk material to make the surface inert, e.g. by regulating hydrophobicity/hydrophilicity, superficial charges, roughness etc. This could be accomplished by creating a superficial coating or micro- or nanostructured surfaces [16]. These modifications could be not sufficient when the implants come in contact with body fluids rich in proteins, that is the most frequent scenario. Proteins rapidly deposit onto the surface, creating a new interface for bacterial contact. Apart from rare cases in which unspecific adhesion of some proteins like albumin compete with the adsorption of pro-adhesive molecules, host proteins (i.e. collagen, fibrinogen, fibronectin, laminin, vitronectin, clumping factor A and B, bone-sialoprotein, elastin, IgG and other components of extracellular matrix - ECM) enhance the adhesion of pathogens via adhesins [17]. Adsorption can be partially prevented by the alteration of the interaction potential, by the use of high potential barriers to slow down the adsorption kinetics, or pre-adsorbing on the material molecules that compete with host pro-adhesive molecules or alter hydrophobic/hydrophilic properties of the surface [18, 19]. Many methods have been explored to obtain such type of surfaces; Table 1.2 lists a set of bioinert surfaces which proved effective in inhibiting pathogens' adhesion at least *in vitro*.

Anti-adhesive polymer coating	**In vitro** tested efficacy		References
	Gram-negative	Gram-positive	
Poly(ethylene oxide) (PEO)	EC, PA	SA, SE, SS	[127, 128]
Pluronic surfactants [copolymers of PEO and poly(propylene oxide) (PPO)]		SE	[129]
Poly(ethylene glycol) (PEG)	EC	SE	[130]
PEG-co-acrylic acid (PEG-AA) microgels		SE	[131]
Poly(l-lysine)/poly(l-glutamic acid) (PLL/PGA) multilayers ending by several PLL/PGA-g-PEG bilayers	EC		[132]
Poly(epsilon-caprolactone) (PCL)/PEG copolymer	BS		[133]
Triethylene glycol dimethyl ether (triglyme)	PA		[134]
Phosphorylcholine (PC)-based polymers	EC, PA	SA, SM	[135, 136]
2-Methacryloyloxyethyl phosphorylcholine (MPC) polymer	PA	SA, SE	[137]
Sulfonated poly(ethylene glycol)-acrylate copolymer	EC	SE	[138]
Polyacrylamide		SS, SA (CA)	[139, 140]
Poly(2-methyl-2-oxazoline) (PMOXA)	EC		[141]
Zwitterionic poly(sulfobetaine methacrylate) (pSBMA)	PA	SE	[142-145]
Poly[N-isopropylacrylamide (NIPAAm)-co-SBMA]	EC	SE	[146]
Zwitterionic phosphonated polyurethane		SA	[147]
PEGylated poly(vinylidene fluoride) (PVDF)	EC, SMl		[148]
Poly[oxy(acetylamino-n-undecylesterthiomethyl] ethylene]	PA	SA, SE, EF	[149]
Oligo(ethylene glycol) (OEG) self-assembled monolayers (SAMs)		SA, SE	[150, 151]
Superhydrophobic fluorosiloxane coatings		SA	[152]
Methacryloyloxydecyl phosphate (MDP)-PEG		SM	[153]
Superhydrophobic xerogel coating from a mixture of nanostructured fluorinated silica colloids, fluoro-alkoxysilane, and a backbone silane	PA	SA	[154]
Silk sericin-functionalized titanium surfaces		SA, SE	[155]
Silica nanoparticles grafted with acrylate and PEG groups	PA (±)	SE	[156]
Methacryloyloxydecyl phosphate (MDP)-PEG		SM	[157]
Peptide-PEG amphiphilic macromolecules		SA, CNS	[158]
Peptide-functionalized poly(l-lysine)-grafted-poly(ethylene glycol) (PLL-g-PEG/PEG-RGD)		SA	[35]
Trimethylsilane		SE	[159]
Silicon nitride (Si3N4)	EC, PA	SA, SE	[160, 161][a]
Polytetrafluoroethylene [a]	PA	SA	[162]

BS, Bacillus subtilis; CA, Candida albicans; CNS, Coagulase-negative staphylococci; EC, Escherichia coli; EF, Enterococcus faecalis; PA, Pseudomonas aeruginosa; SA, Staphylococcus aureus; SE, Staphylococcus epidermidis; SM, Streptococcus mutans; SMl, Stenotrophomonas maltophilia; SS, Streptococcus salivarius.

[a] *In vivo* tested.

Table 1.2. Anti-adhesive coatings (modified from Campoccia et al., 2013; refer to the original publication for references)

Some biomaterials are bactericidal *per se*. Intrinsically antibacterial materials are principally represented by some metals (i.e. silver, copper, zinc and their alloys) and polymeric materials (i.e. chitosan and its derivatives, bioactive glasses), or materials created assembling polymers containing molecules with antimicrobial properties [14]. Unfortunately, metals have the tendency to corrode or release ions, potentially leading to local toxicity, but also accumulation in peripheral organs; moreover, cytotoxicity can interfere with cellular differentiation, which is important for implant integration [20-22]. Chitosan, on the other hand, is only weakly antimicrobial, and various derivatives have been explored to enhance this property [23].

To confer to non-bioactive bulk materials the desired anti-infective properties without compromising desirable raw material characteristics, antibacterial coatings have been developed. These coatings lay at surface-tissue interface, hence possibly modifying host response. As in the case of intrinsically bioactive materials, it is important also in this case to balance antibacterial properties with biocompatibility. Different methods have been explored aimed to create such type of coatings. Antimicrobial coatings effective on contact can be created by grafting or adsorbing non-leachable, well-known antimicrobial molecules on material surface [24]. This type of coatings does not release the active molecule, permitting the use of substances that could be harmful if released in the body. They possess self-sterilizing effect and long-lasting activity, but their effect could be hindered by the coverage of host proteins [25]. There exist also anti-infective coatings producing or releasing nitric oxide (NO) or reactive oxygen species (ROS) [26-29]. These coatings could have potential cytotoxic effects, so the release rate have to be fine-tuned. Another category of bioactive coatings is represented by photoactivable compounds [30].

A well-explored strategy consists in the incorporation of antimicrobial substances in the coating or within the biomaterial (Tab. 1.3). The incorporation can be achieved through different strategies, i.e. the inclusion in the ingredients during material production, absorption onto porous or permeable materials, covalent linking to polymer’s functionalities, incorporation into self-assembled layers, etc. The modality of release is therefore dependent on the strategy used: it could occur through erosion or degradation of the coating or material, hydrolysis of the covalent bond, by diffusion to aqueous phase, etc. Although these bioactive biomaterials are very useful in clinical settings, reducing complications and costs associated with implant infections, attention has to be payed because of the possibility of systemic toxicity and, most importantly, the possibility of spreading of antimicrobial resistance with the massive use of these implants [31, 32]. Moreover, subinhibitory concentrations of antimicrobial substances released after the initial burst can exert a promoting effect on biofilm formation and select resistant strains, which are opposite effects of the pursued ones [33].

Antimicrobial substance	Examples of delivery systems
Chlorhexidine	Chlorhexidine grafting, chitosan, 5-methyl-pyrrolidinone chitosan (MPC), methacrylate and polymethylmethacrylate (PMMA)-based resin cements, 4-acryloyloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butylborane (4-META/MMA-TBB) resin, impregnated hydroxyapatite and hydroxyapatite–chlorhexidine coatings, calcium-phosphate and calcium-fluoride nanocomposites, brushite bone cements, poly-l-glutamic acid and poly-l-lysine functionalized polyelectrolyte films, calcium-phosphate and calcium-fluoride nanocomposites, titanium/polybenzyl acrylate coating
Silver (elemental/ions)	Chitosan–polyphosphate formulation antibacterial alginate film-containing DNA as a carrier, coral hydroxyapatite, dacron vascular prosthesis, caprolactone-l-lactide copolymer, polyamidoamine dendritic diol (PAMAM), dendritic oligourethane (SCDOU), alginate fibres and dressings, polyamide composites, PEG/dopa polymer-based gel, electrodeposited on titania nanotubes, Ag^+-exchanged 11A tobermorite, $Ca_5Si_6O_{16}(OH)_2.4H_2O$, perfluoropolyether-urethane coating, plasma-sprayed wollastonite/silver coatings, silver–alginate coating, polyethylene glycol (PEG) polymers with reactive catechol moieties, plasma-sprayed silver-doped hydroxyapatite coating, polyether–urethane end-capped with silver lactate, TiO2/Ag coatings, Ag-containing plasma polymer coatings, silver–polyethylene oxide (Ag/PEO) nanocomposites, silver-containing colloids of poly(2-methacryloyloxyethyl phosphorylcholine), poly(3-sulfopropylmethacrylate), phenyltriethoxysilane sol–gel coating
Silver sulfadiazine	Polyurethane (PU) polyether–urethane end-capped with silver sulfadiazine, endotracheal tubes coating, ABA-type block copolymers of poly(l-leucine) (PLL) and PEG, bilayer chitosan membranes, alginate microspheres-impregnated collagen scaffold
Silver sulfadiazine + chlorhexidine	Impregnated polymeric catheters, catheters coatings
Triclosan	Coating on polyglactin 910 sutures, polyglycolide threads, poliglecaprone 25, PE, polidioxanone, loaded in pegylated silicone elastomers, chitosan-coated graft, triclosan-loaded nanostructured mesoporous silicon
Benzalkonium	Catheters impregnation, benzalkonium-bonded heparin-coated catheters
5-Fluorouracil	Catheters coating
Methyl and propyl parabens	Polyurethane (PU)
Silver + zinc	Glass polyalkenoate cement (GPC) coatings
Zinc	ZnO films, Zn-incorporated TiO2 coatings, Zn-based glass polyalkenoate cements
Copper	317L stainless steel copper alloy (317L-Cu), Cu-sputtered polyester, Na_2O–CaO–P_2O_5 copper releasing degradable phosphate glass fibres
Peptide nisin	Poly[ethylene oxide]–poly[propylene oxide]–poly[ethylene oxide] (PEO–PPO–PEO) surfactant Pluronic F108
Peptide ponericin G1	(Poly 2)/polyanion/ponericin G1/polyanion)$_{75}$ where polyanion = chondroitin sulfate, alginic acid or dextran sulfate.
Peptide magainin I	Covalently bound to an 11-mercaptoundecanoic acid (MUA) and 6-mercaptohexanol (C6OH) self-assembled monolayer
Cationic antimicrobial peptide β-defensin-2	Coating of functionalized titanium surfaces
Peptide LL-37 (human cathelicidin peptide)	Mesoporous silica
Cationic antimicrobial peptide Tet213 (KRWWKWWRRC)	Conjugated to the copolymers brushes of poly(N,N-dimethylacrylamide-co-N-(3-aminopropyl)methacrylamide hydrochloride) (poly(DMA-co-APMA))
Antimicrobial peptide defensin from *Anopheles gambiae* mosquitoes	Polyelectrolyte multilayer films
Lipopeptides: Pal-Lys-Lys-NH(2) and Pal-Lys-Lys	Soacked Dacron prostheses
Lipopeptide: C_{12}Orn-Orn-Trp-Trp-NH2	Poly(2-hydroxyethyl methacrylate) hydrogels
Functionalized polyanilines (fPANI)	PVA, PE
Bronopol	Coating on PE
Nisin	Brushite bone cement (Nisin F), liposomal nanoparticles
Berberine	Nano-hydroxyapatite/chitosan (n-HA/CS) bone cement
Octenidine	PMMA cement pellets
Lysostaphin	Lysostaphin-functionalized cellulose fibres, adsorbed on polypropylene mesh, chitosan–collagen hydrogel

Table 1.3. Overview of antimicrobial substances delivery systems (modified from Campoccia et al., 2013)

A recent approach to FRBIs is based on nanotechnologies, and in particular on nanostructured materials. The characteristics of surfaces created with these methods can be fine-tuned, with the possibility not only to create antifouling or super-hydrophobic surfaces, but also surfaces able to directly kill microorganisms [34, 35]. Nanotechnologies permit to control the nanotopology of the surfaces, but also the physicochemical form of materials. One of the more explored applications of nanotechnologies is the use of nanoparticles (NPs, i.e. regularly or irregularly shaped particles with at least one dimension smaller than 100 nm) to create thin surface antimicrobial coatings, but they are also used to dope bulk materials or to improve the activity of photoactivated or NO-releasing surfaces [36]. The antimicrobial activity of NPs depends both on intrinsic factors, i.e. concentration, size, shape and chemical composition, and extrinsic factors, i.e. environmental factors, type of microorganisms, growth rate of the pathogen, metabolic and cell cycle phase and the presence of a pre-existing biofilm [37]. Although the mechanism of microbicidal activity of NPs needs more studies to be completely clarified, it seems that this activity is in part exerted by cell membrane damage and production of ROS. NPs can also act onto cellular mechanisms, interacting with proteins or other cellular machineries [38]. NPs can also be coated or derivatized to improve bactericidal properties or to lower toxicity. However, further studies are needed to clarify their toxicology and to exclude local or systemic toxicity and genotoxic or apoptotic effects, which can depend basically on the same factors taken in consideration for the antimicrobial effect [20].

A further approach to combat microbial colonization of implant surfaces is the use of substances with antibiofilm properties. These compounds can alternatively degrade or downregulate the expression of extracellular matrix, kill quiescent bacteria embedded in biofilms, interfere with the QS system and induce biofilm dispersion or reduce biofilm metabolism [39]. Due to the diversity of the organisms involved, these molecules often possess only a limited spectrum of action but, on the other hand, they have limited effects on host cells, with some exceptions (e.g. proteolytic enzymes) [40]. For these molecules, the most promising strategy is the delivery simultaneously with conventional antibiotics [41].

New strategies to prevent FRBIs are being studied. These include the use of iontophoresis, vibroacustic stimulation, bacterial interference, and the use of bacteriophages [14]. There are however some concerns mostly on the use of biological agents. Indeed, the use of implants precoated with non-pathogenic species raises the possibility that they can turn into opportunistic pathogens. On the other hand, apart from the lack of data evaluating the effect of high titre of these viruses in the host, phages exhibit only a limited specificity and can lead to the spread of resistant bacteria, both by selecting them and by transferring antibiotic resistance factors [42]. Moreover, bacteriophages could be inactivated either in the steps of incorporation into a biomaterial or by the pre-exposure to host immune system. There are under study also antisense-peptide nucleic acids (PNAs), which should be able to interfere with bacterial gene expression, such as that of resistance factors, reproduction, survival and biofilm formation, in combination with cell-penetrating peptides [43]. However, the safety of these compounds, regarding principally their *in vivo* degradation and potential mutagenic effects, needs to be assessed. A last approach is the use of active cytokines to modulate local immune response of the host [44]. Also in this case the absence of adverse reactions needs investigations.

1.3 Antimicrobial peptides of the innate immunity

Immunity is defined as resistance to infectious disease. The physiologic function of the immune system, i.e. the cells, tissues and molecules that mediate resistance to infections, is to prevent infections and eradicate existing ones. The immune system is able to distinguish between self and non-self, to recognise and eliminate invading pathogens and altered cells. It divides into two branches, the innate and the adaptive immune system. The innate immune system is the first evolved and is present in all organisms. It represents the early line of defence against pathogens and injured cells. It is rapid, and does not change upon repeated infections. It is composed by physical and chemical barriers, cellular components and proteins. The adaptive immune system has evolved more recently only in vertebrates. It is characterised by immunological memory, i.e. there is an enhanced reaction to pathogens that have already been encountered, and it is therefore highly specific to a particular pathogen, providing long-term protection. Like innate immunity, also adaptive immunity comprises both humoral and cellular components.

Antimicrobial peptides (AMPs, also known as host defence peptides, HDPs, in higher eukaryotic organisms) are components of the innate immune system, conserved among all living species. More than 5000 AMPs have been discovered or synthesized from 1939, when gramicidin was extracted and subsequently isolated from a soil *Bacillus* strain, and found to be effective for the treatment of wounds and ulcers [45-48]. Natural AMPs have been isolated from both prokaryotes and eukaryotes, ranging from protozoan and fungi to plants, insects and vertebrates [49-52]. AMPs are mainly produced by the cells that constitute the first line of innate immune defence, such as epithelia and phagocytic cells. In epithelia, AMPs are secreted both by barrier epithelial cells and glandular structures; the AMPs present in phagocytes are stored in granules, and can act upon intracellular fusion of phagocytic vacuoles with granules, or upon release of the granule content in the extracellular fluid [53]. AMPs can be produced constitutively by these cells or their expression can be induced by microbial molecules and/or cytokines. Their sequence is very different among organisms and species; probably this diversity arises from the microbicidal function of AMPs, and the different immune challenges that each host organism have to face [54]. Most natural AMPs are ribosomally synthesized as pre-propeptides, which are subsequently processed to remove the signal peptide and the prosequence. Precursor sequences typically undergo post-translational modifications like proteolytic processing, glycosylation, amidation, halogenation, phosphorylation incorporation of D-amino acids and cyclization [55], that modify their activity. Generally, the pro-peptide is composed by the active sequence, preceded by a propiece, which is anionic and has been proposed to serve for targeting of the AMP and/or for its correct folding [56]. The precursor sequences are often inactive and undergo proteolytic activation only when needed, possibly protecting the host from potential side effects of the mature AMP. In contrast to the mature peptide, the sequence of the preproregion is often highly conserved within families of AMPs. This suggests the existence of ancestor genes that evolved, through duplication and modification events, to give rise to the AMPs of the same family [57].

AMPs are short peptide sequences (<100 amino acids), generally with a broad spectrum of activity against bacteria and fungi, and some of them against enveloped viruses, parasites and even cancerous cells [58, 59]. As mentioned above, there is not a definite consensus amino acid sequence determining their biological activity. However, despite the great sequence and structure diversity among different AMPs, there are some conserved traits, such as charge, hydrophobicity and amphipathicity. 90% of AMPs have a cationic character, but there is also a 10% of anionic AMPs [60]. Due to their prevalence, positive-charged AMPs (therefore often referred to as CAMPs) are the most studied. Their positive charge (generally +2 to +9) is imparted by the presence of arginine and lysine residues. The hydrophobic residues that confer to the AMPs their amphipathic character, generally account for more than 30% of the total residues. The amphipathic conformation of most AMPs is typically assumed upon interaction with membranes or membrane mimics. Several categorizations have been proposed for AMPs. A well-accepted classification, based on the secondary structure, divides AMPs into four groups: α-helical peptides, β-sheets, mixed structures and extended peptides (Fig. 1.4) [61, 62].

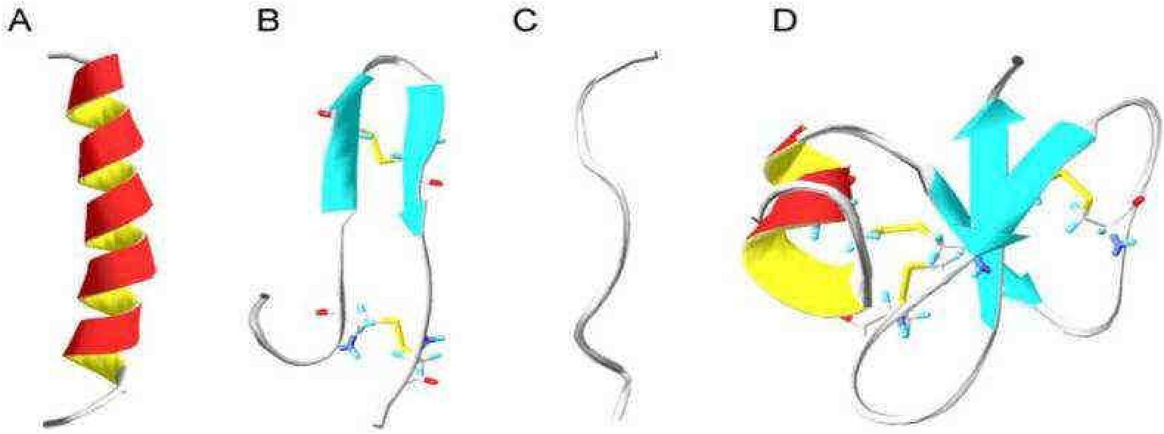

Figure 1.4. Classification of antimicrobial peptides (AMPs). AMPs can be divided on the basis of their secondary structure in four classes: α-helical peptides (e.g. LL-37) (A), β-sheet peptides (e.g. polyphemusin) (B), extended peptides (e.g. indolicidin) (C), mixed-structures (e.g. human β-defensin-2) (D) (modified from Mojoska et al., 2015)

Among the first discovered α-helical peptides, cecropins were found initially in invertebrates. They are the most abundant AMPs of insects. They are 31–39 residues linear α-helical peptides, devoid of cysteine residues but generally having two tryptophan residues at the very first N-terminus and an amidated C-terminus. Cecropins have demonstrated to be active against Gram-negative and Gram-positive bacteria, as well as fungi [63, 64]. Although first isolated and studied in insects, cecropin-like peptides have also been found in the porcine intestine [65] and in marine *protochordata* [66]. Another α-helical AMP found in insects is melittin, a 26-residues hemolytic peptide, found principally in bee venom. It has an hydrophilic C-terminus, while N-terminus is prevalently hydrophobic [67]. Magainins are α-helical AMPs of 23 amino acid residues isolated from the skin of the frog *Xenopus laevis*. First discovered by Zasloff in 1987 [68], in the past years they were well-studied for their ability to form pores in the envelopes of target microorganisms, without important cytotoxicity towards eukaryotic cells [69]. Their wound-healing properties made them among the first AMPs developed for therapeutic purposes [69, 70]. Amphibian AMPs have also been considered promising candidates for clinical use for their biological properties not limited only to antimicrobial activity [71]. In particular, temporins are 10-16 amino acid α-helical peptides, initially isolated from *Rana temporaria* [72, 73]. Also esculentins, isolated from *Rana esculenta* skin secretions [74, 75], have attracted attention for having potent antimicrobial activity without toxic effects on eukaryotic cells [76].

The cathelicidin family of AMPs is composed by AMPs sharing a well-conserved N-terminal prodomain, known as the cathelin domain, for its more than 70% homology to cathelin, an inhibitor of cathepsin L, isolated from porcine neutrophils [77]. However, they greatly differ in the C-terminal antimicrobial domain, that becomes active upon its release from the holoprotein. Based on the structure of the C-terminal domain, cathelicidins are further divided into linear and cyclic molecules. Most cathelicidins are linear, 23-40 amino acid peptides that assume an α-helical conformation in anisotropic environments such as that of biological membranes (e.g. porcine PMAP-36 and -37, rabbit CAP-18, mice CRAMP, human LL-37/hCAP-18, bovine BMAP-27, -28 and -34, ovine SMAP-29 and -34). Other classes of cathelicidins encompass cyclic peptides with one disulfide bond (i.e. cyclic dodecapeptide, a peptide of 12 amino acids, containing two cysteine residues that form a loop); peptides with two disulfide bonds (e.g. protegrins, a group of five 16-18 residue peptides with two intra-chain disulfide bonds and an amidated C-terminus); peptides rich in tryptophan residues (e.g. indolicidin, a 13-mer containing three proline and five tryptophan residues); short tandem-repeat sequences (e.g. bovine bactenecins Bac5 and Bac7, ovine bactenecins OaBac5 and 7.5, porcine PR-39 and prophenins that are rich in proline residues). Cathelicidins are mainly produced by epithelial cells, neutrophils and macrophages [78]. They have been identified in various classes of vertebrates, from mammals to fishes, amphibians and birds [79-91]. Besides their broad-spectrum antimicrobial activity against Gram-positive and -negative bacteria, also including MDR strains [92], cathelicidins are also effective in killing fungi, parasites and enveloped viruses [93-97].

In humans, the only cathelicidin identified is LL-37, an α-helical AMP expressed by epithelial cells of the skin, gastrointestinal tract, epididymis and lungs, and by circulating neutrophils, monocytes, lymphocytes and myeloid bone marrow cells [98, 99]. Bovine cathelicidins BMAP27 and BMAP28, expressed by bovine neutrophils, are other two well-characterized α-helical peptides. They have potent antimicrobial activity towards bacteria and fungi [100], but display also cytotoxicity against mammalian cells [101].

A wide family of β-sheet-structured peptides is represented by defensins. These AMPs have been found in many animals and plants [102]. Mammalian defensins are arginine- and cysteine- rich peptides of 18-45 amino acids. They are produced by monocytes/macrophages, NK cells, B and T cells, neutrophils, Paneth cells, epithelial cells and dendritic cells [103-105]. Based on the arrangement of the three disulfide bonds between their six conserved cysteine residues, they can be further subcategorised into α-, β- and θ-defensins. In humans, there are six α-defensins (HNP1-4 and HD5-6), differentially expressed in different immune cells, and four β-defensins in leukocytes and epithelial cells [106]. Defensins have a wide spectrum of activity, ranging from Gram-positive and -negative bacteria and fungi, to enveloped viruses [107-109]. Plant defensins are 45-54 amino acids AMPs, with a relatively diverse amino acid composition, assuming a conformation composed of a triple-stranded β-sheet with an α-helix in parallel, with four disulfide bridges [110]. Another class of plant AMPs are thionins, proteins consisting of up to 48 amino acids among which arginine, lysine and cysteine predominate. They assume a secondary structure composed of two antiparallel α-helices and an antiparallel double-stranded β-sheet with three or four conserved disulfide linkages [111].

Also bacteria produce AMPs, called bacteriocins. As obvious, each bacterial species produces peptides harmful for other bacteria but not for the producer strain. They are classified into Class I (lanthionine-containing), Class II (non-lanthionine containing) and bacteriolysins (non-bacteriocin lytic proteins) [112]. Several bacteriocins have been studied for the treatment of antimicrobial-resistant bacteria and as food preservatives [113].

Importantly, increasing evidence demonstrate how AMP deficiency, disregulation, or overproduction can aggravate a pre-existing pathological condition and contribute to disease [114]. One example is that of morbus Kostmann, a severe congenital neutropenia, in which the deficiency of LL-37 and α-defensins lead the patients to suffer from recurrent infections and periodontal diseases [115]. On the opposite, a well-known situation in which AMP overexpression is at the basis of a disease is that of psoriasis. It has been demonstrated that patients suffering of psoriatic disease have an abnormal production of AMPs, especially LL-37, β-defensins and S100 proteins, in their keratinocytes [116]. Moreover, LL-37 has been proposed as an autoantigen in psoriasis patients, triggering the activation of T-cells, which in turn produce pathogenic cytokines [117].

1.4 Antimicrobial properties of AMPs

Generally, AMPs target bacterial membrane, and this is the reason for their scarce propensity to induce antimicrobial resistance, because bacteria should mutate generalised structures such as the components of their membranes.

The positive net charge of AMPs determines higher affinity for microbial membranes, that are negatively charged, rather than for zwitterionic mammalian membranes, in which the negative phospholipid head groups are directed towards the interior. Moreover, their hydrophobicity promotes the interactions with the fatty acyl chains. Usually AMPs exert their antimicrobial activity by direct killing of the target microorganism. This is due mainly to their capacity to disrupt the bacterial cytoplasmic membrane, but some AMPs could also interfere with intracellular processes such as DNA and protein synthesis, protein folding, enzymatic activity and cell wall synthesis by binding to negatively charged molecules such as nucleic acids and phosphorylated proteins [118] (Tab. 1.4 and Fig. 1.5). Recently, it has been proposed that an outer membrane protein expressed by Gram-negative bacteria can interact specifically with α-helical AMPs, acting as a receptor for these AMPs [119-121].

An example of AMPs acting on intracellular targets is that of apidaecin and other proline-rich AMPs, such as Bac7, which are able to penetrate into the cytoplasm of bacterial cells without membrane permeabilization and to interact with some molecular targets crucial for bacterial growth, finally leading to cell death [122]. The translocation, at least in these cases, is likely mediated by SbmA, an inner membrane protein predicted to be part of an ABC transporter, that possesses high affinity for proline-rich peptides [123, 124]

Major target	**Specific target/mode of action**	**Example peptides**[a]	**Refs**
External proteins	Autolysin activation	Pep5, nisin	[10]
	Phospholipase A2 activation	Magainin 2, indolicidin	[10]
Outer surface lipids	LPS permeabilization (Gram-negative)	Cecropin P1	[69]
	Lipid II (peptidoglycan precursor)	Defensins, Nisin and other lantibiotics	[34, 35] [70]
Outer membrane proteins (Gram-negative)	Outer membrane protein I (OprI)	SMAP-29, CAP-18	[71]
	LPS-assembly protein D (LptD) inhibition	Protegrin I peptidomimetic analogs	[33]
	Outer membrane protein F (OmpF)	HP(2–20) analog	[72]
Inner membrane	Barrel-stave pore	Alamethicin	[10]
	Detergent micellization	Dermaseptin, cecropin	[10]
	Toroidal pore	Magainin 2, melittin protegrin I	[10] [31]
	Disordered toroidal pore	Magainin analog, melittin	[22]
	Membrane thinning/thickening	PGLa, LL-37	[21]
	Charged lipid clustering	Magainin analogs, Arg-rich peptides	[26]
	Non-bilayer intermediate formation	Gramicidin S	[73]
	Oxidized phospholipid targeting	Temporin L, indolicidin	[27]
	Anion carrier	Indolicidin	[49]
	Non-lytic membrane depolarization	Bovine lactoferricin, daptomycin	[32] [74]
	Electroporation	NK-lysin	[42]
Integral membrane proteins	Proton translocation-related proteins	Clavanin A	[28]
Nucleic acids	DNA (general)	Buforin 2, tachyplesin, indolicidin	[10]
	DNA (covalent interaction)	Indolicidin	[50]
	Branched DNA	WRWYCR	[43]
	RNA (general)	Buforin 2	[10]
Intracellular proteins	DnaK inhibition, GroEL chaperonin	Pyrrhocoricin, drosocin, apidaecin	[10]
	20S proteasome, SH3-containing proteins	PR-39	[46] [47]

[a] Peptides listed are not limited to their associated modes of action here and may employ multiple mechanisms simultaneously.

Table 1.4. Different modes of action of AMPs (modified from Nguyen et al., 2011; refer to the original publication for references)

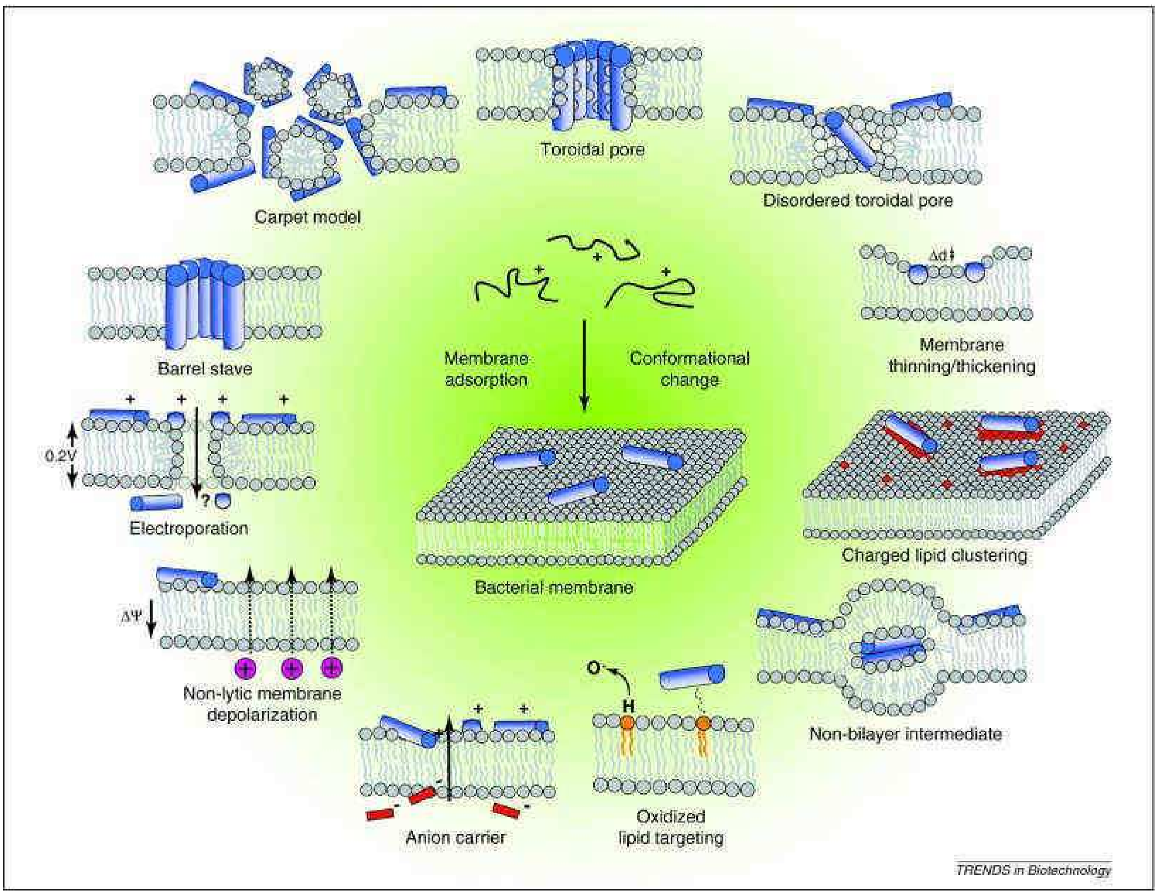

Figure 1.5. AMP action on bacterial membrane. Upon the reaching of threshold concentration and AMP alignment on membrane surface, different events can occur. Peptides can insert across the membrane and lead it to collapse into micelles (carpet model), or they form a membrane-spanning pore (barrel-stave model), or a pore delimited by lipid headgroups and associated peptides (toroidal pore model). The disordered toroidal pore model entails the involvement of fewer peptides, with a stochastic formation of the pore. Anionic lipid clustering causes the leakage of intracellular contents or depolarization. Targeting of oxidized lipids can enhance AMP adsorption to the membrane. Some AMPs are able to determine a potential dissipation without evident damage by binding to small anions across the bilayer. The accumulation of peptides can render the membrane transiently permeable by an increase of potential (electroporation model). AMPs can also affect membrane thickness or induce non-bilayer intermediates. (modified from Nguyen et al., 2011)

The amphipathic α-helix found in many AMPs is the major determinant for the interaction with and lysis of microbial membranes. Hydrophobicity and the propensity to assume this structure determine the selectivity towards bacterial membranes, because too high hydrophobicity and stable helixes can become cytotoxic to mammalian cells [125, 126]. Different models have been proposed for membrane permeabilization exerted by these peptides. All these models necessitate the reaching of a certain threshold concentration of AMPs, which first align on membrane surface thanks to their positive charge, thus allowing the hydrophobic part to interact with the lipid bilayer and insert into it. In the barrel-stave model, peptides aggregate and reorientate, inserting into the membrane with their hydrophobic regions aligned with acyl fatty chains and hydrophilic regions towards the interior, forming a membrane-spanning pore [127]. In the toroidal pore model peptides lying on the membrane force the lipids to fold inwards, forming a pore delimited by lipid headgroups and associated peptides [128]. A revised "disordered" toroidal pore model entails the stochastic formation of the pore, involving fewer peptides [129, 130]. The carpet model was first described for dermaseptin, and entails the association of AMPs on the outer leaflet of the membrane until they reach a critical concentration at which membrane loses electrostatic interactions, collapsing into micelles [131, 132]. There could also occur an AMP-induced clustering of anionic lipids, causing the leakage of intracellular contents or depolarization (lipid segregation) [133]. Targeting of oxidized lipids on the membrane can enhance AMP activity [134]. Some AMPs, such as buforin, can translocate intracellularly without causing membrane disruption and accumulate in the cytoplasm interacting with nucleic acids [135]. Most β-sheet AMPs induce membrane disruption forming toroidal pores or act by non-lytic mechanisms [128, 136-140]. Extended peptides principally act by intracellular targeting [141, 142]. AMPs can cause thinning or remodelling of the membranes; some AMPs are able to bind to small anions across the bilayer, determining a dissipation of membrane potential without evident damage [118]. Conversely, the membrane could become transiently permeable by an increase of potential induced by the accumulation of peptides (electroporation model) [143]. In general, the combination of a cationic region together with an imperfect amphipathicity frequently results in broad spectrum activity [144].

Recently, AMPs elicited interest for their possible use against biofilms. Indeed, they possess characteristics that an ideal antibiofilm drug should have. First, thanks to their permeabilizing mechanism of action, they display a rapid killing ability. Considering that an intact membrane is necessary for bacteria survival, AMPs could kill also slow growing cells such as those present in biofilms. As mentioned before, their mechanism of action renders them less prone to induce resistance, being at the same time active against multidrug-resistant strains. Moreover, AMPs often act also in different modes, i.e. interfering with metabolic processes or intracellular targets; this not only lowers the incidence of resistance, but also allow them to exert their action on different biofilm sub-populations and microbial species. Additionally, environmental factors present specifically in the biofilm or surrounding milieu, such as acidic pH, can modulate the activity of some AMPs [145-147]. For some AMPs there is evidence for their ability to interfere with QS, biosynthesis of ECM or regulatory pathways [148-151]. Figure 1.6 highlights some of the ways AMPs can interfere with biofilm formation, and table 1.5 lists some peptides demonstrating antibiofilm activity [152, 153]. Not all AMPs are equally active against biofilms and the different microbial species they are composed of, and therefore they could be used in combination with molecules that possess different specificity and mode of action. Many studies were performed to find synergistic effects of AMPs with other compounds, because this would permit to reduce possible side effects and lower active antimicrobial concentration, and therefore the amount administered. [152, 154-156].

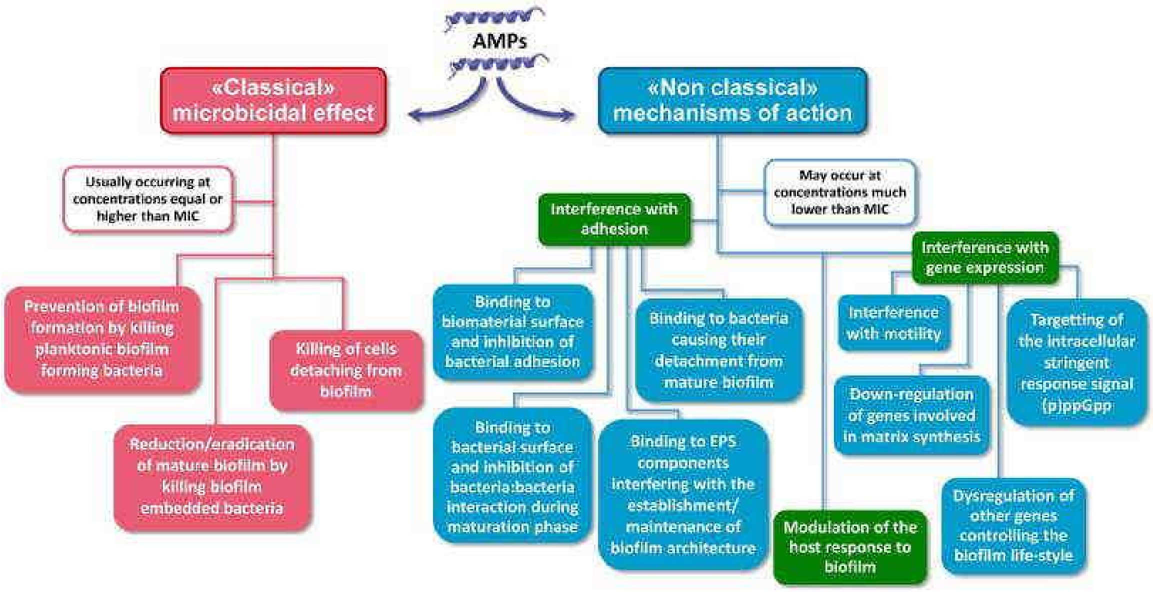

Figure 1.6. Mechanisms of AMP interference with biofilm formation (modified from Batoni et al., 2015)

Name	Organism	Antibiofilm activity A	G	P	Minimal active concentration (μM*)	Reference
MUC7 20-mer	*S. mutans*			X	25	Wei *et al.* (2006)
C16G2	*S. mutans*			X	25	Eckert *et al.* (2006b)
M8-33	*S. mutans*			X	25	Eckert *et al.* (2006b)
M8G2	*S. mutans*			X	25	Eckert *et al.* (2006b)
AAP2	*S. mutans*			X	40	Li *et al.* (2010)
Lys-a1	*S. mutans*		X		7.55	Da Silva *et al.* (2013)
Indolicidin	*S. aureus*		X		0.08	Mataraci & Dosler (2012)
				X	335.78	
		X			0.84	
Nisin	*S. aureus*		X		0.05	Mataraci & Dosler (2012)
				X	183.09	
		X			0.46	
Cecropin A (1-7)-Melittin	*S. aureus*	X			0.45	Mataraci & Dosler (2012)
			X		0.04	
				X	361.55	
$F_{2,5,12}W$	*S. epidermidis*		X		10	Molhoek *et al.* (2011)
				X	10	
Hepcidin 20	*S. epidermidis*	X			50	Brancatisano *et al.* (2014)
G10KHc	*P. aeruginosa*			X	23.45	Eckert *et al.* (2006a)
1026	*P. aeruginosa*		X		1.27	De la Fuente-Núñez *et al.* (2012)
1037	*P. aeruginosa*		X		4.07-123.70	De la Fuente-Núñez *et al.* (2012)
LL-25	*P. aeruginosa*		X		10	Nagant *et al.* (2012)
LL-31	*P. aeruginosa*		X		5	Nagant *et al.* (2012)
LL7-37	*P. aeruginosa*		X		5	
NRC-16	*P. aeruginosa*		X		16	Gopal *et al.* (2013)
KR-12	*A. baumannii*	X			81.48	Feng *et al.* (2013)
			X		81.48	
KR-20	*A. baumannii*	X			25.94	Feng *et al.* (2013)
			X		25.94	Feng *et al.* (2013)
KS-30	*A. baumannii*	X			17.57	Feng *et al.* (2013)
			X		17.57	Feng *et al.* (2013)
KSL-W	*C. albicans*		X		19.12	Theberge *et al.* (2013)

A, inhibition of adhesion; G, inhibition of growth; P, eradication of preformed biofilm.
*Minimal concentration determining 50% reduction of biofilm compared with untreated control.

Table 1.5. Biofilm-active AMPs (modified from Di Luca et al., 2014; refer to original publication for references)

1.5 Immunomodulatory properties of AMPs

Apart from their direct antimicrobial activity, AMPs display also immunomodulatory properties, mediated by the interaction with immune cells such as monocytes, dendritic cells, T cells, and epithelial cells. Indeed, AMPs can alter inflammatory signalling pathways, therefore modulating pro- and anti-inflammatory responses [157]. Moreover, they can recruit effector cells by acting as chemokines themselves or inducing chemokine production [158, 159] and enhance intracellular and extracellular bacterial clearance [160]. Among the properties of AMPs there is the capability to induce macrophage differentiation and the maturation of polarized dendritic cells [161]. Other properties include the promotion of wound healing, modulation of autophagy, apoptosis and pyropoptosis (Figure 1.7) [162-167].

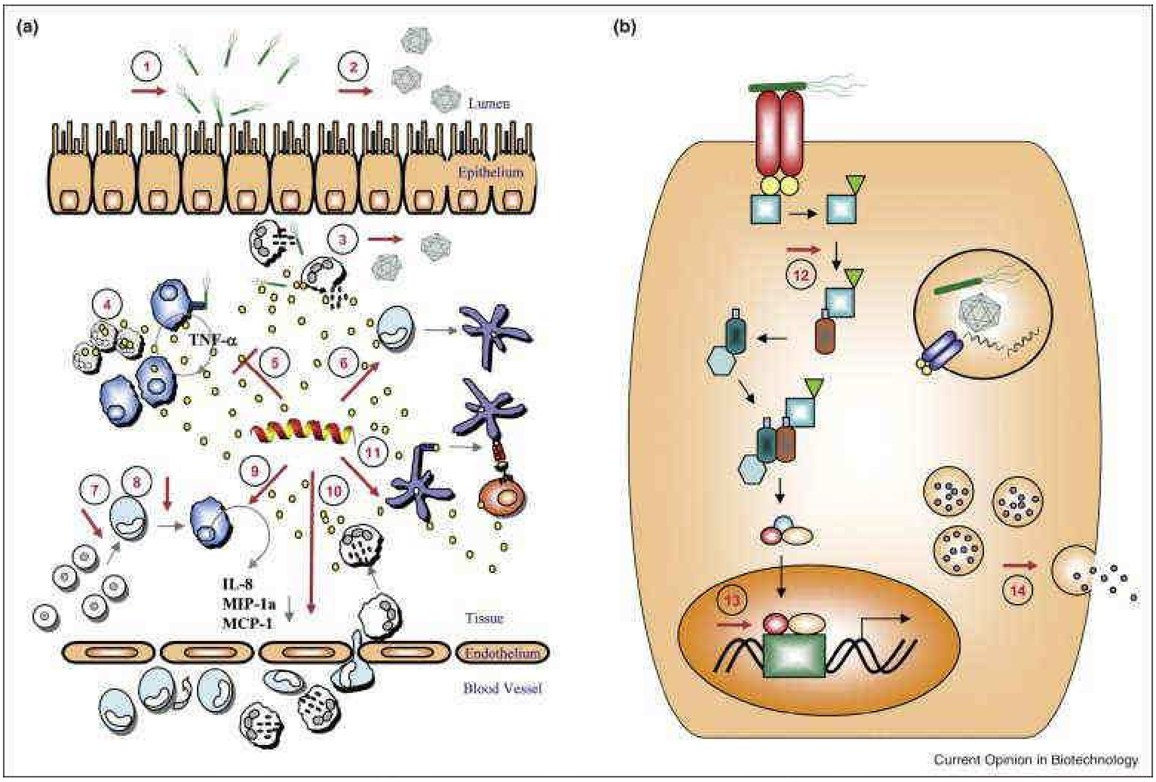

Figure 1.7. Immunomodulatory properties of AMPs. AMPs can exert their immunomodulatory effects at systemic (a) or single-cell (b) level. They can impede the interaction between host cell membranes and invading bacteria (1) or viruses (2). AMPs can stimulate the release of neutrophil secretory vesicles content (3) or block the expression of pro-inflammatory molecules and inhibit neutrophil apoptosis (4 and 5). Peptides can drive the differentiation of dendritic cells (6) and enhance their expression of co-stimulatory molecules and IL-12 (11). AMPs are able to stimulate the maturation and differentiation of monocytes to macrophages (7 and 8) and their release of effector molecules (9). AMPs, along with cytokines, stimulate chemotaxis and migration of leukocytes towards the infection site (10). AMPs can act at various levels in the signalling cascade of immune cells (12), altering gene transcription (13), production of many effector molecules and cellular degranulation (14). (modified from Hamill et al., 2008)

The immunomodulatory properties of AMPs have been studied in particular in the human context. Hence, extensive studies have been done on human defensins and LL-37, the sole human cathelicidin. This latter peptide has demonstrated a large plethora of immunomodulatory effects. Therefore, the presented disquisition on AMP immunomodulatory properties will focus on the effects observed for LL-37, with some exceptions.

One of the first immunomodulatory properties observed for LL-37 was its ability to exert a regulatory activity on immune responses, balancing between pro- and anti-inflammatory effects. In fact, it can lower proinflammatory responses to lipopolysaccharide (LPS) and lipoteichoic acid (LTA), responsible for septic shock, in particular cytokine production. Direct binding to those amphiphilic, negatively charged, bacterial components, is not the only way by which LL-37 exerts this action [168, 169]. Indeed, it can modulate TLR-mediated responses, in particular NF-κB pathways and inhibiting the translocation of this regulator to the nucleus [157], but also it can bind to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to the promotion of p38 mitogen-activated protein kinase (MAPK)- activated responses [170]. On the other hand, by interacting with multiple pathways, such as phosphoinositide 3-kinase (PI3K), MAPK, and NF-kB, LL-37 can enhance the production of interleukin (IL)-1β, to which it synergizes to enhance the recruitment of monocytic cells [171].

LL-37 can enhance chemotaxis both in an indirect and direct mode. Indirectly, chemotaxis can be promoted through the enhanced expression of chemokines such as CXCL8/IL8 and CCL2/MCP1 by monocytes/macrophages, epithelial cells, mast cells, and T cells [158]. LL-37 can act also as chemokine itself, attracting neutrophils and eosinophils, but this effect occurs *in vitro* only at high concentrations, well above physiological ones [159]. Looking to other peptides, it has been shown that the intraperitoneal injection of BMAP28 in mice promotes neutrophil recruitment and increases their phagocytic ability [172]. In addition, also defensins have been demonstrated to be chemotactic for monocytes, polymorphonuclear leucocytes and T-Cells [173].

Besides direct bacterial killing, LL-37 has been demonstrated to exert its antimicrobial activity through neutrophil- or mast cell- derived extracellular traps (NETs and MCETs). These structures are complexes of extracellular components such as DNA and granular antimicrobial molecules, which forms with the expulsion of nuclear components and proteins from granules acted by neutrophils and mast cells upon stimulation by bacteria, activated platelets, inflammatory stimuli [160]. LL-37 and HNPs have been found among the components of these extracellular traps [174]. Within NETs bacteria were thought to growth in sessile mode, so that AMPs probably can kill bacteria by means of their antibiofilm activity [150, 175].

Interestingly, LL-37 was also found to be able to induce dendritic cells and macrophages differentiation, bridging innate and adaptive immune system. LL-37, used in combination with GM-CSF and IL-4 *in vitro*, can enhance the

endocytic activity of dendritic cells and their costimulatory molecule expression, and modify the expression of phagocytic receptors and their function [161].
Besides the action on the innate immune system, LL-37 and its murine analogue, CRAMP, have been demonstrated to exert a direct effect on cells of adaptive immunity such as B and T cells, and the ability to modulate IgG production and stimulate the recognition of bacterial DNA by peripheral blood mononuclear cells (PBMCs) [176, 177].

LL-37 can aid wound healing, triggering the reconstitution of ECM by inducing chemotaxis of epithelial cells and the production of metalloproteinases [178]. On the other hand, its deficiency reduce the re-epithelialization of chronic wounds [166]. This activity seems to be correlated with an activation of PI3K/AKT and MAPK pathways, mediated through epidermal growth factor receptor (EGFR) and formyl peptide receptor-like-1 (FPRL1) [179, 180]. Also β-defensins can stimulate keratinocyte migration by the activation of STAT1 and STAT3 pathways through EGFR [181].

Autophagy consists in the degradation and recycling of cellular components to preserve cellular energy, and it has been proposed to be activated also by intracellular pathogens [182]. LL-37, through the mediation of vitamin D3, is able to induce autophagy by activating autophagy-controlled genes [183].

LL-37 can also modulate apoptosis, (non-inflammatory programmed cell death) both in a positive and negative manner, depending on the cellular type. Indeed, it can stimulate apoptosis in infected epithelial cells, leading to an enhanced clearance of pathogens, while inhibit it in neutrophils through purinoceptor 2 (P2X7) and G-protein-coupled receptors, promoting immune response [165]. LL-37 can also inhibit LPS/ATP-induced pyropoptosis (cellular death induced by pathogen- and damage-associated molecules) in macrophages [167].

Table 1.6 summarizes immunomodulatory activities of mammalian AMPs [184].

Cell or tissue type	Peptide production and activity	References
Hematopoietic cells		
Monocytes and macrophages	LL-37 and β-defensins are monocyte chemoattractants *in vitro* and *in vivo*. LL-37 has anti-endotoxic activity, induces chemokine production, promotes IL-1β secretion, but inhibits inflammatory responses to certain	[32, 61, 62]
Neutrophils	LL-37 and defensins are produced by neutrophils, stored within neutrophil granules and play an important microbicidal role in phagolysosomes. When released extracellularly, LL-37 acts as a neutrophil chemoattractant, inhibits neutrophil apoptosis, reduces pro-inflammatory cytokines and promotes both chemokine induction and the antimicrobial functions of	[63, 64]
Mast cells	Mast cells are important producers of LL-37 in the skin. LL-37 and β-defensins are mast cell chemoattractants and promote mast cell degranulation	[65, 66]
Conventional dendritic cells	Defensins and cathelicidins are dendritic cell (DC) chemoattractants. LL-37 promotes differentiation of monocyte-derived DCs, but inhibits DC maturation and activation by TLR-ligands. β-Defensin 2 might promote DC activation as an endogenous TLR4 ligand. The adjuvant activities of defensins and cathelicidins in vivo might be mediated in part through their activity on DCs	[67 - 69]
Plasmacytoid dendritic cells	LL-37 in complex with DNA oligonucleotides strongly induces IFNα production by plasmacytoid DCs. This activity might contribute to the pathology of psoriasis	[70]
Epithelial cells		
Keratinocytes	LL-37 promotes keratinocyte migration and production of IL-8, inhibits keratinocyte apoptosis, modulates responses to TLR ligands, and might have wound healing activities in the skin. Altered proteolytic processing of hCAP18 and LL-37 has been implicated in the pathology of rosacea	[54, 71, 72]
Bronchial epithelium	LL-37 acts on bronchial epithelial cells to stimulate cytokine and chemokine production and promote apoptosis	[73, 74]
Intestinal epithelium	α-Defensins are produced by Paneth cells and their microbiocidal activity plays an important role in the immune defenses of the gut. Reduced α-defensin production might contribute to Crohn's disease. LL-37 promotes mucin production and survival of intestinal epithelial cells	[75, 76]
Other cells		
Vascular endothelium	LL-37 induces activation and proliferation of vascular endothelium, promoting angiogenesis	[77]
Mesenchymal stromal cells	LL-37 acts as a chemokine for mesenchymal stromal cells and promotes the production of various cytokines, as well as VEGF and MMP2; this can contribute to angiogenesis and tumor progression	[57]
Cancer cells	LL-37 promotes migration and proliferation of lung, ovarian and breast cancer cells and LL-37 production by cancer cells in vivo promotes tumor growth. However, LL-37 also augments the anti-cancer therapeutic activity of CpG oligonucleotides	[78, 79]

Table 1.6. Immunomodulatory properties of mammalian AMPs (modified from Easton et al., 2009; refer to original publication for references)

In the perspective of using AMPs in implant surgery, it would be important to remember that LL-37 has been demonstrated to promote the differentiation of monocytes into bone-forming cells and induce the expression of bone morphogenetic proteins in macrophages [185, 186]. Moreover, it has demonstrated to be able to block osteoclastogenesis, at least *in vitro* [187]. In addition, it has been shown that human β-defensins can differently stimulate the proliferation of MG-63 osteoblast-like cell line *in vitro*, and they may positively affect the differentiation of these cells [188].

1.6 The potential of AMPs as therapeutic agents

The use of AMPs in clinical sets is particularly attractive. In fact, during the last 30 years, there has been a discovery void in antibiotics (Fig. 1.8) [189]. At the same time, pathogens increasingly acquired mechanisms of drug resistance, leading to the risk of returning to a pre-antibiotic era [189]. In this scenario, the use of AMPs has been regarded with even much interest, for their broad-spectrum activity, diverse potential applications, i.e. antibiotics, alone or in combination, immunomodulators, endotoxin-neutralizing compounds, but also for their scarce propensity to induce antimicrobial resistance. Concerns have been proposed for the possibility that the acquisition by pathogens of mechanisms of AMP resistance could lead to a reduced susceptibility to the innate immune system. However, resistance selection for AMPs is far more slow than for conventional antibiotics. There is a study demonstrating the acquisition of resistance of *E. coli* and *P. fluorescens* to Pexiganan in 6-700 generations when exposed continuously to elevated doses of AMP [190]. However, this scenario is quite different to the real one, and it is not possible to estimate the time required for resistance acquisition. Moreover, the use of peptides in over-the-counter products (polymyxin B, gramicidin S) and foods (nisin) did not affect the susceptibility of microorganisms towards neither peptides themselves nor towards the immune system [191].

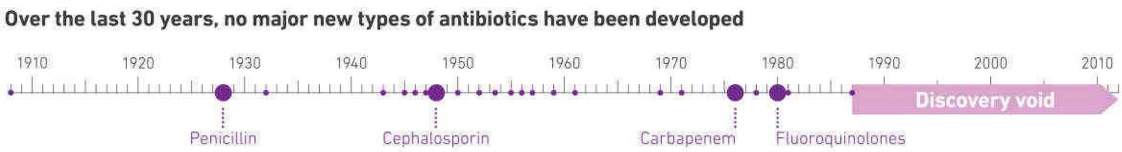

Figure 1.8. Time-line of antibiotic development. (modified from http://www.who.int/topics/drug_resistance/en/)

Table 1.7 enlists some of the advantages and disadvantages of AMPs over conventional antibiotics [192].

Property	Conventional antibiotics	Cationic antimicrobial peptides
Spectrum of activity	Bacterial infections (often selective)	Bacterial, fungal and viral infections; septicaemia; and/or inflammation
Uptake	Specific mechanisms	Relatively non-specific: based on charge. Self-promoted uptake
Targets	Usually one dominating target or class of targets (e.g. penicillin-binding proteins, topoisomerases, ribosomes)	Relatively less specific (possibly multiple targets in any given cell)
Resistance rate and mechanism	Resistance development at frequencies of 10^{-7} to 10^{-10}, or after a few passages at sub-MIC. Resistance caused by mechanisms such as reduced uptake or increased efflux, chemical modification or degradation of antibiotic, or altered target	Resistance generally cannot be directly selected. Needs multiple passages on sub-MIC concentrations to induce resistance. Resistance caused by mechanisms such as an impermeable outer membrane or specific proteases (can be overcome by incorporating D-amino acids or backbone alterations)
Additional activities	No	Include anti-endotoxic and/or boosting of innate immunity
Pharmacokinetics	Varies but once per week antimicrobials under development	Short systemic half-life owing to proteolytic degradation
Toxicology	Antibiotics tend to be one of the safest groups of pharmaceuticals	No known topical toxicities; systemic toxicity issues remain undefined
Manufacturing costs	Can be inexpensive (e.g. $0.8 per gram for aminoglycosides)	Expensive ($50–400 per gram)

Table 1.7. Differences between AMPs and conventional antibiotics (modified from Marr et al., 2006)

Although AMPs display many advantages over conventional antibiotics, several drawbacks have slowed their development as therapeutics [192]. The first great impediment that strongly limits the possibility of AMPs to substitute conventional antibiotics is the high cost of production. It has been calculated that a gram of a synthetic AMP would cost about $50-400, five to twenty times the cost of conventional antibiotics. Considering that the daily dose for AMP treatment would be 1mg/kg, the cost of an entire cycle of treatment would result very high, strongly impacting on healthcare system and/or patient finances [193]. However, the increasing interest into this type of molecules for therapeutic use, the improvement of synthesis technologies (for example microwave-based peptide synthesis), and the investment by big companies would permit to reduce costs of reagents and procedures, rendering industrial production more feasible. An alternative production method explored in the past years is recombinant expression in microorganisms resistant to the produced peptide. Costs significantly lower but, for the required yields, million litres of fermentation mixture are required, rendering this way less affordable [194].

Another concern on the use of AMPs as therapeutics is toxicity, being these compounds active on the membranes. Peptides are selective for bacterial membranes for their composition, i.e. the presence of negatively charged phospholipids, membrane potential and the absence of cholesterol, but this selectivity is not complete. So, peptides, in particular if administered systemically, could be harmful also for host cells. In this regard, peptide modifications in order to improve the therapeutic index have been done. In general, the haemolytic activity of AMPs is correlated to high hydrophobicity, amphipathicity and helicity, while bactericidal activity is less affected by these parameters [195, 196]. Being some AMPs able to translocate into the cells for their similitude to nuclear localization signal peptides, also other toxicities not related to membrane interaction should be considered, for example the possibility to induce apoptosis, mast-cell degranulation or extracellular DNA transfer [54].

Also pharmacokinetic and pharmacodynamic matters should be taken into consideration. In particular, the tendency of some peptides to aggregate, their sensitivity to salt and serum conditions, and in particular their short half-life *in vivo*, due to proteolytic degradation. For these reasons, most of the clinical trials involving AMPs regarded their topical use [193]. To address this issue, many strategies have been proposed, intervening both on formulation and on peptide structure [197, 198]. Amongst various modification proposed, introduction of D-amino acids, amidation at the N-terminus, the use of non-natural amino acids, use of peptidomimetics, cyclization or a combination of them are the mostly explored solutions. Table 1.8 resumes some of the strategies used for the development of AMPs as therapeutics [54].

Activity	Critical need	Strategies
Screening	Larger variety of peptides	Peptide arrays for increased diversity New natural lead molecules Peptide-like (mimetic) approaches Non-natural amino acids Screen for both antimicrobial and immune-modulating activities
Toxicity	Understand mechanisms of toxicity	Toxicology in animal models Assess subtle toxicities Toxicogenomics
Pharmacology	Improve half-life *in vivo*	Peptidomimetics Modified and/or d-amino acids New formulations (for example, liposomal) Immune-modulating peptides may not require regular dosing
Cost of goods	Lower the cost of expensive therapeutics	Make shorter analogs that work Recombinant manufacturing processes Natural sources (for example, lantibiotics) Immune-modulating peptides may require smaller doses Local administration
Efficacy	Improve activities in the context of model infections	Realistic animal models of disease In vitro assays should match *in vivo* realities (for example, physiological conditions) Develop *in vitro* methods of predicting effective immune modulation

Table 1.8. Approaches for the design and development of AMPs (modified from Hancock et Al., 2006)

For all these reasons, only few AMPs have reached clinical trials and are in clinical development (Tab. 1.9) [199]. An emblematic case is that of Pexiganan (Magainin Pharmaceuticals, since renamed Genera). This analogue of magainin 2 was the first AMP developed commercially for the topic treatment of diabetic-foot ulcers under the name of Locilex™. Phase III clinical studies demonstrated its efficacy in wound healing, with very few toxicity [200]. Despite this, in 1999 FDA rejected the approval of its commercialization because it did not demonstrate sufficient advantages over the standard cares. To date, the most advanced antimicrobial peptides are Omiganan pentahydrocloride, an indolicin-based AMP developed by Migenix (Canada) for the prevention of catheter-related infections [201-203], and $rBPI_{21}$ (Opebacan, NEUPREX®), a modified recombinant fragment of BPI proposed in an injectable formulation for the treatment of meningococcal sepsis [204].

Further effort for the development of effective and stable formulations for AMPs is surely needed to render these clinically attractive molecules products for the pharmaceutical market.

Synthetic AMP	Parent AMP/Source	Clinical Use	Stage of Clinical Trial	References
Pexiganan acetate (MSI-78)	Manganin derivative	Antinfective, treatment of diabetic foot ulcers, used as topical antibiotic	Phase IIII	[294]
hLF1-11 (AM Pharma)	Derivative of lactoferricin	Fungal and bacterial infections that develop during neutropenia in haematopoietic stem cell transplants (HSCT)	Phase I/ II	[299, 302] (https://clinicaltrials.gov/ct2/show/NCT00430469)
Omiganan pentahydrocholoride	Indolicidin derivative	Bactericidal, antifungal, catheter associated infections Papulopustular Rosacea	Phase IIII b In Phase IIII (rosacea)	[295] http://clinicaltrials.gov/ct2/show/NCT01784133) NCT01784133 [437]
Opebacan	BPI derivative	Endotoxaemia	Phase I/II	[325]
Iseganan (IB-367)	protegrin-1 derrivative	In treatment of oral mucositis of patients receiving radiation therapy in head and neck cancer.	Phase IIII	[288, 328]
Brilacidin/PMX-30063 (PolyMedix)	Defensin mimetic	Used in treatment of Acute Bacterial Skin and Skin Structure Infections (ABSSSI) by *S. aureus*	Phase II	[288]
PAC113 (Pacgen Biopharmaceuticals)	Deived from Histatin5 active peptide	In treatment of oral candidiasis in HIV seropositive individuals	Phase IIb	[303] https://clinicaltrials.gov/ct2/show/ NCT00659971
rBPI21	BPI derivative	Meningitis	Phase IIIb	[298]
AP214	α-MSH derivative	Sepsis, postsurgical Organ failure	Phase II	[327] https://clinicaltrials.gov/ct2/show/ NCT00903604
CZEN-002	Derived from melanocyte stimulating hormone	In treatment of vulvovaginal candidiasis	Phase IIb	[310-312]
RDP58 (Genzyme)	Derivative of HLA class I, semisynthetic peptide only immunomodulatory in nature	Oral therapy for IBD	Phase II trial through	[335]
DiaPep277 (DeveloGen)	Immunomodulatory in nature, 24 mer synthetic peptide derived from dominant autoantigen of HSP60	Diabetes mellitus Type1	Phase II complete, in Phase III	[337] https://clinicaltrials.gov/ct2/show/ NCT00644501 http://www.isrctn.com/ISRCTN55429664
IMX942 (Inimex)	Inndolicidin and IDR1 derivative	Immunomodulatory function. No antimicrobial property, prevents infection in patients undergoing chemotherapy	Phase II	[438]
EA230 (Exponential Biotherapies)	Tetrapeptide derived from β chain of HCG, anti-inflammatory, antiseptic	Protection against renal failure, prevention of sepsis	PhaseII	[439]

Table 1.9. Synthetic AMPs in clinical trial (modified from Dutta et Al., 2016; refer to original publication for references)

1.7 AMPs in antimicrobial coatings

Due to their abovementioned favourable properties, AMPs have been considered for the incorporation on biomaterial surfaces, to obtain medical devices resistant to infections. Immobilisation can be achieved either by physical methods, such as adsorption or layer-by-layer assembly, or chemical methods, i.e. covalently bound.

In the layer-by-layer approach AMPs are embedded between a variable number of layers composed by polyionic polymers. Despite the high loading reachable with this method, an efficient release method has to be used to permit the diffusion of the peptides residing in deeper layers outwards the material. Moreover, the diffusion is largely influenced by layer factors, such as tortuosity, thickness and the interactions between peptides and polymers [205]. The establishment of a bacterial layer on the top of the surface, for a too low initial loading or other reasons, can further impede the release of the AMP.

The possible scarce long-term stability of materials bearing physically immobilized AMPs can be overcome by using chemical methods for the covalent binding of peptides [206-209]. Moreover, immobilization can help to overcome the short half-life and cytotoxicity of soluble AMPs [210, 211]. Evidently, the surfaces have to bear reactive functional groups to allow the linkage; non-reactive surfaces can be functionalized with appropriate reactive groups, and eventually a spacer can be added. Table 1.10 gives an overview of the reported AMP immobilization strategies [212]. One of the most explored methods to coat surfaces with reactive groups is the use of self-assembled monolayers (SAMs). This technique allows the functionalization with the required reactive group and spacers of optimized length.

AMP	Substrate	AMP immobilization strategy	Microorganisms assessed
Magainin 2 and Related synthetic amphiphilic peptides	Polyamide resin (pepsin K)	Directly synthesized on polyamide resin, after immobilization through their C-terminal amino acids. •AMP orientation was controlled •Short spacer, with 2- or 6-carbon chains, was used •Stability to heat was studied •no AMP release was observed	*Escherichia coli* ATCC 35695; and ATCC 25922, *Staphylococcus aureus* ATCC 25923 and ATCC 6538, *Klebsiella pneumoniae* ATCC 4352, *Bacillus subtilis* ATCC 6051, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 6275, and *Pseudomonas aeruginosa* ATCC 27853.
Magainin I	Non-fouling copolymer brushes based on different percentages of:2-(2-methoxyethoxy)ethyl methacrylate (MEO_2MA)/hydroxyl-terminated oligo(ethylene glycol) methacrylate (HOEGMA)	Peptide immobilized by its C-terminal amino acid The process consisted of a previous incorporation of a cysteine residue on the C-terminal of magainin, to be reacted with the polymeric brushes via PMPI (*N*-(*p*-maleimidophenyl)isocyanate) •AMP orientation was controlled •Brushes were used as spacers •Different AMPs densities was tested	*Listeria ivanovii*, *Bacillus cereus*
Magainin I	Mixed OH/COOH-terminated self-assembled monolayers (SAMs)	Immobilization by the free AMP amines after activation of the COOH groups of the SAM with NHS/EDC •AMP orientation was not controlled •No spacers •No AMP release was observed	*L. ivanovii*, *Enterococcus faecalis* and *S. aureus*
Magainin-derived MK5E and KLAL	PEGylated TentaGel S, HypoGel 400 and HypoGel 200 resin beads	C-terminally immobilized peptides were achieved by standard solid-phase peptide synthesis and Fmoc (9-fluorenylmethoxycarbonyl)-chemistry N-terminal and side-chain immobilization were achieved by thioalkylation and oxime formation •AMP orientation was controlled •Different AMPs densities were tested •Effect of PEGylated spacers was tested •The haemolytic effect was assayed	*E. coli* strain DH5α, *B. subtilis* strain DSM 347
Melimine	Commercial contact lenses (etafilcon A)	Immobilization by the free AMP amines using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide •AMP orientation was not controlled •No spacers	*P. aeruginosa* 6294, *P. aeruginosa* ATCC 15442, *S. aureus* Saur31, *S. aureus* CK5, *S. pneumoniae* 010
Melimine	Glass coverslips	Immobilization through the free AMP amines using two different strategies: •Using EDC after previous activation of the OH groups of the glass surface with 4-azidobenzoic acid (ABA) and irradiation with UV-light (320 nm) •As above but using 4-fluoro-3-nitrophenyl azide (FNA) instead of ABA •AMP orientation was not controlled •No spacers •Different AMP densities were tested	*S. aureus* strain 38, *P. aeruginosa* PA01
Cathelin LL37	Silanized titanium surfaces:Using glycidyloxypropyl triethoxysilane (epoxy silane)3-aminopropyl triethoxysilane(amino silane)	•N-maleimidopropionic acid succinimide ester •R-N-hydroxysuccinimidyl-*ö*-maleimidyl-PEG •Effect of AMP orientation was tested •Effect of PEGylated spacers was tested •Different AMPs densities were tested	*E. coli* strain K12
E14LKK	Oxidized polyethylene films (ox-PE)	Immobilization by the terminal AMP amine (using protected E14LKK side chains amines) with and without PEGylated spacer onto ox-PE using 1-ethyl-3-(3-aminopropyl)-carbodiimide •AMP orientation was controlled •Effect of PEGylated spacers was tested	*E. coli* ATCC 25922
FKVKFKVKFK	PEG-PS Resin beads	Peptide–resin conjugates were synthesized by using Fmoc (9-fluorenylmethoxycarbonyl)-chemistry. To investigate the effect of the resin on the activity, the β-sheet peptide was conjugated with MBHA–resin	*S. aureus* ATCC 6538, *Micrococcus luteus* ATCC 9341, *P. aeruginosa* ATCC9027, *E. coli* ATCC 25922
6K8L	PEG-PS resin beads	The peptide was synthesized by solid-phase peptide synthesis on a PEG-modified polystyrene resin (PEG-PS) using Fmoc (9-fluorenylmethoxycarbonyl)-chemistry. The antimicrobial activity of the peptide–resin conjugate was evaluated against different microorganisms.	*B. subtilis* (wild-type PB2, 168 Marburg strain27), *E. coli* O157: H7 ATCC 33150, *Kluyveromyces marxianus*, *L. monocytogenes* ATCC 689426, *P. fluorescence*, *Salmonella typhimurium* H 3380 phage type DT 104, *Serratia liquefasciens* and *S. aureus* ATCC 13566.
122 variant peptides of 2 starting sequences: Bac2A and Indolicidin	Cellulosebifunctional resin TGS (1–6-dichloro-1- Beta-dideoxy-Beta-fructofuranosM-chloro-4-deoxy-galactopyranoside) NH_2/RAM (p-[(R,S)-a-[1-(9H-fluren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phen-oxyacetic acid)Microtiter plate	Peptides were: •directly synthesized on a cellulose support using a cellulose-amino-hydroxypropyl ether (CAPE) linker chemistry or; •directly synthesized onto a bifunctional resin or; •directly bound to the microtiter plate via biotin-streptavidin interaction. •AMP orientation was controlled •No spacers	Mini-Tn5-luxfliC::lux CDABE strain H1001 of *P. aeruginosa* PAO1, *S. aureus* ATCC 25923, *C. albicans* (lab isolate).

Table 1.10. Outline on described AMP immobilization strategies (modified from Costa et Al., 2011)

Generally, covalently immobilised AMPs have lower antimicrobial activity as compared to their soluble counterparts [206, 210, 213]. For this reason, a fine-tuning of some parameters, such as peptide surface concentration, spacer length and flexibility, and AMP orientation, is important to obtain an effective coating.

Regarding surface concentration, besides an adequate density of reactive groups, also coupling conditions and steric hindrance effects are fundamental to obtain a peptide coverage sufficient to exert the antimicrobial effect [214]. However, concentration is not a key factor for the activity of tethered AMPs [210].

Spacer length is often pivotal for the retention of antimicrobial activity. It has been demonstrated that the immobilisation of some AMPs (e.g., LL-37 and nisin) without a spacer completely abolishes their antimicrobial activity [215, 216]. This could be due to the fact that a long spacer should permit peptide interaction and insertion into bacterial membranes. However, it is still not clear if tethered AMPs act as their soluble counterparts by permeabilizing bacterial membranes. Indeed, some peptides, e.g. magainins, linked to a polyamide resin through a short spacer (2-6 carbon atoms), retain their killing ability, probably creating an electrostatic imbalance in bacterial surface [207, 217]. Spacer flexibility is important to permit a sufficient lateral mobility to the bound AMP. A polymer frequently used as spacer both on SAMs and functionalised polymer resins is polyethylene glycol (PEG). It possesses bacterial anti-adhesiveness properties [218-220] and its flexibility permits a high mobility of bound peptides. Moreover, its water-swelling property aids the maintenance of AMP antimicrobial activity [213]. The degradation of the spacer, that can lead to the loss of the active peptide, can be prevented by using stabilised polymers, thus prolonging the efficacy of the biomaterial [221].

The orientation of the immobilised AMP could also influence its activity; in particular, attention should be taken to avoid the attachment of the AMP through a residue important for its activity. Orientation can be controlled by using chemo-selective reactions [222, 223] or by synthesizing the peptide directly on the support [55, 224-226]. Importantly, studies have shown that the ability of the immobilised peptides to assume their secondary structure, i.e. α-helix or β-sheet, is fundamental for maintaining their antimicrobial activity [207, 213].

A fundamental issue in the development of biomaterials for clinical applications, is their biocompatibility. The concept of biocompatibility has been revised several times since the introduction of implantable devices. One of the most complete definitions of biocompatibility refers to it as "[…] the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimising the clinically relevant performance of that therapy" [227]. From this definition it is evident that the absence of toxic effects is only a part of the whole, surely important but not sufficient. Both harmful and favourable effects could depend both on the material itself and its coating, taking also into account the variability among patients, that can modify the response elicited by the implant [228]. In this context, AMPs could be useful tools for improving biocompatibility, not only for their potential to prevent harmful reactions, in particular those triggered by pathogens, but also for their abovementioned ability to perform a beneficial effect on osseointegration.

2. Aim of the study

Health-care associated infections (HCAIs), and in particular foreign body-related infections (FBRIs) are among the most feared complications in clinical settings. These infections are associated with substantial morbidity and high economic burden. In particular in orthopaedics, most of FBRI are caused by biofilm-forming multidrug resistant (MDR) strains of *Staphylococcus* spp, posing relevant challenges to clinical management and urging the need to develop effective methods to prevent implant-associated infection.

The antimicrobial peptides (AMPs) of the innate immunity have increasingly attracted attention as promising candidates in this regard. Indeed, AMPs display potent and broad spectrum antimicrobial activity, also including MDR strains and microbial biofilms. At difference with conventional antibiotics, these molecules show low propensity to select resistant mutants. Additionally, some of them are endowed with immunomodulatory and osteo-inductive properties. This makes AMPs attractive candidates for the development of innovative infection-resistant biomaterials.

This study aimed to investigate the antimicrobial and anti-biofilm activities of selected α-helical AMPs *in vitro* under relevant pathophysiological conditions, and their effects on osteoblast cells, to identify promising candidates for the development of novel AMP-based biomaterials.

Of the five AMPs chosen for the study, we were able to select three peptides with a good antimicrobial activity against *S. epidermidis* and *S.aureus* reference strains and 24 clinical isolates derived from orthopaedic surgery. None of the peptides affected the viability and the differentiation of osteoblast cells in culture. The properties of these peptides were further investigated in conditions mimicking the pathophysiological environment of an orthopaedic implant (i.e. in the presence of human serum, hyaluronic acid and synovial fluid).

One peptide candidate was then selected to evaluate its antimicrobial properties upon immobilization on solid support. To this aim, commercial agarose resin beads, coated with streptavidin, were used, exploiting the high affinity of the biotin-streptavidin interaction and, accordingly, the peptide molecule was biotinylated. In addition, peptide modifications useful for its detection and quantification were performed. The main goal was to verify whether the immobilized peptide holds antimicrobial efficacy and, additionally, to specify the orientation of the immobilized molecule and, possibly, to verify whether a spacer is needed or not.

The appropriately biotinylated peptide derivatives have been immobilized on the streptavidin-coated resin beads and the antibacterial activity and the effects on host cells of the immobilized peptides were assessed.

Based on the results presented in this thesis, a promising peptide candidate suitable for the immobilization onto a clinically-usable biomaterial, for the prevention of biomedical-device related infections, has been identified.

3. Results

3.1 Peptides under study

A preliminary selection among a large number of potential peptide candidates was made to focus the study on a smaller group of the most promising peptides. These should ideally be endowed with potent antimicrobial activity and be devoid of toxic effects towards host cells, at least at microbicidal concentrations. In addition, their amino acid sequences should be easy to assemble by standard peptide synthesis procedures. In order to select the peptides with the best activity against Gram-positive bacteria, in particular *Staphylococcus* species, a database collecting all available data obtained in the research group about more than hundred native and synthetic antimicrobial peptides was first exploited. The survey of the antimicrobial properties of 71 natural AMPs and their derivatives (i.e. 53 cathelicidins, 15 defensins and other 3 peptides), and of 46 *in silico*-designed AMPs [229], led to the selection of two naturally occurring peptides and three synthetic derivatives, which are listed in Table 3.1. BMAP27 and BMAP28 [100] are bovine members of the cathelicidin AMP family. Their antimicrobial spectrum includes Gram-negative and Gram-positive bacteria, also including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecalis* (VREF) strains [230], bovine mastitis isolates [231], and various pathogenic fungal spp [93, 232]. Both are linear peptides with the N-terminal region, encompassing residues 1-18, predicted to assume an α-helical conformation, whereas in the C-terminal region this structure is unlikely due to the presence of proline residues (Fig. 3.1). This C-terminal region is rich in hydrophobic residues and is considered as a major determinant of cytotoxicity towards eukaryotic cells [100]. The Edmundson wheel projections of BMAP27 and BMAP28, reported in Figure 3.1, illustrate the amphipathic character of these two AMPs. BMAP27(1-18) and BMAP28(1-18) are BMAP analogs with sequences corresponding to residues 1-18 of BMAP27 and BMAP28, respectively. Their antimicrobial effects are comparable to those of the parent peptides [100] and, in addition, their cytotoxic potential is greatly reduced by the absence of the C-terminal hydrophobic tail. P19(9/G7) is a synthetic AMP, developed on the basis of a consensus sequence for amphipathic α-helical antimicrobial peptides, by using computer-aided rational design [229]. All the selected peptides are highly cationic and have the C-terminus amidated (Tab. 3.1).

Peptide	Origin	Sequence
BMAP27	cow	GRFKRFRKKFKKLFKKLSPVIPLLHL-NH_2
BMAP27(1-18)	fragment	GRFKRFRKKFKKLFKKLS-NH_2
BMAP28	cow	GGLRSLGRKILRAWKKYGPIIVPIIRI-NH_2
BMAP28(1-18)	fragment	GGLRSLGRKILRAWKKYG-NH_2
P19(9/G7)	in silico-design	GLLKKIGKKAKKALKKLGY-NH_2

Table 3.1. Origin and sequence of the five selected AMPs. The C-terminus is amidated.

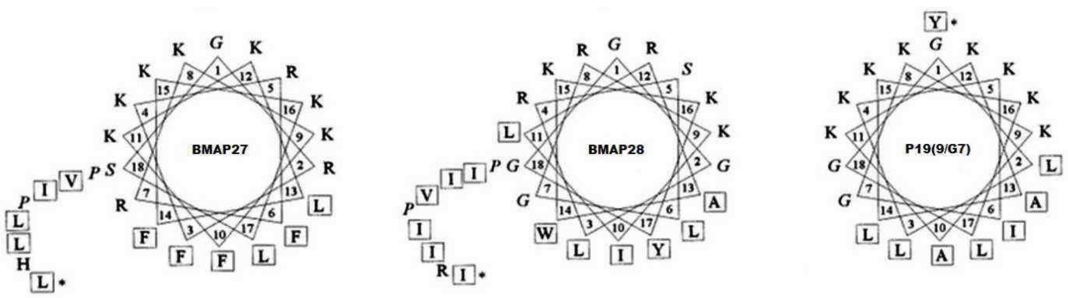

Figure 3.1. Edmundson wheel projections of BMAP27, BMAP28 and P19(9/G7), illustrating the amphipathic character of the α-helical region. The hydrophobic residues are boxed, while the hydrophilic neutral residues are in italics. Asterisk (*) indicates the amidated C-terminus

3.2 Antimicrobial activity in standard conditions

The five selected peptides (i.e BMAP27, BMAP28, BMAP27(1-18), BMAP28(1-18) and P19(9/G7)) where tested *in vitro* for their antimicrobial activity, according to CLSI standards [233], against three reference strains and 27 clinical isolates derived from orthopaedic patients, in comparison with two conventional antibiotics used in orthopaedic surgery, i.e. cefazolin and linezolid. The two antibiotics were chosen for their use in clinical practice and for their different mode of action. In particular cefazolin, which is the most commonly used antibiotic in orthopaedic surgery, is a first-generation cephalosporin antibiotic and therefore acts by inhibiting the synthesis of the bacterial cell wall, resulting in a bactericidal effect [234]. Linezolid, typically used for the treatment of serious infections caused by Gram-positive bacteria that are resistant to other antibiotics, is a member of the oxazolidinone class of drugs and acts as a protein synthesis inhibitor, blocking the initiation of protein production, therefore resulting in most of the cases in a bacteriostatic effect [235].

The reference strains included the biofilm-forming *Staphylococcus epidermidis* ATCC 35984, the non-biofilm-forming *Staphylococcus epidermidis* ATCC 12228, and *Staphylococcus aureus* ATCC 25923. The isolates included 8 *Staphylococcus epidermidis* (one was methicillin-resistant, MRSE), 8 *Staphylococcus aureus*, 1 *Staphylococcus hominis*, 1 *Staphylococcus caprae*, 1 *Staphylococcus capitis*, 2 *Streptococcus agalactiae*, 1 Group G *Streptococcus* and 2 *Enterococcus faecalis*. Data are reported in Table 3.2 as Minimum Inhibitory (MIC) and Minimum Bactericidal (MBC) concentrations. For *S. epidermidis* and *S. aureus* clinical isolates, MIC_{50} and MIC_{90} values are also reported.

All peptides were effective at micromolar concentrations against most strains, with the exception of *E. faecalis*, which was only susceptible to BMAP28. It is interesting to note that all the peptides showed slightly higher MIC values against the biofilm-forming *S. epidermidis* ATCC 35984, with respect to the non-biofilm-producing *S. epidermidis* ATCC 12228. Considering *S. aureus* and *S. epidermidis* species, no significant differences between clinical isolates and the corresponding reference strains were observed. Although a wider MIC range was registered for the three shorter peptides, the MIC_{50} and MIC_{90} values indicate that low micromolar peptide concentrations were effective in most of the cases. All the peptides displayed overall slightly higher MIC values against *S. aureus* isolates and were highly active against the strains of *S. epidermidis*. On a molar basis, the MIC values of the AMPs were in general comparable to those obtained with cefazolin and linezolid. Notably, the MRSE was resistant to conventional antibiotics but susceptible to all the peptides tested (Tab. 3.2, MIC values reported with an asterisk). In most cases the MBC values for the peptides and cefazolin were very close to MIC values, indicating a bactericidal mode of action, while MBC values were not detectable for linezolid, thus confirming its bacteriostatic effect towards these species (Tab. 3.2).

Reference Strains	BMAP27	BMAP28	BMAP27(1-18)	BMAP28(1-18)	P19(9/G7)	Cefazolin	Linezolid
	MIC (µM)						
S. epidermidis ATCC 12228	1	1	1	1	1	1	3
S. epidermidis ATCC 35984	2	2	2	2	4	4	6
S.aureus ATCC 25923	2	2	4	2	16	0,6	6
	MBC (µM)						
S. epidermidis ATCC 12228	1	1	1	1	1	1	48
S. epidermidis ATCC 35984	2	2	2	2	4	8	48
S.aureus ATCC 25923	4	2	8	4	32	0,6	24

Clinical Isolates (n° of isolates)	BMAP27	BMAP28	BMAP27(1-18)	BMAP28(1-18)	P19(9/G7)	Cefazolin	Linezolid
	MIC (µM)						
Staphylococcus epidermidis (8)	0,5-2 (1*)	1-2 (2*)	0,5-4 (1*)	1-2 (1*)	0,5-32 (1*)	0,6-18 (>18*)	<0,4->95 (3*)
Staphylococcus aureus (8)	2-4	1-4	2-16	2-8	16->64	0,6-2	6
Other *Staphylococcus spp* (3)	0,5-1	1-2	0,5-1	0,5-1	0,5-1	0,6-4	3
Enterococcus faecalis (2)	8-32	4	>64	64	>128	>18	6
Streptococcus spp (3)	1-2	0,5-4	1-2	1-2	0,5-2	0,3	1,5-3
	MBC (µM)						
Staphylococcus epidermidis (8)	1-2 (1*)	1-2 (2*)	0,5-4 (1*)	0,5-4 (1*)	0,5-64 (0,5*)	0,6-18 (>18*)	n.d.
Staphylococcus aureus (8)	2-4	1-4	2-16	2-8	16->64	1-4	n.d.
Other *Staphylococcus spp* (3)	0,5-1	1-2	0,5-1	0,5-1	0,5-1	1->18	n.d.
Enterococcus faecalis (2)	8->32	4	>64	>64	>128	>18	n.d.
Streptococcus spp (3)	1-2	2-4	1-2	1-2	0,5-2	1	n.d.
	MIC_{50} (µM)						
Staphylococcus epidermidis (8)	1	2	1	1	1	2	3
Staphylococcus aureus (8)	2	2	4	4	16	1	6
	MIC_{90} (µM)						
Staphylococcus epidermidis (8)	1	2	1	1	2	9	6
Staphylococcus aureus (8)	2	2	8	4	32	2	6

Table 3.2. Antimicrobial activity of AMPs and conventional antibiotics in standard conditions. Values reported with an asterisk (*) refers to the methicillin resistant *S. epidermidis*; n.d.: not detectable; MIC_{50}: MIC value at which ≥50% of isolates are inhibited; MIC_{90}: MIC value at which ≥90% of isolates are inhibited.

In the era of rising incidence of antibiotic-resistance, several studies have focused on possible synergistic effects between AMPs and the currently used antibiotics as a mean to lower the effective concentration of clinically used antibiotics and, in turn, lower the probability to give rise to this phenomenon [236]. The rationale is based on the distinct target of AMPs, i.e. the plasma membrane, with respect to that of conventional antibiotics. In the case of α-helical AMPs, their membrane-permeabilizing effect could facilitate the action of antibiotics acting on an intracellular target. To point out possible synergistic effects between AMPs under study and the antibiotics, checkerboard experiments were performed. Pairwise combinations of BMAP27(1-18) (as representative AMP) and cefazolin or linezolid, all in the range of 4 x MIC to 1/32 x MIC, were tested against the reference strains of *S. aureus* and *S. epidermidis*. These experiments however highlighted only an additive effect (FIC indices in the range of 1-2) between the AMP and the antibiotics (data not shown).

3.3 Antibiofilm activity

In the context of orthopaedic surgery, it is mandatory to prevent microbial biofilm formation on implant surfaces. Indeed, bacteria grown in the biofilm mode are far more resistant to antibiotics with respect to those grown in planktonic state, thus often causing recurrent infections and eventually leading to the loss of the implant.

The antibiofilm activity of the AMPs under study, also in comparison with conventional antibiotics, was tested against *S. epidermidis* ATCC 35984. Bacterial suspensions were allowed to form biofilm by 18 h incubation at 37°C, in the presence of several concentrations of AMPs or antibiotics, above and below their MICs. After this time the supernatants containing free floating bacteria were removed and the adherent cells carefully washed with PBS. The viability of the adherent bacteria was then assessed by the PrestoBlue® metabolic assay. Results are reported in Figure 3.2 as percent viability with respect to biofilm formed in the absence of any agent.

Results indicate that, at MIC values, all the peptides produced a higher than 95% reduction of biofilm viability, while they were scarcely effective at concentrations below this value. This observation likely suggests that bacteria were killed by the peptides before they could attach to the surface and form biofilm. Notably, cefazolin inhibited biofilm formation at concentrations well above its MIC (almost complete inhibition at 4 x MIC), while linezolid was active also below this value (about 70% inhibition at half-MIC) (Fig. 3.2).

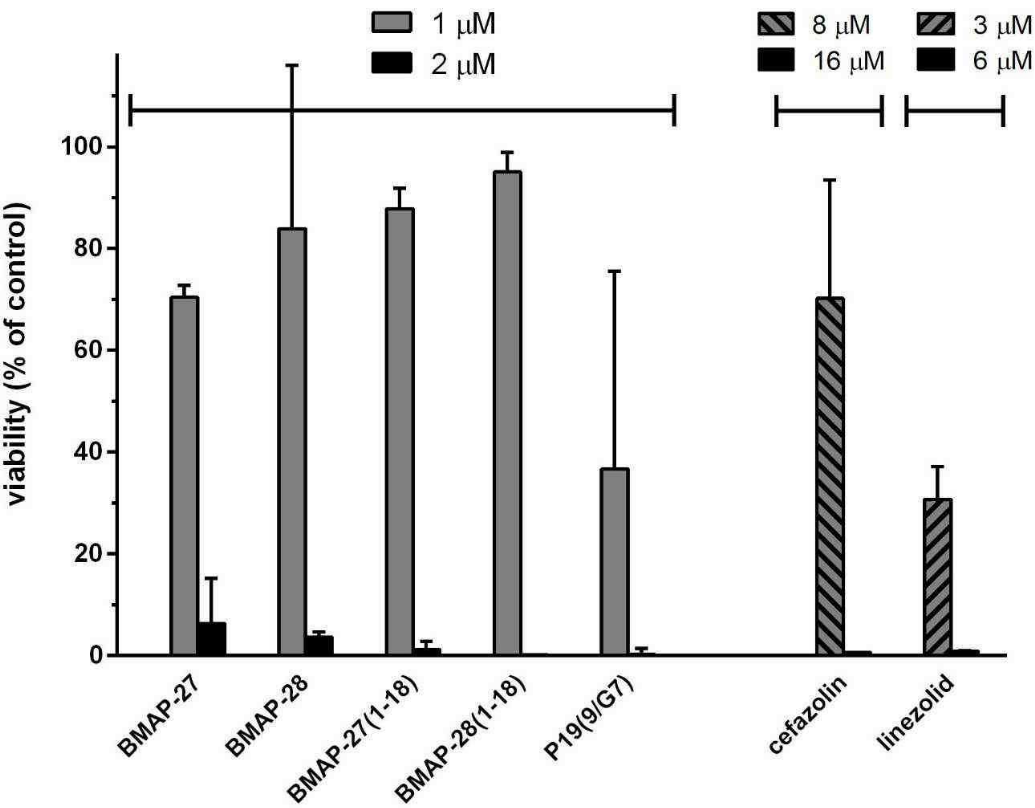

Figure 3.2. Inhibition of biofilm formation. *S. epidermidis* ATCC 35984 ($1 \cdot 10^7$ CFU/mL) was incubated at 37°C in flat-bottom 96 wells polystyrene plates in the presence and absence of different concentrations of AMPs or antibiotics. Viability of adherent bacterial cells was measured following 24 h incubation by the PrestoBlue® metabolic assay, and expressed as percentage of viability as compared to untreated bacteria. The means ± SD of at least two independent experiments are reported.

3.4 Lipopolysaccharide and lipoteichoic acid neutralization

Infected indwelling medical devices can cause sepsis, a worrisome condition due to an excessive and dysregulated inflammatory response [237]. Toxic microbial components, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) of Gram-negative and Gram-positive bacteria, respectively, act as potent agonists of Toll-like receptors (TLR), present on monocytic cells [238], stimulating the production of various inflammatory mediators [239].

To evaluate the ability of AMPs to neutralize the pro-inflammatory effects of these factors, the murine macrophage cell line RAW 264.7, a well-established cellular model, was used. Among the effects produced specifically by LPS and LTA in these cells, the induction of the enzyme nitric oxide synthase can be assessed by measuring the release of nitric oxide and its oxidation products by using the Griess assay. This simple and cost-effective method is suitable for rapid screening of AMPs endowed with LPS- and/or LTA-neutralizing properties.

Hence, RAW 264.7 cells were incubated with 100 ng/mL LPS from *E. coli* O111:B4 or 5 µg/mL LTA from *S. aureus* for 24 h at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated (h.i.) Fetal Bovine Serum (FBS) (complete medium) in the presence of different concentrations of the selected AMPs. Peptides and bacterial components were added to cells simultaneously. The lipopeptide polymyxin B, which binds to these bacterial factors with high affinity [240, 241], served as positive control. Following 24 h incubation, the nitrite concentration in the culture media was measured spectrophotometrically and the results, expressed as percentage with respect to cell samples incubated with LPS or LTA alone, are reported in Figure 3.3.

The data obtained indicate that both full length BMAPs are able to inhibit LPS-induced nitrite release at very low concentrations (0.5-1 µM, Fig. 3.3a), while higher peptide concentrations (4 µM, Fig. 3.3b) are required to achieve a similar inhibition in LTA-stimulated cells. The three shorter peptides seemed overall less effective, with some differences. BMAP28(1-18) displayed better inhibition of LPS-induced nitrite release (90% inhibition at 2-4 µM, Fig. 3.3a), and BMAP27(1-18) seemed more active against the LTA-induced nitrite production (60% inhibition at 4 µM, Fig. 3.3b). A 70% and roughly 30% inhibition, respectively, of LPS- and LTA-induced nitrite release, was observed with 8 µM P19(9G/7) (Fig. 3.3a and b). However, the results could not be ascribed to a decreased number of viable cells as none of the peptides proved toxic towards RAW 264.7 cells at the concentrations and under the conditions used (90-95% cell viability after 24 h incubation with 4 µM peptide alone and in the presence of 100 ng/ml LPS, data not shown). Anyhow, all the issues concerning cytotoxicity of the AMPs under investigation have been addressed more specifically in the following chapters.

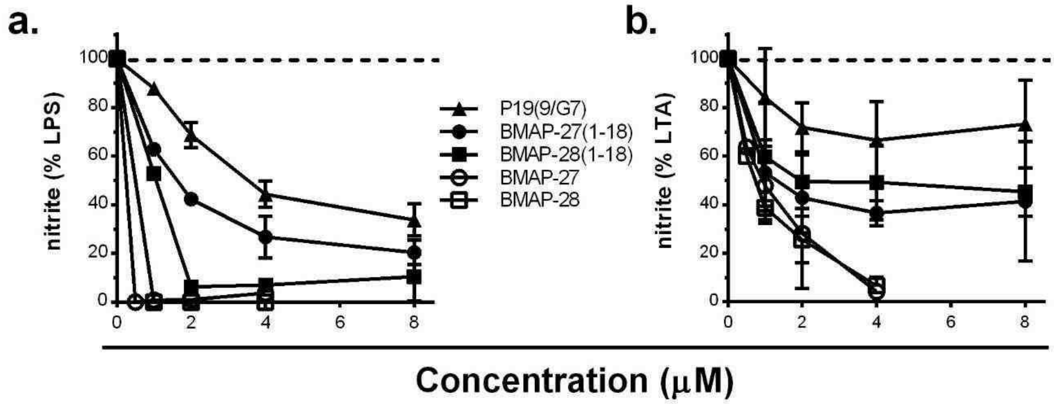

Figure 3.3. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) neutralization. RAW 264.7 cells were incubated at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated (h.i.) Fetal Bovine Serum (FBS) (complete medium) in the presence of 100 ng/mL LPS from *E. coli* O111:B4 (a) or 5 μg/mL LTA from *S. aureus* (b) and increasing concentrations of AMPs. Nitrite production was measured after 24 h incubation by the Griess assay, and expressed as percentage of nitrite released as compared to LPS or LTA alone. The means ± SD of two independent experiments are reported.

3.5 Effects on viability and differentiation of osteoblast cells

The effects of the AMPs under study on host cells that normally reside in the joint environment were next investigated as some AMPs are reported to induce osteoblast differentiation [185] and proliferation [188]. In order to develop AMP-based implantable devices, the impact of these molecules on the principal physiological processes of osteoblast cells has to be assessed. To this aim, the human osteosarcoma-derived MG-63 cell line was used. These cells are at a less differentiated stage of osteoblastic maturation when compared to other osteosarcoma-derived cell lines (e.g. SaOS2, U2OS) [242], are responsive to osteogenic stimuli such as the glucocorticoid dexamethasone [243] and are thus suitable for investigation of early differentiation events [244].

Potential short- and long-term toxic effects of the peptides on these cells were first evaluated by testing plasma membrane integrity and global cell metabolic activity under standard culture conditions. MG-63 cells were incubated for 1 h at 37°C in the presence of increasing concentrations of AMPs in complete culture medium. The activity of the cytosolic enzyme lactate dehydrogenase (LDH) in the cell culture supernatants was then measured as an indicator of impaired plasma membrane integrity. The results, expressed as percentage of total cellular LDH activity, are reported in Figure 3.4a. In an additional set of experiments carried out under almost identical conditions, the metabolic activity of MG-63 cells, incubated with the peptides for 24 h, was measured by using the Prestoblue® metabolic assay. The results, expressed as percent cell viability with respect to untreated cells, are shown in Figure 3.4b.

Data reported in Figure 3.4 show that the (1-18) BMAP fragments and P19(9/G7) are safe for MG-63 osteoblast cells up to 10 μM. Conversely, marked impairment of cell viability and massive LDH release was evident with 10 μM BMAP27 and BMAP28. It should be noted however that these two latter peptides were not toxic to osteoblast cells in the range of their microbicidal concentrations (1.25-5 μM, Fig. 3.4a and b).

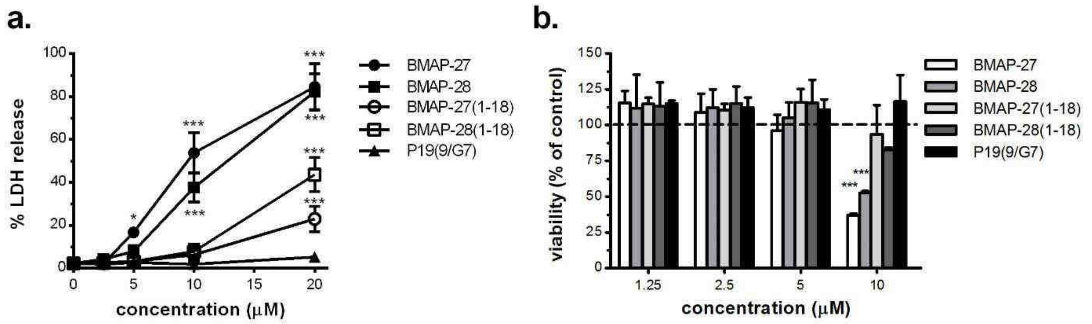

Figure 3.4. Effects on viability of osteoblast cells. MG-63 osteoblast cells were incubated in the absence and presence of increasing concentrations of the indicated peptides in complete medium. a. LDH activity was measured in the culture supernatants after 1 h incubation, and expressed as percentage of total cellular LDH activity. The means ± SD of three independent experiments are reported. b. Prestoblue® metabolic assay was performed after 24 h incubation. Viability was calculated as percentage of untreated cells, and is the mean ± SD of three independent experiments performed in triplicate.
*: p<0,5; **: p<0,01; ***: p<0,001; one way ANOVA and Bonferroni posthoc test.

To evaluate the effects of the AMPs under investigation on the process of osteogenic differentiation, MG-63 cells were incubated for 7 days in osteogenic medium (i.e. serum-free DMEM supplemented with 10 nm β-glycerophosphate and 50 μg/mL ascorbic acid) in the presence and absence of 2 μM each AMP. 100 nm dexamethasone served as positive control (Fig. 3.5a). Separate cell samples were incubated with combinations of 100 nm dexamethasone and 2 μM each BMAP27(1-18), BMAP28(1-18), and P19(9/G7) (Fig. 3.5b). The osteogenic medium, containing AMPs and/or dexamethasone, was changed every two days. Following 7-day incubation, cell culture supernatants were removed, and cells were resuspended in water and lysed by osmotic shock and sonication. The activity of alkaline phosphatase (ALP) as a well-characterized early osteogenic marker was then measured in total cell lysates and normalized to protein content of each sample. Data shown in Figure 3.5 are expressed as fold increase with respect to basal ALP activity (Fig. 3.5a), and as percent with respect to ALP activity induced by dexamethasone alone (Fig. 3.5b). The full-length BMAPs exhibited some toxicity to MG-63 cells, as deduced by decreased protein content of the lysates (data not shown), likely due to the absence of serum. The three shorter peptides, though not toxic, were not able to induce osteogenic differentiation *per se*. Most important, they did not negatively affect the differentiation induced by dexamethasone, suggesting that they could be used for orthopaedic applications without adverse effects on osteoblast differentiation.

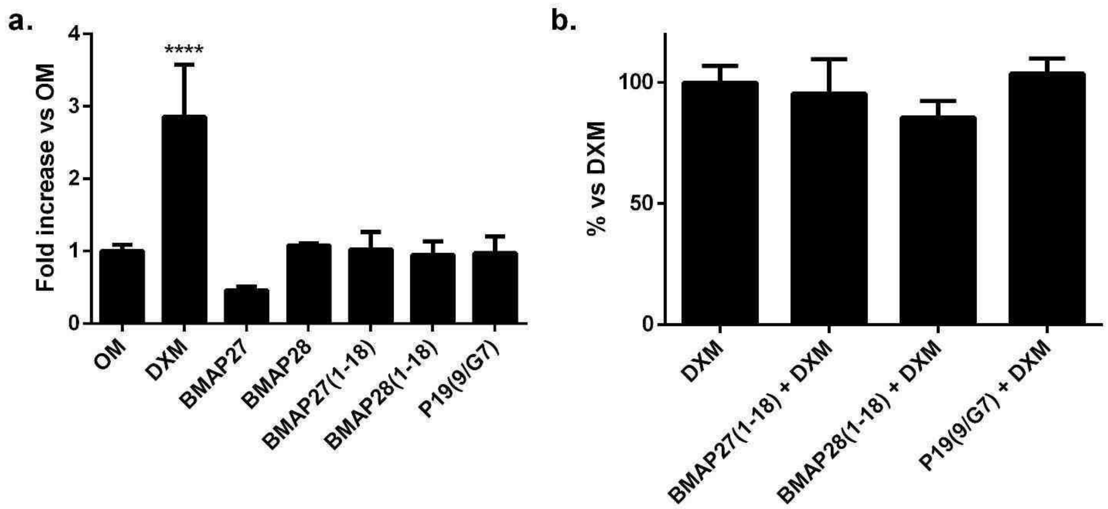

Figure 3.5. Effects on differentiation of osteoblast cells. MG-63 osteoblast cells were incubated in osteogenic medium (OM - serum-free DMEM supplemented with 10 nm β-glycerophosphate and 50 µg/mL ascorbic acid) in the absence and presence of 2 µM AMPs or 100 nm dexamethasone (a), or a combination of them (b). Alkaline phosphatase (ALP) activity as a marker for osteoblast differentiation was measured in the cell lysates following 7 days of incubation at 37°C, and expressed as fold increase with respect to basal ALP activity (a), and as percent with respect to ALP activity induced by dexamethasone alone (b). The means ± SD of at least two experiments performed in duplicate are reported. OM: osteogenic medium; DXM: dexamethasone.
*: p<0,5; **: p<0,01; ***: p<0,001; ****: p<0,0001; one way ANOVA and Bonferroni posthoc test.

Based on the results of these experiments, the three shorter AMPs were thus selected for further characterization.

3.6 Efficacy under physio-pathological conditions and selection of peptide candidate

Although displaying excellent antimicrobial activity in standard conditions, AMPs may perform in a significantly different manner in the presence of body fluids, e.g. plasma or serum. Several organic and inorganic components of biological fluids, present in the specific applicative context, could affect the biological activities of AMPs to different extents [245-249].

To address this issue, HPLC experiments aimed at assessing the chemical stability of selected AMPs in the presence of human serum, were first performed. BMAP27(1-18) and BMAP28(1-18) were incubated for 3 h at 37°C in 20% normal human serum, collected from healthy donors. Some experiments were also carried out in heat inactivated serum. Sample aliquots were collected at various time points and analysed by reverse phase chromatography after removal of serum proteins by acid precipitation (see the Materials and Methods section for a more detailed description). Unfortunately, due to inconsistent results it was not possible to gather some useful information from these experiments. We speculated that the critical step could be the precipitation of serum proteins that probably led to a simultaneous but not uniform precipitation of the AMPs, giving contrasting results among different experiments. Individual variability among different serum samples might also have contributed to increasing complexity.

To verify how the selected peptides were affected by the presence of serum, in the next set of experiments bacterial growth inhibition kinetics were performed in the presence of commercially available pooled normal human serum (HS) and, for comparison, in the presence of bovine (FBS) serum, which is routinely used in cell culture. Serial dilutions of BMAP27(1-18), BMAP28(1-18) and P19(9/G7) were assayed against *S. epidermidis* ATCC 35984 at 37°C in 50% Mueller-Hinton broth (MH), in the presence and in the absence of 25% HS or FBS. Bacteria were added to the wells containing sera simultaneously with the AMPs or following 3 h pre-incubation of AMPs with serum. Both sera have been heat inactivated (h.i.) to avoid possible serum-mediated killing of bacteria. Bacterial growth was monitored for 24 h by measuring optical density at 600 nm with a microplate reader.

Graphs in Figure 3.6 show the end-point results at 24 h calculated as percentage of growth inhibition as a function of peptide concentration, whereas single selected growth kinetics are shown in Figure 3.7. Results indicate that none of the peptides was impaired by co-incubation with each serum and, in the case of BMAP27(1-18), complete growth inhibition was achieved at lower peptide concentrations with respect to the control (Fig. 3.6a, b and c). In this regard it is important to note that bacterial growth was not inhibited but rather enhanced in the presence of serum alone without peptides (Fig. 3.7c), thus excluding possible antimicrobial effects of serum components. However, upon pre-incubation the presence of human and, to a lesser extent, bovine serum had an evident impact on the activity of the AMPs (Fig. 3.6e and f). In this condition, peptide concentrations leading to complete growth inhibition increased considerably, though to different extents with the different AMPs, with BMAP27(1-18) being the less affected. Concerning this latter AMP, it is interesting to note that the peptide concentration corresponding to its MIC value, as determined in previous assays (i.e. 2 μM), was sufficient to completely prevent bacterial growth for at least six hours (Fig. 3.7b) and to achieve a broadly 50% growth inhibition at 24 h incubation (Fig. 3.6e).

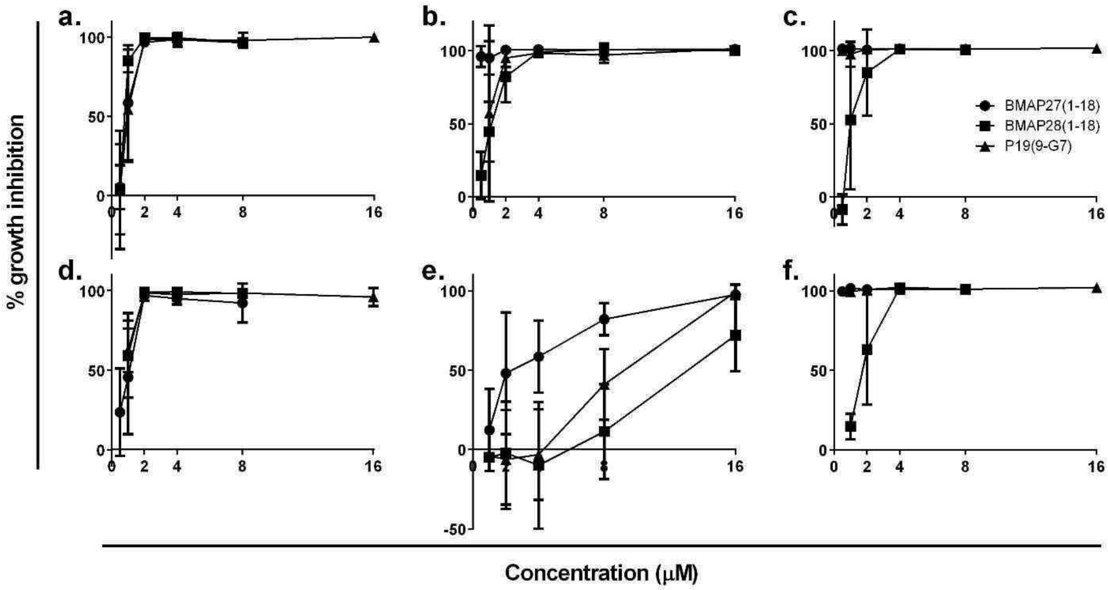

Figure 3.6. Peptide inhibition of bacterial growth in the presence of serum. *S. epidermidis* ATCC 35984 ($1{\cdot}10^7$ CFU/mL) was incubated at 37°C for 24 h with the peptides in 50%MH broth in the absence (a, d) or presence of 25% human (b, e) or fetal bovine serum (c, f) and bacterial growth was monitored by measuring the optical density of the suspension at 600 nm. Bacteria were added simultaneously (a-c) or after 3 h pre-incubation (d-f) of the AMPs in the selected media. Data were calculated as percentage of growth inhibition at 24 h with respect to untreated bacteria (0%), and are the mean ± SD of three independent experiments.

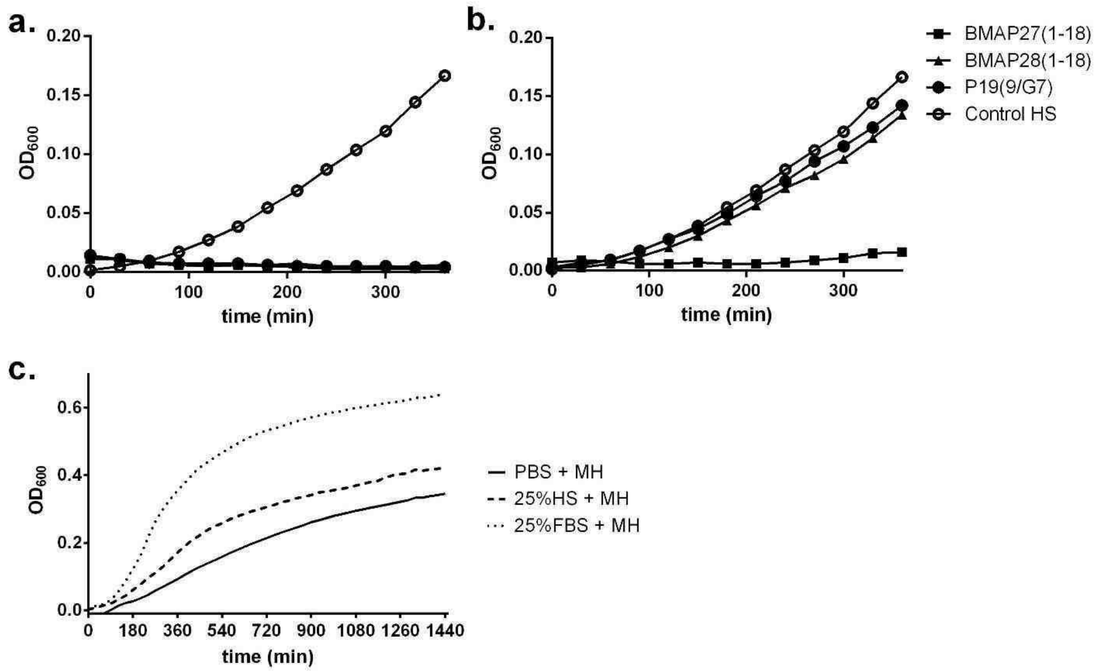

Figure 3.7. Bacterial growth kinetics in the presence of serum. a-b. *S. epidermidis* ATCC 35984 was incubated as described in Fig. 3.6 in 50% MH supplemented with 25% human serum (HS) in the absence and presence of 2 µM AMPs and growth kinetics were monitored by measuring the optical density of the suspension at 600 nm for 6 h. Bacteria were added simultaneously with the peptides (a) or after 3 h of peptide incubation (b). c. *S. epidermidis* ATCC 35984 was incubated in 50% MH broth in the absence or presence of 25% HS or FBS and optical density of the suspension was monitored for 24 h. One representative experiment out of three is reported in each graph.

The application potential of BMAP27(1-18), BMAP28(1-18) and P19(9/G7) in arthroplasty was further investigated by examining their antimicrobial effects under culture conditions most resembling the prosthetic joint environment.

Specifically, the antimicrobial activity against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923 was tested in the presence of hyaluronic acid (HA), which represents the major component of synovial fluid [250]. Being negatively charged, HA could interact with the AMPs that are cationic, thus interfering with their antimicrobial activity. To address this issue, growth inhibition kinetics were performed in the presence of 0.5 and 3 mg/mL HA, corresponding to the concentrations present in joints of arthroprosthetic patients and healthy subjects, respectively [251, 252]. The results are reported in Figure 3.8 as percent of growth inhibition at 24 h incubation with respect to untreated bacteria. In addition, representative selected growth kinetics are shown in Figure 3.9. It is important to note that HA *per se* did not inhibit bacterial growth (Fig. 3.9d) and affected peptide activity against *S. epidermidis* only slightly (Fig. 3.8b and c and 3.9c).

Neither concentration of HA affected the activity of the AMPs against *S. aureus* to a significant extent (Fig. 3.8e and f)

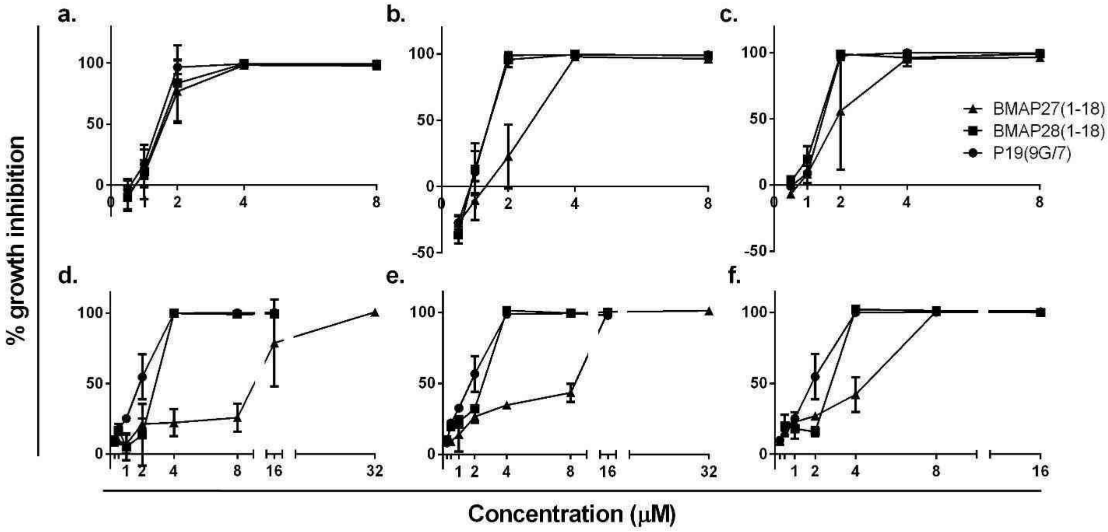

Figure 3.8. Peptide inhibition of bacterial growth in the presence of hyaluronic acid (HA). *S. epidermidis* ATCC 35984 (a-c) and *S. aureus* ATCC 25923 (d-f) were incubated with the peptides at 37°C for 24 h in 50% MH broth in the absence (a, d) or presence of 0.5 mg/mL (b, e) and 3 mg/mL (c, f) HA and bacterial growth was monitored by measuring the optical density of the suspensions at 600 nm. Data were calculated as described in Fig. 3.6, and are the mean ± SD of three independent experiments.

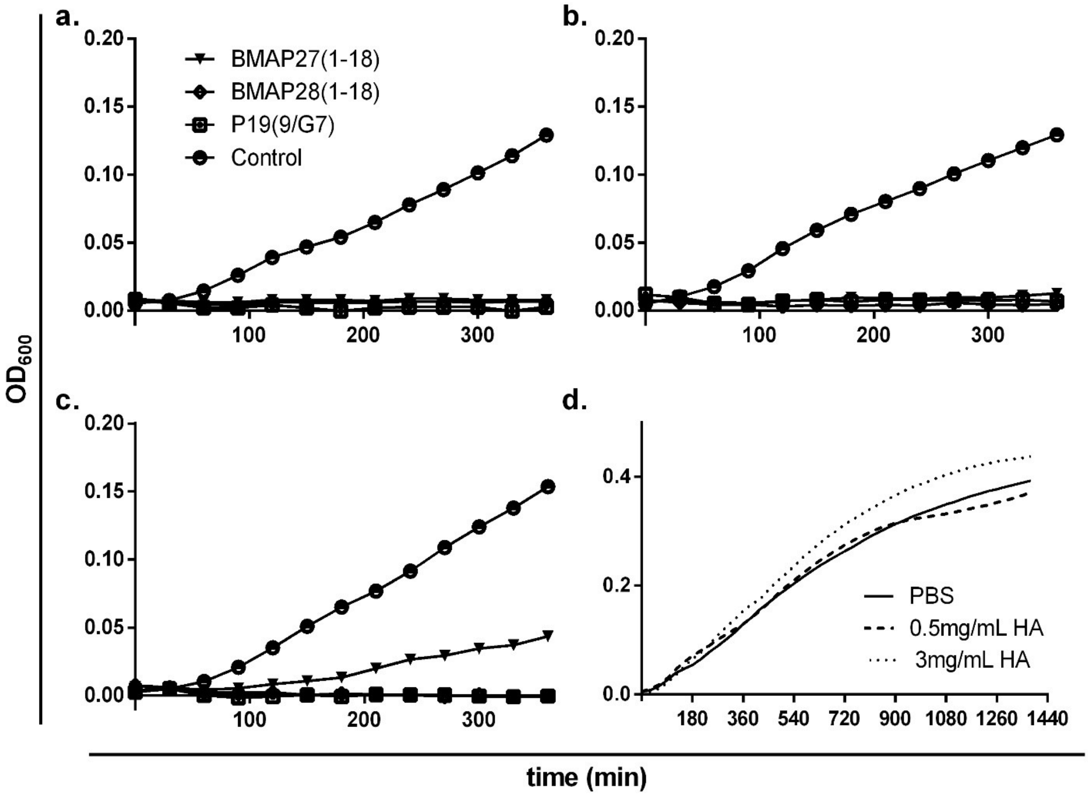

Figure 3.9. Bacterial growth kinetics in the presence of hyaluronic acid. a-c. *S. epidermidis* ATCC 35984 was incubated as described in Fig. 3.8 in 50% MH alone (a) or supplemented with 0.5 mg/mL (b) and 3 mg/mL (c) HA in the absence and presence of 2 µM AMPs and growth was followed by measuring optical density of the suspension at 600 nm for 6 h. d. *S. epidermidis* ATCC 35984 was incubated in 50% MH broth in the absence or presence of HA and optical density of the suspension was measured for 24 h. One representative experiment out of three is reported in each graph.

The effects of AMPs on viability of osteoblast cells in the presence of HS and HA were also addressed. MG-63 cells were incubated for 1 h at 37°C in the presence of increasing concentrations of AMPs in culture medium (DMEM) supplemented with 10% and 25% HS, or in Hank's balanced salt solution (HBSS), supplemented with 10% HS with and without 0.5 mg/mL HA. The LDH activity in cell culture supernatants was then measured as described for Figure 3.4 and the results, expressed as percentage of total cellular LDH activity, are shown in Figure 3.10.

Based on these data, it is clear that none of the three peptides tested was toxic to MG-63 cells at microbicidal concentrations (i.e. up to 10 µM), and the presence of hyaluronic acid did not exert any additional effect (Fig. 3.10d). The release of LDH, indicating damage to cytoplasmic membrane, became evident only at peptide concentrations higher than 20 µM and 40 µM, in the presence of 10% and 25% HS, respectively (Fig. 3.10, upper panels). It is also evident that cells perform better in nutrient-rich culture medium than in HBSS, despite serum supplementation (Fig. 3.10, panels on the left).

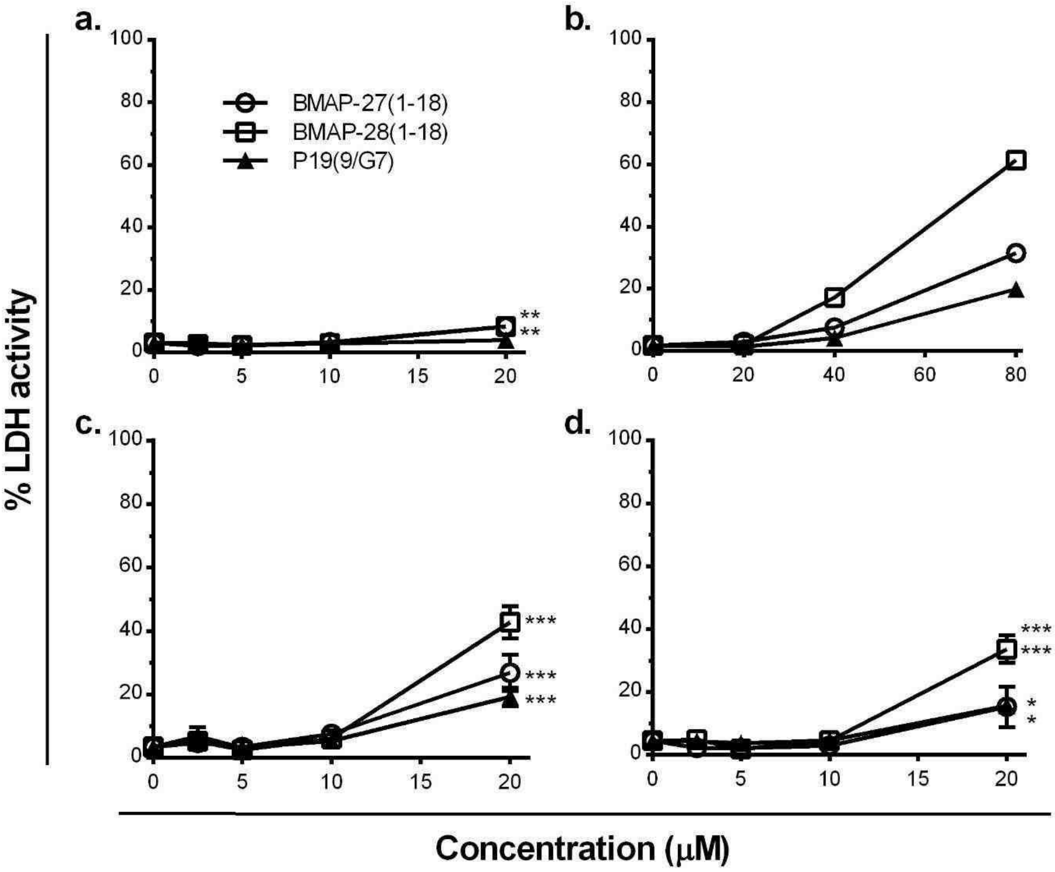

Figure 3.10. Effects on viability of osteoblast cells in the presence of human serum and hyaluronic acid. (a-b) MG-63 osteoblast cells were incubated in the absence and presence of increasing concentrations of the indicated peptides in DMEM supplemented with 10% (a) or 25% (b) HS. (c-d) Cells were incubated in the absence and presence of increasing concentrations of the indicated peptides in HBSS supplemented with 10% HS alone (c) or with 10% HS and 0.5 mg/mL HA (d). LDH activity was measured in the culture supernatants after 1 h incubation, and expressed as percentage of total cellular LDH activity. Except for panel b, where one representative experiment is shown, the means ± SD of three independent experiments are reported.
*: p<0,5; **: p<0,01, ***: p<0,001; one way ANOVA and Bonferroni posthoc test.

To further characterize the therapeutic potential of the AMPs under study, their antimicrobial activity was tested against *S. aureus* ATCC 25923 in the presence of synovial fluid (SF) from orthopedic patients. However, the availability of synovial fluid samples was limited and in many instances their suitability was questionable for technical reasons. Moreover, the effect of SF alone on bacterial growth was highly variable. Indeed, the majority of the samples inhibited bacterial growth *per se*, thus impeding to perform growth kinetics with the AMPs (Fig. 3.11d). Despite these limitations, one experiment was performed in the presence of a SF sample permitting detectable bacterial growth. The results of this preliminary experiment shown in Figure 3.11 indicate that the antimicrobial efficacy of the peptides under study was not inhibited but rather improved by SF (Fig. 3.11a-d).

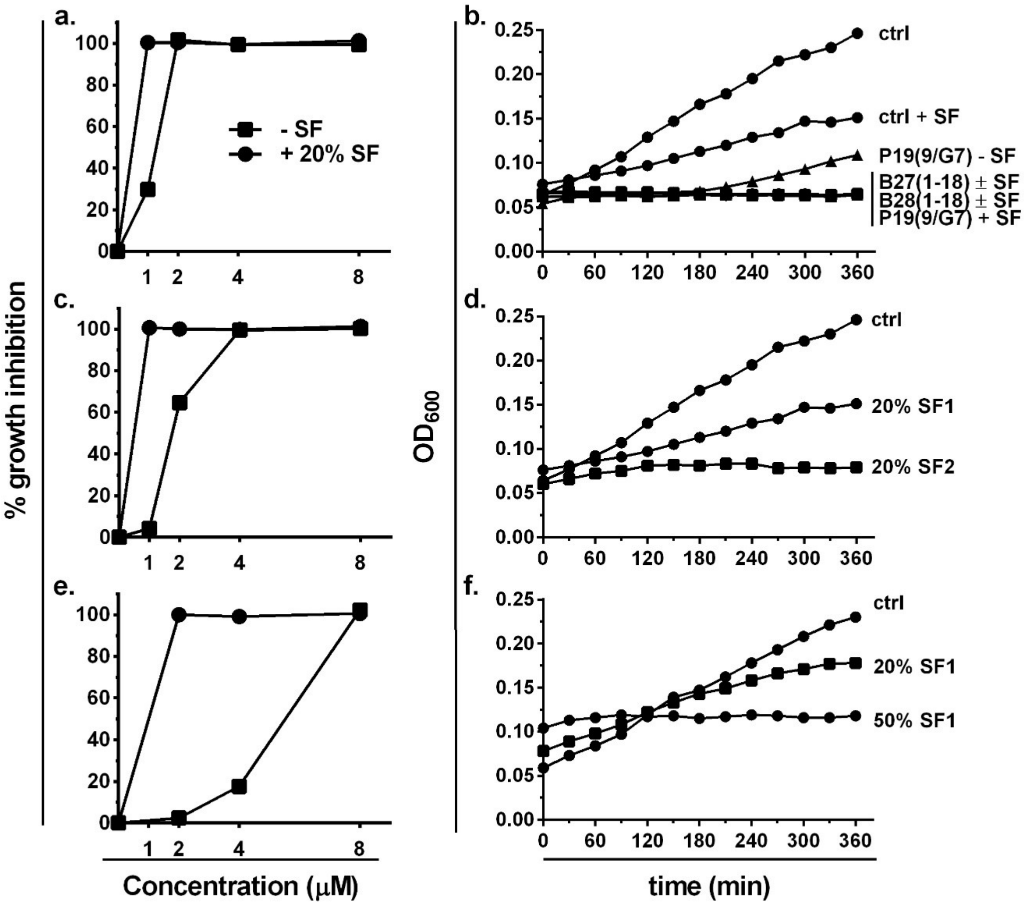

Figure 3.11. Peptide inhibition of bacterial growth in the presence of synovial fluid (SF). a, c, e. *S. aureus* ATCC 25923 ($2.5 \cdot 10^5$ CFU/mL) was incubated with BMAP27(1-18) (a), BMAP28(1-18) (c) and P19(9/G7) (e) at 37°C for 24 h in 20% MH broth in the absence or presence of 20% SF and the optical density of the suspension at 600 nm was then monitored. Data were calculated as described in Fig.3.6. b, d, f. Bacterial growth kinetics in the presence of SF. b. *S. aureus* ATCC 25923 was incubated in 20% MH broth in the absence or presence of 20% SF and peptides and optical density of the suspension was monitored for 6 h. d. Bacteria were incubated in the absence and presence of 20% SF from two different patients. f. The sample of SF permitting detectable growth was then tested at two different concentrations. One single experiment is reported.

Considering globally the results thus far obtained, the functional characterization of peptide candidates was narrowed to BMAP27(1-18). This peptide displayed good antimicrobial and antibiofilm activity and was not cytotoxic against osteoblast cells at microbicidal concentrations. Notably, when compared to the other two peptide candidates, its antimicrobial activity was affected by human serum to a lesser extent. The effect of human serum was investigated more in depth in the next set of experiments with the aim to answer the question whether the reduction of antimicrobial activity in serum was due to proteolytic degradation of the peptide by serum proteases or other hydrolytic enzymes, or to unspecific sequestration of the peptide by serum components like serum albumin or lipoproteins. To this aim, BMAP27(1-18) was compared to its enantiomer all-D-BMAP27(1-18), i.e. a peptide with the same amino acid sequence but composed entirely of D-amino acids and therefore resistant to enzymatic degradation. It is demonstrated in the case of α-helical AMPs that their D-enantiomers are equally able to interact with bacterial membranes and kill susceptible microorganisms as their natural counterparts [253, 254]. At difference with the natural peptide, the all-D enantiomer is presumed to adopt a left-handed α-helical conformation, as confirmed by the specular dichroic spectra shown in Figure 3.12.

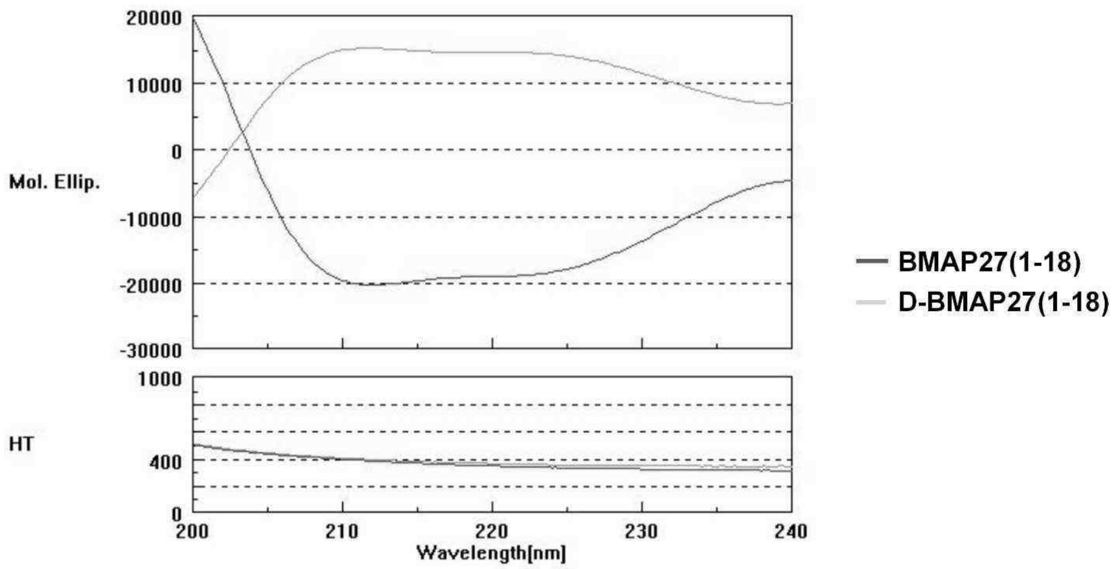

Figure 3.12. Structural properties of BMAP27(1-18) and of the D-enantiomer. CD spectra were recorded at 20 µM concentration in 10 mM phosphate buffer, pH 7.4, in the presence of 50% trifluoroethanol (TFE). Data are the average of three scans and are reported as mean residue elipticity.

The ability of both peptides to inhibit growth of *S. epidermidis* ATCC 35984 was investigated in parallel in the presence of human and bovine serum, either heat inactivated or not. The results shown in Figure 3.13 confirmed that the antimicrobial activity exerted by the two peptides was highly comparable in standard culture medium (Fig. 3.13a and b), and it was somehow improved by co-incubation with human and bovine serum to a very similar extent for both peptides with no appreciable differences after heat inactivation (Fig. 3.13c-j).

However, marked differences were observed when the peptides have been pre-incubated with sera prior to the addition of bacteria. Specifically, pre-incubation with human and bovine serum produced a more evident decrease in the activity of BMAP27(1-18) (Fig. 3.13c and g), with respect to the enantiomer (Fig. 3.13d and h), suggesting the involvement of a stereospecific interaction such as for example that of an enzyme. Accordingly, in the case of fetal bovine serum the decrease in the antimicrobial activity of BMAP27(1-18) (Fig. 3.13g) was far less evident after heat inactivation (Fig. 3.13i). In the case of human serum, however, the results suggest that unspecific binding of the AMPs by serum components may also be involved because the all-D enantiomer was also affected by pre-incubation in inactivated serum (Fig. 3.13c-f).

Importantly, neither human nor bovine serum inhibited bacterial growth *per se*, regardless whether heat inactivated or not (Fig. 3.14).

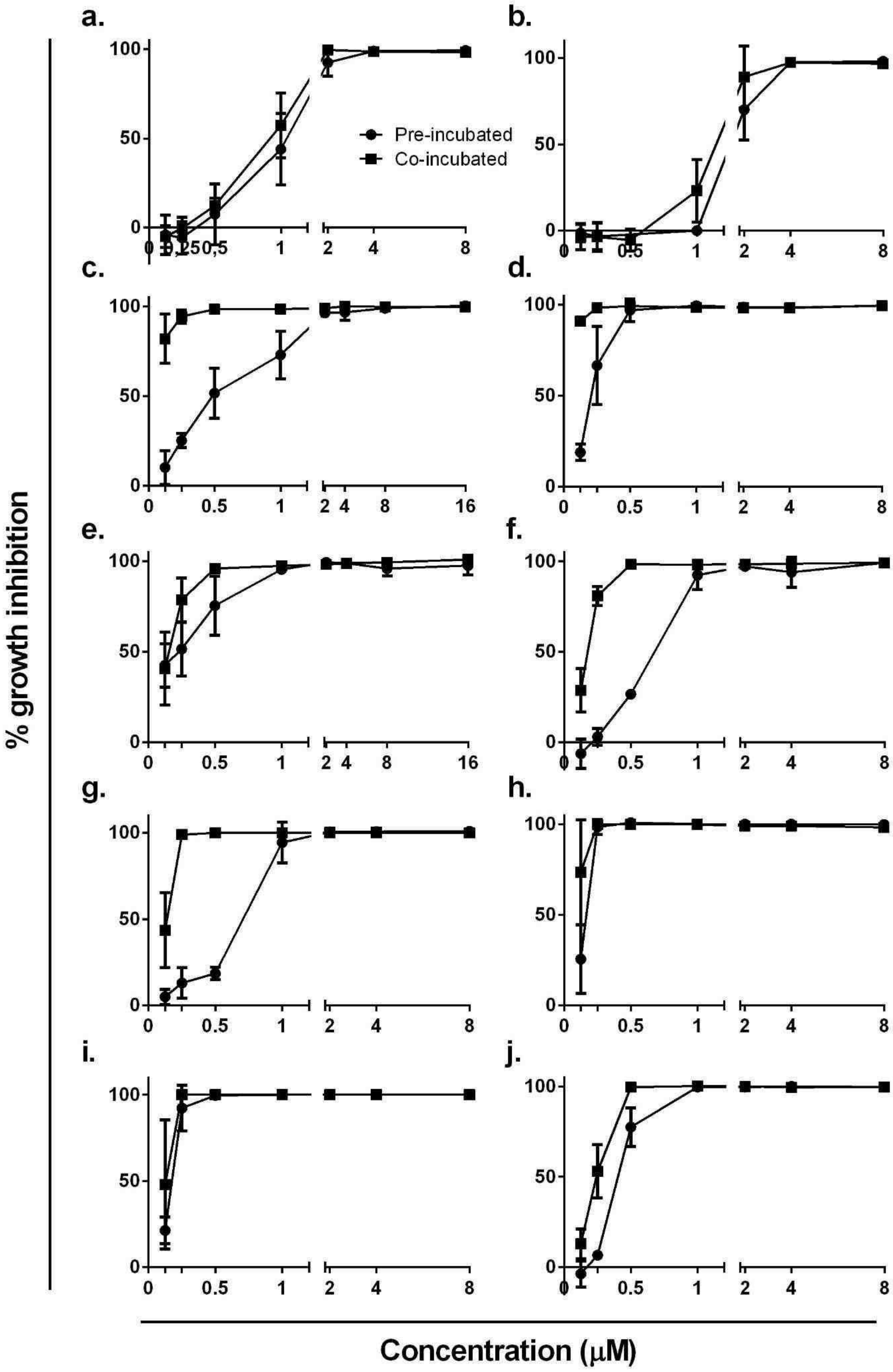

Figure 3.13. Effects of different sera on the antimicrobial activity of BMAP27(1-18) and of the all-D enantiomer. *S. epidermidis* ATCC 35984 ($2.5 \cdot 10^5$ CFU/mL) was incubated at 37°C with BMAP27(1-8) (a, c, e, g, i) and D-BMAP27(1-18) (b, d, f, h, j) for 24 h in 50% MH broth in the absence (a-b) or presence of 25% HS (c-d), 25% h.i. HS (e-f), 25% FBS (g-h) and 25% h.i. FBS (i-j). Bacterial growth was monitored by measuring optical density of the suspension at 600 nm. Bacteria were added to the suspension simultaneously with AMPs (co-incubated) or after 3 h pre-incubation of the AMPs in the selected media (pre-incubated). Data were calculated as reported in Fig. 3.6, and are the mean ± SD of three independent experiments.

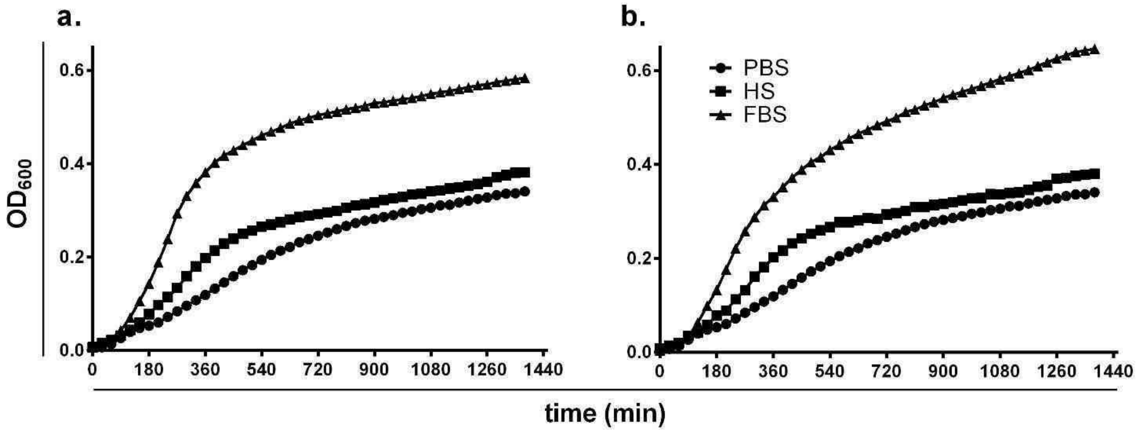

Figure 3.14. Bacterial growth kinetics in the presence of sera. *S. epidermidis* ATCC 35984 was incubated as described in Fig. 3.13 in 50% MH broth in the absence or presence of HS or FBS (a) and 25% h.i. HS or FBS (b) and optical density of the suspension was measured for 24 h. One representative experiment out of three is reported in each graph.

3.7 Efficacy of BMAP27(1-18) upon immobilization on solid support

Different strategies may be considered in order to develop AMP-based biomaterials. With the aim to make the surface of biomaterials refractory to bacterial colonization, the most promising peptide candidates should be immobilized onto the material surface. Covalent immobilization could have several advantages over a system releasing the active molecules. Indeed, covalent immobilization could sustain the antimicrobial activity for a longer time with respect to a diffusible molecule [208, 212]. Moreover, covalently bound peptides could be less prone to degradation [255]. Conversely, a possible loss of antimicrobial activity upon chemical immobilization on a solid surface could be of great concern. Several reports addressed this issue suggesting the addition of a spacer between the surface and the peptide to allow effective interaction of the immobilized peptides with bacterial membranes.

In order to assess if BMAP27(1-18) maintains activity upon immobilization, the peptide was bound to a solid support by exploiting the extremely high affinity of streptavidin for biotin. Biotin-streptavidin interaction is one of the strongest among biological molecules, with a k_d of ≈10^{-14} mol/L, close to that of a covalent bond. For this purpose, a commercial agarose resin, functionalised with streptavidin, was used, and the peptides BMAP27 and BMAP27(1-18) were biotinylated at the N-terminus as described in the Materials and Methods section.

The full length peptide was used for comparison as published data reported on its activity upon immobilization, but without specifying the orientation of the immobilized peptide molecule [256]. With respect to the full length peptide, the backbone of BMAP27(1-18) is 24-atoms shorter due to the lack of the C-terminal tail. Hence, this latter AMP has been biotinylated with biotin or with a biotinylated short polyethylene glycole (PEG) spacer, with length comparable to that of the missing tail. Prior to their immobilization on resin, the three biotinylated peptides, i.e. biot-B27, biot-B27(1-18) and biot-PEG-B27(1-18), were compared to the parent peptides for their activity against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923 in solution. As shown in Table 3.3, the covalent addition of the biotin at the N-termini of BMAP27 and BMAP27(1-18) did not affect the activity against *S. aureus* and the addition of the biotinylated PEG_2 spacer affected it only slightly, whereas the activity against *S. epidermidis* was not affected at all.

	MIC (µM)	
Peptide	***S. epidermidis* ATCC35984**	***S. aureus* ATCC25923**
BMAP27	1	2
biot-B27	1	2
BMAP27(1-18)	1	2
biot-B27(1-18)	1	2
biot-PEG-B27(1-18)	1	4-8

Table 3.3. Antimicrobial activity of biotinylated BMAP27 and BMAP27(1-18) against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923.

The biotinylated derivatives of BMAP27 and BMAP27(1-18) have been immobilized on Sepharose-streptavidin resin by overnight incubation with resin beads in PBS at 4°C. As a reference, a separate resin sample was incubated in parallel with biotin. Extensive washing steps with PBS were performed to remove any residual biotinylated molecule in solution from resin supernatants. The procedure is described in more detail in the Materials and Methods section. The antimicrobial efficacy of the three immobilized peptides was tested against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923. The resin beads, functionalized with biot-B27, biot-B27(1-18) and biot-PEG-B27(1-18), and with biotin alone, suspended in 50% MH, were incubated for 1 h at 37°C with each bacterial strain. Comparable volumes of each resin supernatant were tested in parallel. Bacterial viability was then assessed with the PrestoBlue® metabolic assay and CFU counts. The results reported in Figure 3.15 show that in the case of resins functionalized with biot-B27 and biot-B27(1-18), a 5% resin suspension was sufficient to virtually sterilize both bacterial suspensions. Conversely, the resin functionalized with biot-PEG-B27(1-18) appeared somehow less effective, especially against *S. aureus* (Fig. 3.15b). However, at a more concentrated resin suspension (25%), the antimicrobial efficacy of this resin was markedly improved, achieving complete inhibition of *S. epidermidis* and almost 80% viability reduction of *S. aureus* (not shown). Considering their very similar MIC values in solution, this latter finding likely reflects a lower resin substitution in the case of biot-PEG-B27(1-18), with respect to the resins functionalized with biot-B27 and biot-B27(1-18). As expected, the supernatants of the functionalised resins did not display any antimicrobial effect, thus confirming that the drop in bacterial viability, as assessed by the metabolic dye, and bacterial killing, as seen by CFU counts, have to be ascribed to the immobilized peptides on the resin and not to soluble peptides released in the medium.

The resins functionalized with the biotinylated peptides have also been tested for possible toxicity towards MG-63 cells by measuring LDH release in the culture media after 1 h incubation of cells at 37°C in the presence of 5% resin suspensions. As shown in Table 3.4, the effects of these particles on LDH release were negligible.

In addition, to verify the stability of the functionalized resins, the antimicrobial activity was measured against *S. epidermidis* under identical conditions as above after 4-week incubation of the resins at 4°C. As seen in Figure 3.16, bacterial viability was strongly impaired in the presence of functionalized resin beads, whereas resin supernatants did not affect it at all.

Overall, these data indicate that the immobilized peptides are microbicidal and not cytotoxic. In addition, the strong interaction of biotin with streptavidin renders the resin functionalization very stable under standard laboratory conditions.

This very simple approach proved useful to qualitatively assess the antimicrobial efficacy of the immobilized peptides. However, for the improvement of the immobilization procedure, a quantitative comparison of different peptide analogues and different immobilization procedures would be necessary. In this regard, the use of labelled peptides in order to allow their detection during the incorporation steps could be of help. To this purpose, fluorescent derivatives of BMAP27(1-18) were synthesized and their functional characterization, both in solution and upon immobilization, is reported in the next part of the thesis.

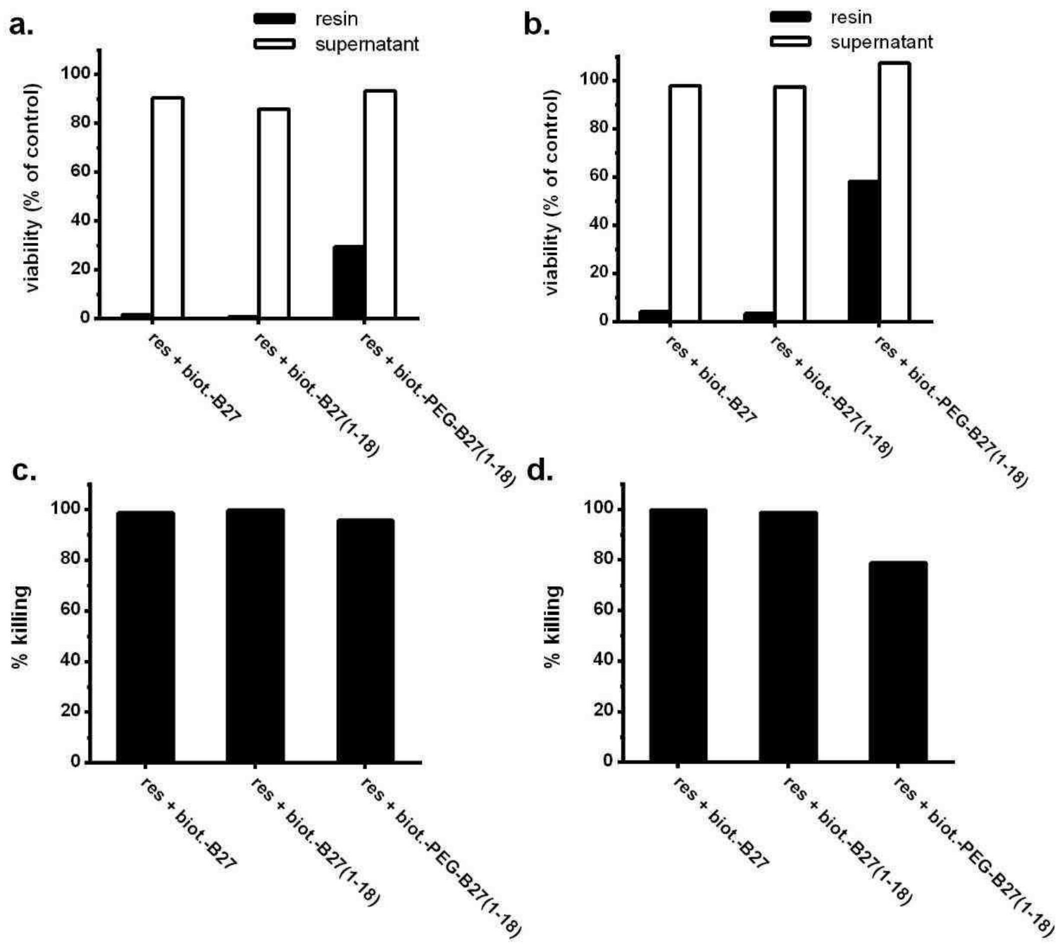

Figure 3.15. Antimicrobial effects of biotinylated AMPs immobilised on Sepharose-streptavidin resin. Sepharose-streptavidin resin beads were functionalised with the biotinylated derivatives of BMAP27 and BMAP27(1-18) as reported in the text. *S. epidermidis* ATCC 35984 ($1 \cdot 10^7$ CFU/mL) (a,c) and *S. aureus* ATCC 25923 ($1 \cdot 10^7$ CFU/mL) (b, d) were incubated with the indicated 5% resins or with their respective supernatants for 1 h in 50% MH broth at 37°C under agitation. a-b. Bacterial viability was evaluated by the PrestoBlue® metabolic assay on both resin- and supernatant-treated bacteria and reported as percent viability with respect to the control resin (i.e. functionalised with biotin alone) and its supernatant, respectively. c-d. CFU counts were performed on the same samples. Killing was calculated as percent with respect to bacteria incubated with the control resin. One representative experiment out of two is reported.

	% LDH release
Control	3,58
Resin + biotin	1,66
Resin + biot-B27	4,94
Resin + biot-B27(1-18)	3,04
Resin + biot-PEG-B27(1-18)	0,51

Table 3.4. Effects of the resins on MG-63 osteoblast cells. Data were calculated as percentage of total cellular LDH. One representative experiment out of two is reported.

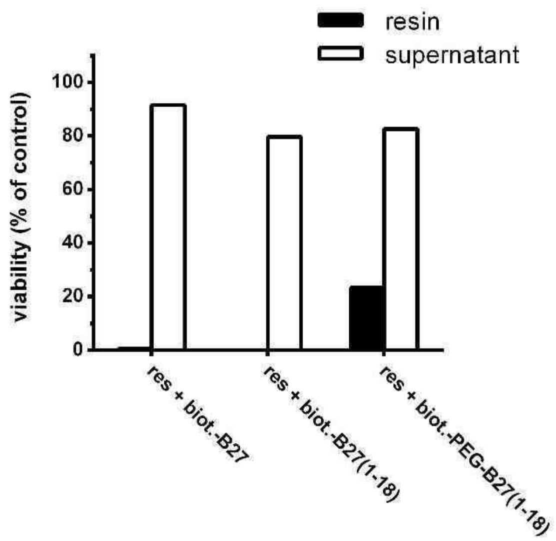

Figure 3.16. Antimicrobial activity of the immobilised AMPs upon prolonged storage at 4°C. The samples reported in Fig. 3.15 were assessed for their antimicrobial activity after 1-month storage at 4°C. *S. epidermidis* ATCC 35984 ($1{\cdot}10^7$ CFU/mL) was incubated with the indicated 5% resins or with their respective supernatants for 1 h in 50% MH broth at 37°C under agitation. Bacterial viability was evaluated by the PrestoBlue® metabolic assay on both resin- and supernatant-treated bacteria and reported as percent viability with respect to the control resin (i.e. functionalised with biotin alone) and its supernatant, respectively. One representative experiment out of two is reported.

3.8 Cyano-phenylalanine-derivatives of BMAP27(1-18)

The peptide BMAP27(1-18) was labelled with a fluorescent probe by synthesizing a derivative with the four phenylalanine residues substituted by cyano-phenylalanine (Phe_{CN}). The Phe_{CN}-substituted analogue was named BMAP27(1-18)Phe_{CN}. Phe_{CN} is a non-natural amino acid, endowed with convenient spectroscopic properties, used in protein binding and folding studies [257]. Upon excitation at 240 nm, this amino acid emits fluorescence with a maximum at 300 nm. The replacement of Phe residues with Phe_{CN} would render the peptide BMAP27(1-18) fluorescent by keeping the structural modifications at a minimum.

The structural properties of the analogue could be deduced by comparing the HPLC profiles (Fig. 3.17a 17b) and Circular Dichroism (CD) spectra (Fig. 3.17c and d) of the two peptides. The shorter retention time of the Phe_{CN} analogue reflects its major hydrophilic character with respect to the parent peptide. On the other hand, the analysis by CD spectroscopy indicates that both peptides, unstructured in aqueous environment, adopt an α-helical conformation in the presence of trifluoroethanol, thus undergoing a conformational transition that is typical for linear, membrane-active AMPs. Hence, we expected that the functional properties of BMAP27(1-18) should not be much affected by the replacement of Phe residues with Phe_{CN}.

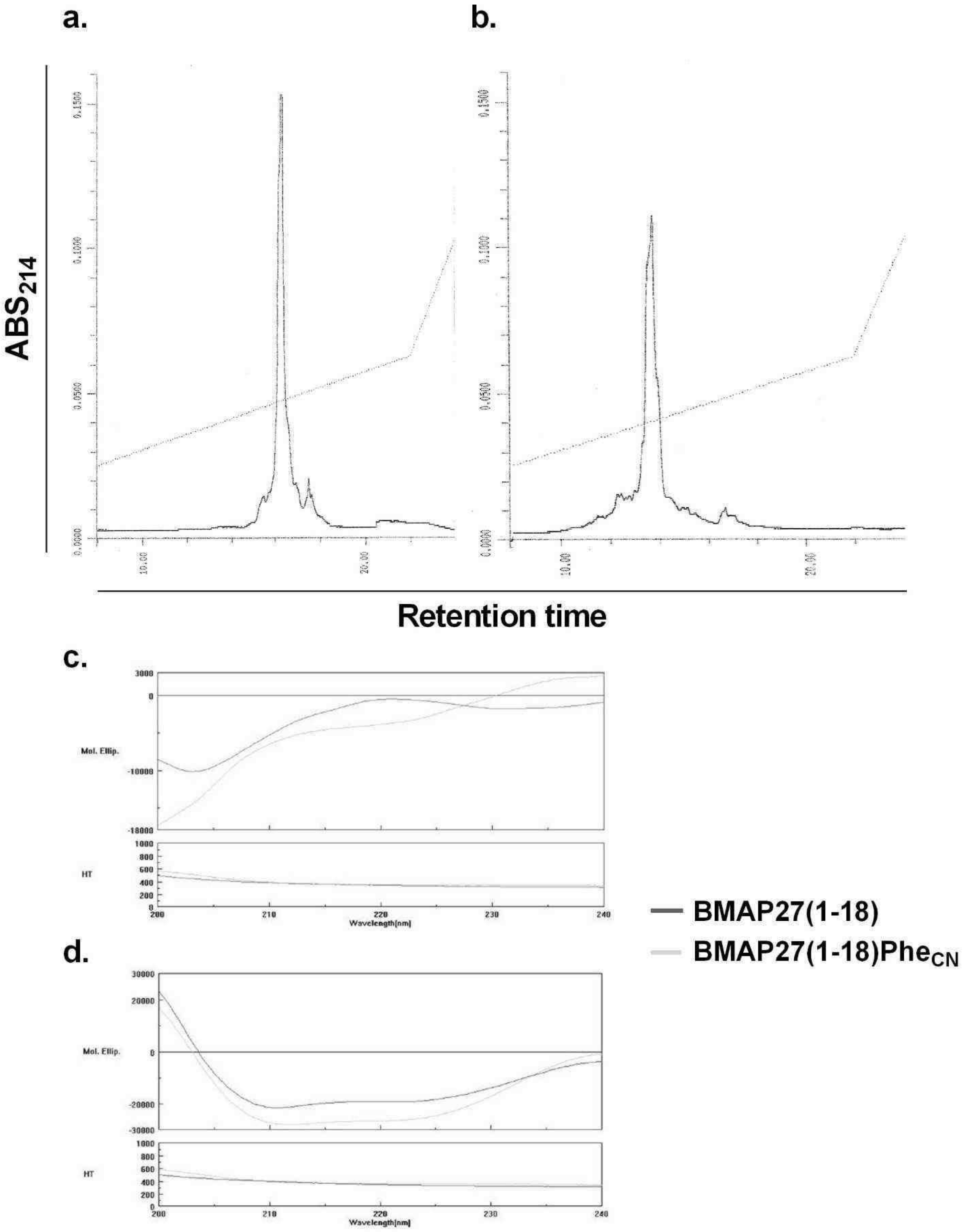

Figure 3.17. Structural properties of BMAP27(1-18) and of the cyano-phenylalanine (Phe_{CN}) derivative. a-b. HPLC profile of BMAP 27(1-18)Phe_{CN} (b) in comparison to BMAP27(1-18) (a). c-d. CD spectra were recorded at 20 µM concentration in 10 mM phosphate buffer, pH 7.4, in the absence (c) and presence (d) of 50% trifluoroethanol (TFE). Data are the average of three scans and are reported as mean residue elipticity.

The Phe_{CN} analogue was then compared to BMAP27(1-18) for its antimicrobial activity against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923 in standard conditions and against *S. epidermidis* ATCC 35984 in the presence of serum and hyaluronic acid.

As reported in Table 3.5, the two peptides showed roughly comparable antibacterial activity in terms of MIC values, with the cyano-derivative being slightly less active. In particular, a two-fold and a four-fold increase in MIC values was observed against *S. epidermidis* ATCC 35984 and against *S. aureus* ATCC 25923, respectively.

	MIC (µM)	
Peptide	***S. epidermidis* ATCC35984**	***S. aureus* ATCC25923**
BMAP27(1-18)	1-2	2-4
BMAP27(1-18)Phe_{CN}	2-4	8-16

Table 3.5. Antimicrobial activity of BMAP27(1-18) and of the Phe_{CN} derivative against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923.

The effect of serum was evaluated similarly to experiments reported in the previous chapters by monitoring bacterial growth kinetics in the presence of BMAP27(1-18) or BMAP27(1-18)Phe_{CN} either co-incubated or pre-incubated with each serum. The end-point results at 24 h, calculated as percent growth inhibition, are reported in Figure 3.18, whereas representative kinetics are shown in Figure 3.19. Data indicate a basically lower antimicrobial activity for the Phe_{CN} analogue, although only slightly higher peptide concentrations inhibited bacterial growth almost completely (Fig. 3.18a and d). Major differences between the two peptides have been observed after pre-incubation with human serum. While bovine serum affected peptide activity only marginally, the activity of the Phe_{CN} derivative was affected by human serum to a greater extent with respect to the native peptide. The effect, less evident during the early phases of bacterial growth (Fig. 3.19), became clearly evident at 24 h incubation (Fig. 3.18e).

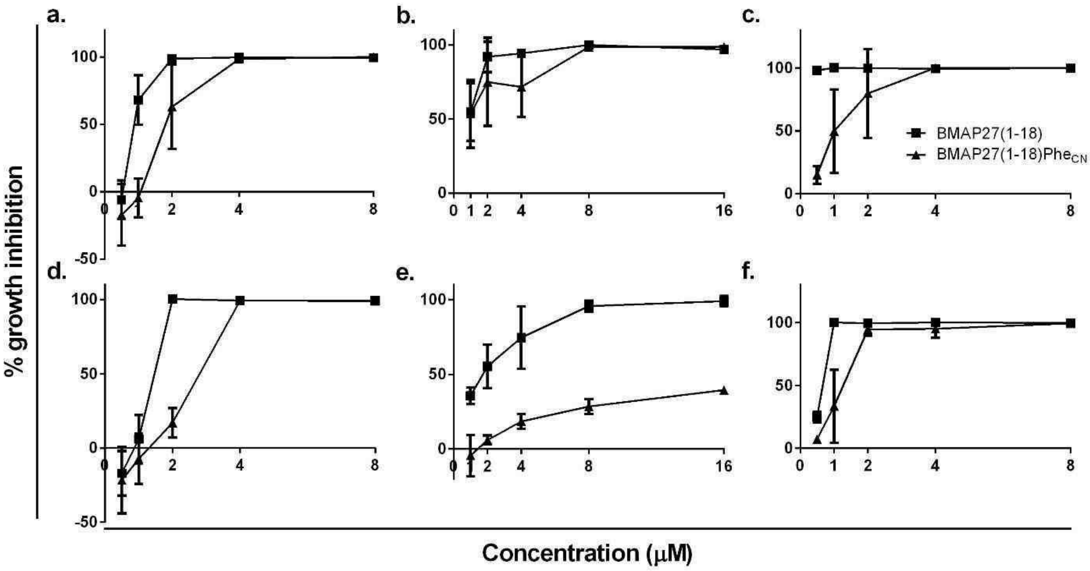

Figure 3.18. Effects of different sera on the antimicrobial activity of BMAP27(1-18) and the Phe$_{CN}$ derivative. *S. epidermidis* ATCC 35984 ($2.5 \cdot 10^5$ CFU/mL) was incubated with peptides at 37°C for 24 h in 50% MH broth in the absence (a, d) or presence of 25% HS (b, e) or FBS (c, f). Growth was monitored by measuring the optical density of the suspension at 600 nm. Bacteria were added to the suspension simultaneously with AMPs (a-c) or after 3 h pre-incubation of the AMPs in the selected media (d-f). Data were calculated as reported in Fig. 3.6, and are the mean ± SD of three independent experiments.

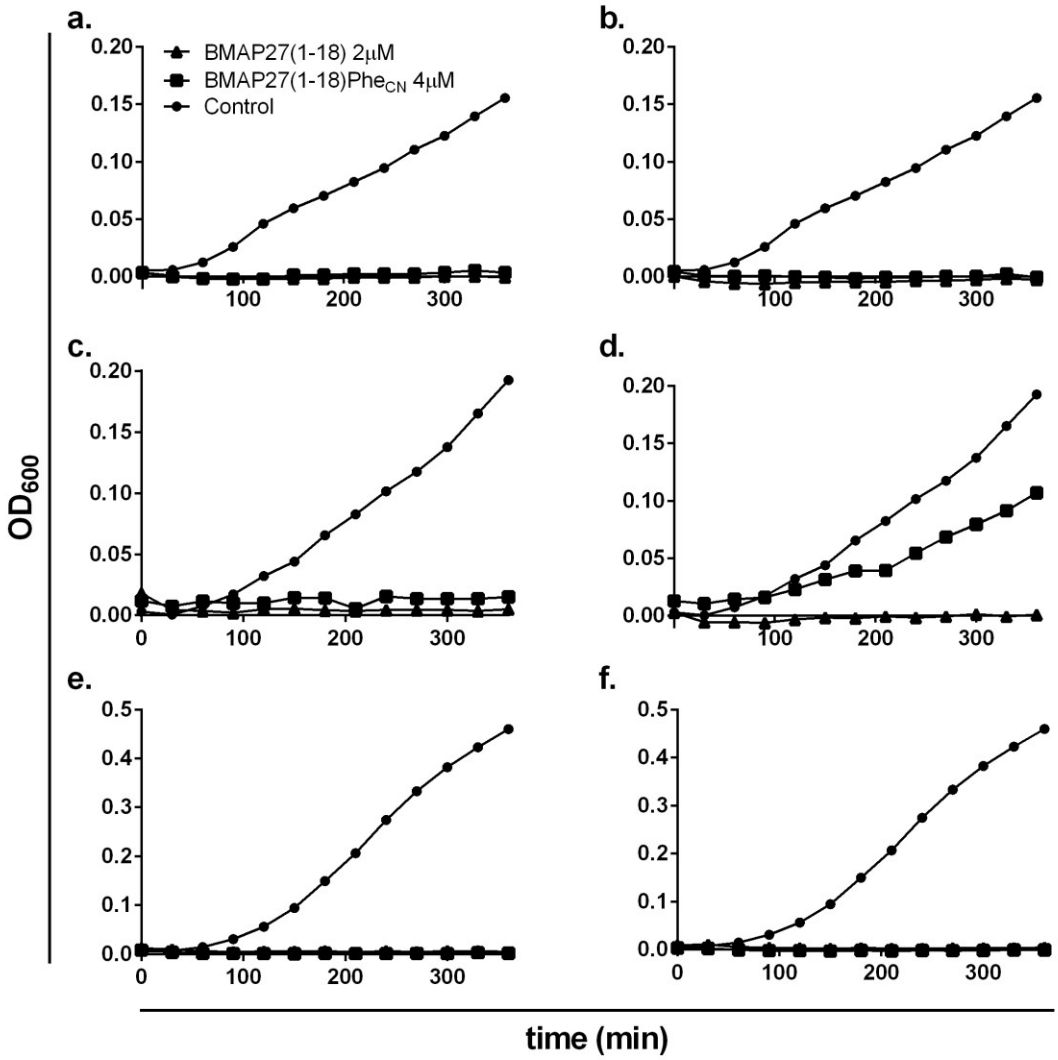

Figure 3.19. Bacterial growth kinetics in the presence of sera. a-b. *S. epidermidis* ATCC 35984 ($2.5 \cdot 10^5$ CFU/mL) was incubated in 50% MH alone (a, b) or supplemented with 25% HS (c, d) or FBS (e, f) in the absence and presence of AMPs at concentrations corresponding to MIC values and optical density of the suspension was monitored for 6 h. Bacteria were added simultaneously with the peptides (a, c, e) or after 3 h peptide incubation (b, d, f). One representative experiment out of three is reported.

The antimicrobial activity of the labelled peptide analogue against *S. epidermidis* ATCC 35984 was also tested in the presence of 0.5 and 3 mg/ml HA. The results in Figure 3.20 show that the higher concentration of hyaluronic acid influenced the cyano-derivative to a greater extent respect to the parent peptide (Fig. 3.20d). Results are reported in Figure 3.20a as percentage growth inhibition at 24 h, with single selected growth kinetics shown in Figure 3.20b-d

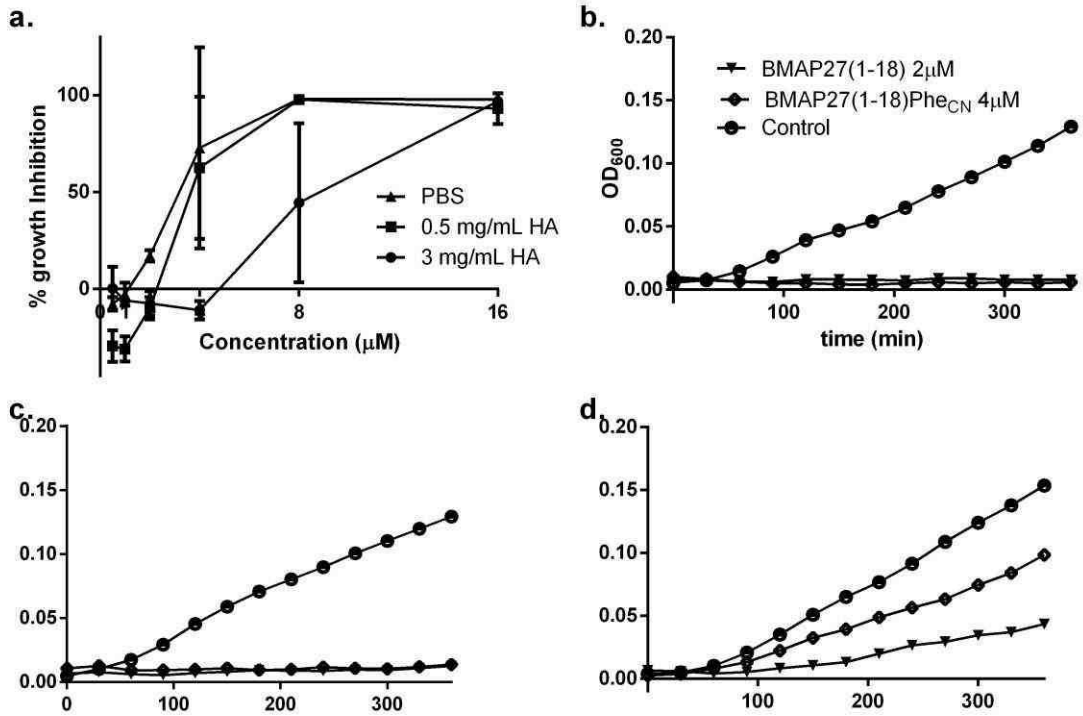

Figure 3.20. Bacterial growth inhibition in the presence of HA. a. *S. epidermidis* ATCC 35984 ($2.5 \cdot 10^5$ CFU/mL) was incubated with BMAP27(1-18)Phe$_{CN}$ at 37°C for 24 h in 50% MH broth in the absence or presence of HA and growth was monitored by measuring the optical density of the suspension at 600 nm. Data were calculated as reported in Fig. 3.6, and are the mean ± SD of three independent experiments. b-d *S. epidermidis* ATCC 35984 was incubated in 50% MH alone (b) or supplemented with 0.5 mg/mL (c) and 3 mg/mL (d) HA in the absence and presence of AMPs at their MIC concentrations and optical density of the suspension was measured for 6 h. One representative experiment out of three is reported.

Possible cytotoxic effects of BMAP27(1-18)Phe$_{CN}$ were analysed in comparison with BMAP27(1-18) on MG-63 cells by LDH release and PrestoBlue® metabolic assay. The experiments were carried out in the same conditions as described in the previous chapter (3.5 Effects on viability and differentiation of osteoblast cells, page 44). Based on the results obtained by both assays, the Phe$_{CN}$ derivative proved safe to osteoblast cells at least as much as the parent BMAP27(1-18) (Fig. 3.21).

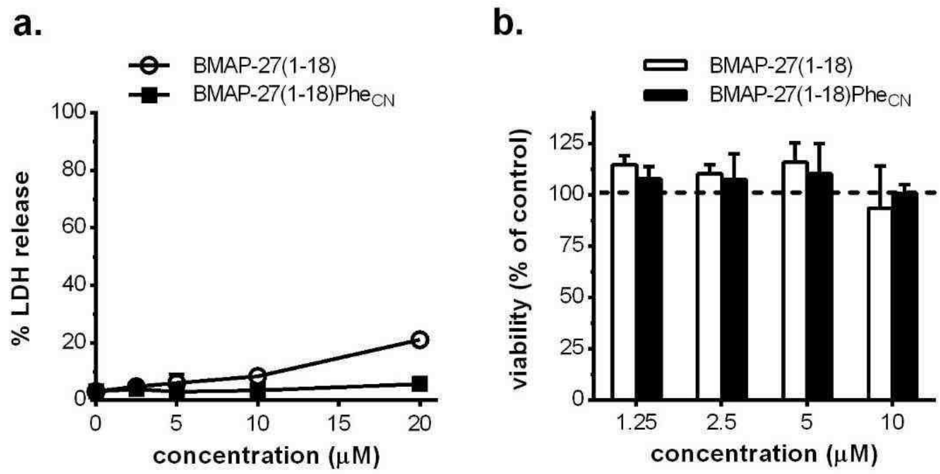

Figure 3.21. Effects of BMAP27(1-18)Phe$_{CN}$ on viability of osteoblast cells. A. MG-63 osteoblast cells were incubated in the absence and presence of increasing concentrations of the indicated peptides in DMEM supplemented with 10% FBS. LDH release was measured as reported in Fig. 10. The means ± SD of two independent experiments are reported. b. Prestoblue® metabolic assay was performed after 24 h incubation. Viability was calculated as percentage of untreated cells, and is the mean ± SD of three independent experiments performed in triplicate.

In the previous chapter, we were able to demonstrate that BMAP27(1-18), immobilized on solid support through interaction of the biotinylated N-terminus with the resin-bound streptavidin, maintains antimicrobial activity. However, it is not known whether the activity of the immobilised AMP is based on the same mode of action of the soluble counterpart. Moreover, the antimicrobial efficacy appeared to be attenuated upon insertion of a short spacer (PEG$_2$) between the peptide molecule and biotin. Therefore, one could speculate whether the presence of a spacer is needed and, in general, whether the N-terminus has importance for antimicrobial activity. To investigate these aspects in more detail, both in solution and upon immobilization, the analogue BMAP27(1-18)Phe$_{CN}$ was further modified.

In order to verify what influence would have a large chemical moiety attached to one end of the peptide molecule on its activity in solution, the N- or the C-terminus of the peptide was alternatively capped with a 3 kDa PEG moiety. For this purpose, two cysteinylated analogues, *Cys*BMAP27(1-18)Phe$_{CN}$ and BMAP27(1-18)Phe$_{CN}$*Cys*, with the cysteine residue placed at the N- and the C-terminus, respectively, have been synthesized and purified. The PEGylation was carried out by 1 h incubation at room temperature of each cysteinylated analogue with PEG$_{3000}$, carrying a maleimide function, in 0.1 M sodium phosphate buffer, pH 7.4. After a HPLC purification step, the antimicrobial activity of the PEGylated derivatives was determined in standard conditions (Table 3.6) and in the presence of serum (Fig. 3.22).

Results clearly show that the PEGylation at either end of BMAP27(1-18)Phe$_{CN}$ affected the antimicrobial activity in solution to the same extent. Respect to BMAP27(1-18)Phe$_{CN}$, the two PEGylated analogues displayed four-fold increased MIC values against both *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 35984 (Tab. 3.6), indicating that capping of the N- or C-terminus with PEG$_{3000}$ is equivalent for the activity of this peptide in solution.

	MIC (µM)	
Peptide	***S. epidermidis* ATCC35984**	***S. aureus* ATCC25923**
BMAP27(1-18)Phe$_{CN}$	2-4	8-16
BMAP27(1-18)Phe$_{CN}$-PEG	8-16	32
PEG-BMAP27(1-18)Phe$_{CN}$	8-16	32

Table 3.6. Antimicrobial activity of the PEGylated Phe$_{CN}$ derivatives against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923.

The influence of human serum on PEGylated AMPs was investigated by monitoring the growth kinetics of *S. epidermidis* ATCC 35984 in the presence of BMAP27(1-18)Phe$_{CN}$-PEG either co-incubated or pre-incubated with each serum. Data reported in Figure 3.22 show that bacterial growth inhibition was negatively affected by the presence of human and fetal serum to a very similar extent (Fig. 3.22), and preincubation of the PEGylated peptide with both sera also produced similar reduction in antimicrobial efficacy (Fig. 3.22b and c).

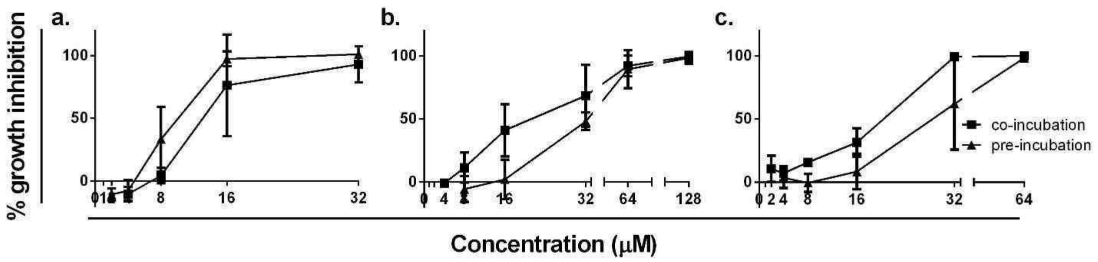

Figure 3.22. Effects of different sera on the antimicrobial activity of PEGylated BMAP27(1-18)Phe$_{CN}$. *S. epidermidis* ATCC 35984 ($2.5 \cdot 10^5$ CFU/mL) was incubated with BMAP27(1-18)Phe$_{CN}$-PEG at 37°C for 24 h in 50% MH broth in the absence (a) or presence of 25% HS (b) or FBS (c) and growth was monitored by measuring the optical density of the suspension at 600 nm. Bacteria were added to the suspension simultaneously with AMPs (co-incubation) or after 3 h of pre-incubation of the AMPs in the selected media. Data were calculated as reported in Fig. 3.6, and are the mean ± SD of three independent experiments.

As a preliminary approach to study the mode of action of immobilised AMPs, we asked ourselves whether the AMPs under investigation are equally active when immobilized on solid support through the N- or through the C-terminus. To answer this question the two cysteinylated BMAP27(1-18)Phe$_{CN}$ peptides were coupled to Sepharose-streptavidin resin in two steps. During the first step, resin beads were incubated for 2 h at room temperature with a large excess of a biotinylated reagent, carrying a maleimide function spaced by a short PEG (maleimide-PEG$_2$-biotin), in PBS. After extensive washings with PBS, the resin beads, functionalized with the maleimide groups, were incubated overnight at 4°C with the N- and C-terminally cysteinylated BMAP27(1-18)Phe$_{CN}$ peptides. A maleimide-functionalized resin sample was incubated in parallel with cysteine as a reference. After coupling, all resin samples underwent extensive washings with PBS to remove the peptide/cysteine in excess.

The fluorescence of the resin samples and their respective supernatants was measured at the end of the incorporation procedure (Tab. 3.7). Data are reported as relative fluorescence units (RFU). It is evident that only the resin samples functionalized with B27(1-18)Phe$_{CN}$, either through the N-terminus or the C-terminus, emitted fluorescence, and with a very similar signal intensity. Conversely, the fluorescence intensity of the resin functionalized with cysteine, as well as the intensity of resin supernatants were at or even below the background level (Tab. 3.7).

The antimicrobial activity of the resins, loaded with the AMP either through the N-terminus (Cys-B27(1-18)Phe$_{CN}$) or through the C-terminus (B27(1-

18)Phe_{CN}-Cys, and with cysteine, was measured against *S. epidermidis* ATCC 35984 with the PrestoBlue® metabolic assay as described in the previous chapter (3.7 Efficacy of BMAP27(1-18) upon immobilization on a solid support, page 58).

It is evident from Figure 3.23 that 25% resin suspensions proved quite effective, although bacterial inhibition was not complete (roughly 80%) in the case of the C-terminally-immobilised peptide. This may be due to a difference in peptide activity or simply to a different resin loading. However, based on the highly similar fluorescence intensity, this latter hypothesis is unlikely. Although the application of a calibration curve constructed with the labelled peptide in solution would have many limitations, at least a rough estimate of peptide concentration in resin samples was possible, resulting in a very similar, approximately 5-7 μM concentration for both resin-bound derivatives.

In addition, the resins bearing these two immobilized derivatives were less effective as compared to the resins functionalized with biot-B27(1-18) and biot-PEG-B27(1-18), as the same level of growth inhibition was obtained at a more concentrated resin suspension. This observation could also be explained by lower resin loading, or by the intrinsically lower activity of the Phe_{CN} analogues, or both. Most important, the reduction of bacterial viability was clearly due to the antimicrobial action of the immobilized peptides, because resin supernatants were not fluorescent and did not exert any effect, suggesting that the immobilized AMPs were firmly bound to the support and kept antimicrobial activity.

	Fluorescence intensity (RFU)	
	Resins	**Supernatants**
Cys	-4648	346
B27(1-18)Phe_{CN}-Cys	16428	278
Cys-B27(1-18)Phe_{CN}	17293	85

Table 3.7. Fluorescence intensity (RFU) of the Sepharose-streptavidin resin samples, functionalized with the Phe_{CN} derivatives or with cysteine, and their respective supernatants. Background fluorescence has been subtracted.

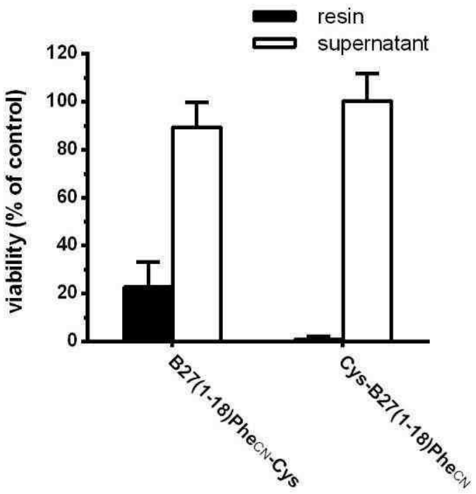

Figure 3.23. Antimicrobial effect of BMAP27(1-18)Phe$_{CN}$ immobilised on Sepharose-streptavidin resin. *S. epidermidis* ATCC 35984 ($1 \cdot 10^6$ CFU/mL) was incubated with the indicated 25% resin or the respective supernatants for 1 h in 50% MH broth at 37°C under agitation. Bacterial viability was evaluated by the PrestoBlue® metabolic assay on both resin- and supernatant- treated bacteria and reported as percent viability with respect to the control resin (i.e. functionalised with cysteine alone) and its supernatant, respectively. Data are the mean ± SD of two independent experiments.

4. Discussion

Despite the improvements achieved during the last decades in the prevention of surgical infections, implant-related infections are still a partially unmet clinical challenge. Indeed, although they are not the most frequent cause of implant loss, infections are surely the most threatening, being associated with substantial morbidity and high economic burden. Moreover, these infections are often difficult to treat, frequently involving MDR strains and the formation of biofilm on implant surfaces. In the years, biomedical research has focused on the development of anti-infective biomaterials for the prevention of biomedical device-related infections. AMPs are considered promising alternatives to conventional antibiotics, given their broad-spectrum antimicrobial activity and their immunomodulatory properties. A considerable part of the research on AMPs is now focused on immobilization of natural AMPs and their synthetic derivatives on various supports of medical interest.

The main goal of the present study was to identify an antimicrobial peptide, effective against Gram-positive species, to be utilized for the development of novel, AMP-based anti-infective biomaterials for arthroplasty. The most suitable candidate should be effective under conditions resembling those encountered in revision surgery, and be devoid of cytotoxicity or other negative effects on relevant host cells. In this regard, the potential of five candidate AMPs was evaluated in the present thesis (Tab. 3.1). These starting molecules have been selected from a previously constructed database collecting data obtained by the research group on structural and antimicrobial properties of a large number of different native and synthetic AMPs. The first selection criterion took into consideration the activity against Gram-positive microorganisms, which are isolated from roughly 70% of HCAIs [12]. An additional criterion were the structural properties of the peptides in order to select simple molecules from a chemical point of view, that should meet the requirement of low manufacturing costs for cost-effective large scale production.

The first step in the identification of the peptide candidate was the comparison of the activities of the selected AMPs against clinically relevant bacterial strains (Tab. 3.2). To this aim, AMPs under study have been tested both against *S. epidermidis* and *S. aureus* reference strains and a panel of Gram-positive bacteria isolated from cases of hip- and knee-prosthesis revision and other orthopaedic surgery at the Valdoltra Hospital in Slovenia and at the main hospital of Udine. Isolates included *S. epidermidis* and *S. aureus*, as well as other less frequent staphylococci and streptococci isolates. All BMAPs displayed similar low micromolar MIC values against most of the strains in standard conditions. These data are consistent with previous findings demonstrating the broad spectrum antimicrobial activity of BMAP27, BMAP28 and their analogues, also against clinical isolates [93, 230, 231, 258]. This wide activity spectrum is mainly due to the mechanism of action of these peptides, which involves membrane perturbation, leading to membrane permeabilization or its depolarization [100, 258, 259]. Conversely, P19(9/G7) showed a clearly lower activity against *S. aureus* as compared to the other tested peptides. This may be partially ascribed to a slightly different mode of action between BMAPs and P19(9/G7). Although all these peptides target bacterial membrane, P19(9/G7) presumably does not cause pore formation. Indeed, when Pag et al. characterized their synthetic analogues of the P19 series, they observed a mechanism of membrane depolarization more consistent with the hypothesis that these peptides can disorganize functional processes and alter cellular response pattern by low-affinity interactions with membrane components, and their antimicrobial effects varied greatly among tested strains [229]. Although most clinical isolates proved susceptible to the AMPs under investigation, we found that the two *E. faecalis* strains were scarcely or not susceptible, with the exception of BMAP28 and partially BMAP27 (Tab. 3.2). This finding was unexpected as in a previous report both full length BMAPs were highly effective against 10 *E. faecalis* isolates, including vancomycin resistant (VREF) strains with MIC ranges 2-16 µM and 1-8 µM for BMAP27 and BMAP28, respectively, whereas both shorter derivatives displayed significantly higher MIC values [230]. It is interesting to note, however, that in the cited work all the BMAP peptides displayed lower MIC values against VREF isolates than against antibiotic-susceptible *E. faecalis. E. faecalis* is generally susceptible to cationic antimicrobial peptides but seems to be prone to acquire resistance to this class of antimicrobials, and the acquisition of resistance towards one cationic AMP can also lead to cross-resistance towards other peptides of the same type [260-262].

In our case, we do not know what therapies have been administered to the patients from which they have been isolated, and therefore, given also the small sample studied, we cannot exclude that the two strains, for unknown reasons, acquired a feature that conferred them resistance to the tested AMPs. Notably, in our hands both *E. faecalis* isolates were not susceptible to the cephalosporin cefazolin. Comparing the activity of AMPs with conventional antibiotics, peptides demonstrated comparable antimicrobial activity on a molar basis (Tab 3.2). Notably, peptides were effective also against the methicillin resistant *S. epidermidis* (MRSE), and this is consistent with the different mode of action of AMPs with respect to conventional antibiotics. Indeed, cationic AMPs are known to act on membrane features that could be difficult for microorganisms to modify. Moreover, the result seen for BMAP 28 can be considered in line with that of Takagi et al. demonstrating the ability of BMAP28 to kill methicillin resistant *S. aureus* [263].

The selected peptides have been also tested against staphylococcal biofilm as biofilm formation on implants renders difficult and problematic the treatment of infections, mainly for the increased resistance of bacteria when embedded in this type of structure. Adhesion of bacteria to the surface of implanted devices is the first step towards the formation of a mature biofilm that, once formed, is difficult to eradicate. Thus our study focused on the initial phase of biofilm formation that occurs in the first 18-24h of growth [264]. Results indicate that the selected AMPs were able to inhibit the formation of staphylococcal biofilm on polystyrene plates at concentrations corresponding to MIC and above (Fig. 3.2). This presumably indicates rapid killing of bacteria before their deposition and attachment to the surface. Therefore, the selected AMPs at MIC concentrations can be effective in the prevention of bacterial colonization of the implant. To note, cefazolin, which is the antibiotic commonly used in orthopaedic prophylaxis, showed a similar inhibition only at 4 folds its MIC. In a previous study, BMAP27 and BMAP28 were tested for their anti-biofilm activity against clinical isolates from cystic fibrosis patients [265]. The present results are only partially comparable to those obtained by Pompilio et al., where the authors found that BMAP28 inhibited formation of biofilm by a *S. aureus* isolate at concentrations close to MIC values (½ MIC, ¼ MIC), whereas BMAP27 was not effective at these concentrations. The discrepancies between the two studies could be related to the different *staphylococcus* species. In addition, one should be aware of the great variability in biofilm-forming capacity between reference strains and clinical isolates and among isolates themselves [266].

Beyond killing of microorganisms, AMPs have the additional property to bind lipopolysaccharide (LPS) and lipoteichoic acid (LTA), the main envelope components of Gram-negative and Gram-positive bacteria, respectively [186, 267], and to neutralize their pro-inflammatory effects. This is an important and distinctive feature of AMPs, since conventional antibiotics are not able to counteract these bacterial components, but rather stimulate their release from lysed bacteria [268]. LPS and LTA can adhere to surfaces, and have been demonstrated to remain as contaminants on implant surfaces after sterilization, generating inflammation and negatively affecting implant osseointegration by impairing osteoblast differentiation [269]. Hence, AMPs could favour implant integration also by blocking the unwanted reactions promoted by these toxic bacterial components. Moreover, endotoxin can adhere to titanium surfaces [270] and contaminate the wear particles derived from the implant parts. These particles can induce inflammation, leading to aseptic loosening of the prosthesis. Contaminating endotoxin has been shown to strongly increase the proinflammatory activity of wear particles [271]. Hence, we evaluated the ability of the selected AMPs to bind LPS and LTA by measuring the inhibition of NO production by RAW264.7 cells in response to stimulation with these two bacterial components. We found that the two full length BMAPs were able to neutralize the LPS-stimulated NO production in the low micromolar concentration, while the two truncated BMAPs were somehow less effective (Fig. 3.3a). Considering the experimental design, peptide-mediated effects on macrophages could reasonably be ruled out as the peptides were added to cells simultaneously with LPS. Hence, it appears more likely that the effects observed depend on the interaction of the peptides with endotoxin. It is possible that the C-terminal tail of the full length BMAPs act in a cooperative way with the N-terminal part in the binding of LPS, making the interaction stronger. This was already described by Tack et al. for SMAP29 and CAP18, the BMAPs orthologs from sheep and rabbit, respectively [57, 272]. These two peptides have been demonstrated to have two LPS binding sites that cooperate intramolecularly (i.e. involving the N- and C-terminal LPS-binding sites of the same peptide molecule) in a synergistic way [272]. It is interesting to note that the authors identified the higher affinity LPS-binding domain in the N-terminal portion [272]. Given the high structural homology of bovine BMAPs with the ovine congener, it is reasonable to assume that BMAP27(1-18) and BMAP28(1-18) are endowed with a high affinity LPS binding site. This would explain why these two analogs at slightly higher concentrations respect to the parent peptides are able to partially neutralize LPS. Data are consistent with previous results obtained by the research group with a different method. The ability of BMAP27 and BMAP28 to neutralize LPS at concentrations comparable to those found in this thesis has been demonstrated by using the *Limulus* amebocyte lysate (LAL) assay [267, 273]. Moreover, Lee et al., using the same cellular system, found inhibition values similar to ours for both BMAP27 and BMAP27(1-18), although in our hands these two peptides were a bit more effective in inhibiting LPS-stimulated nitrite production. Regarding LTA-stimulated cells, a general impression was that all the AMPs proved less effective, although it would be hard to make a direct comparison with their LPS-neutralizing ability. In this regard, it is worth to remind that RAW macrophages were stimulated with a higher concentration of LTA than LPS (5 µg/ml *vs*. 100 ng/ml, respectively) to obtain a comparable NO production. This would help to explain the apparent minor affinity of the peptides for this bacterial component. In particular, P19(9/G7) was only marginally effective in the inhibition of nitrite production, and truncated BMAPs reached a 50% inhibition at the higher concentrations used (Fig. 3.3b). For full-length BMAPs, data obtained for BMAP28 are consistent with a previous publication reporting complete inhibition of LTA-stimulated nitrite release by 4 µM peptide [274]. Furthermore, Takagi et al recently demonstrated a direct interaction of LTA with surface-immobilized BMAP28 by using the quartz crystal microbalance (QCM) technique [275].

Finally, it is important to note that under these experimental conditions none of the AMPs under study proved toxic to RAW 264.7 macrophages as assessed by a metabolic dye assay.

Cytotoxicity is one of the major drawbacks in the therapeutic development of AMPs. The most potent, wide spectrum α-helical peptides are endowed with structural properties that enable the interaction with bacterial membrane components. At higher than microbicidal concentrations, however, peptide interaction with host cell membranes can occur, depending on peptide itself and incubation conditions. In the present thesis this issue was addressed by focusing on osteoblast cells for the application context we pursued. To this end, possible cytotoxic effects of the peptides were evaluated on MG-63 osteoblast-derived cell line by measuring the release of the cytoplasmic enzyme LDH after a short incubation time (1 h), and cell viability with a metabolic dye after a longer incubation period (24 h) (Fig. 3.4a and b, respectively). The LDH assay indicates cell membrane damage, as it measures the release of a cellular component, that occurs rapidly after the addition of a cytotoxic, membrane-active peptide. The absence of LDH release can therefore indicate absence of gross membrane disruption, but may not be sufficient to exclude other types of cell death, such as apoptosis, that can occur without gross membrane permeabilization. Therefore, the use of PrestoBlue®, which determines cell metabolic activity, would permit to detect possible toxic effects, delayed in time, that go beyond membrane permeabilization. Indeed, BMAP27 and BMAP28 have been characterized in previous reports for their ability to induce apoptosis in various cell types [101]. Moreover, the human cathelicidin LL-37 has been recently reported to reduce cell number and viability of MG-63 osteoblast-like cells by inducing apoptosis [276]. The results of both types of analysis revealed that full-length BMAPs were safe to MG-63 cells in the range of their microbicidal concentrations (1.25-5 μM), and became clearly toxic at 10 μM and above. On the contrary, the truncated BMAPs and P19(9/G7) were not toxic to osteoblast cells up to 10 μM. These data confirm previous data of the research group and others reporting improvement of the therapeutic potential of the shorter N-terminal BMAP analogues respect to the parent full length BMAPs [100, 259]. Other investigators obtained a similar improvement in target cell selectivity by designing a series of alanine-substituted analogues of BMAP27 and BMAP28 [277, 278].

Recently, the human β-defensins have been shown to be able to differently affect osteoblast cells. The osteoblast-like MG-63 cells showed transcription levels of osteogenic markers increased to various extent upon incubation with hBD-1, hBD-2, and hBD-3 [188]. Moreover, the latter two defensins significantly increased in these cells the enzymatic activity of alkaline phosphatase (ALP), a marker of early osteoblast differentiation. This finding prompted us to investigate whether the peptides under study have some osteoinductive properties. As a first step in this direction we measured ALP activity of MG-63 cells upon treatment with AMPs or with the differentiation stimulus dexamethasone (Fig. 3.5). In these experiments, however, BMAP27 and BMAP28 confirmed to be somehow cytotoxic, probably due to the prolonged, 7 day incubation with cells continuously exposed to the peptides in osteogenic medium without serum. On the other hand, the three shorter peptides demonstrated neutral towards osteoblast differentiation, without any toxic effects. Indeed, they were not able to induce differentiation *per se* but were not toxic to the cells and did not impair osteoblast differentiation when incubated with cells together with dexamethasone (Fig 3.5b). The three shorter AMPs, namely BMAP27(1-18), BMAP28(1-18) and P19(9/G7), were thus selected for further characterization under experimental conditions most resembling those of the prosthetic environment.

In the pharmacological development, testing of compounds in conditions most resembling those of the intended applicative context is mandatory. This applies even more to antimicrobial peptides as their potent *in vitro* activity observed under standard laboratory conditions can be inhibited to various extent by components present in the biological fluids such as blood serum. Peptide stability in normal human serum has been addressed by incubation of AMPs in serum, either heat inactivated or not, at 37°C, followed by RP-HPLC analysis. However, these experiments did not give any useful information due to inconsistent results, likely because the precipitation of serum proteins by trichloroacetic acid (TCA) addition caused also a non-uniform precipitation of the peptide molecule, suggesting that peptide was entrapped by some serum proteins. Haines et al. addressed this issue by incubating BMAP27(1-18) in human blood, serum or plasma, in the presence of specific affinity purified anti-BMAP antibodies [95]. After overnight incubation at 4°C, they were able to immunoprecipitate the intact peptide from blood and plasma, as confirmed by mass spectrometry, but not from serum. They proposed the increased proteolytic activity of serum to be responsible of peptide degradation. It should be noted, however, that the serum they used has not been heat inactivated. Several other investigators addressed this issue by testing the antimicrobial activity of various AMPs in the presence of serum both from humans or other animal species, either heat inactivated or not. Despite different experimental settings, in general the antimicrobial activity was found decreased in the presence of serum [70, 278, 279]. In this thesis, the effect of serum was evaluated by monitoring bacterial growth kinetics in the presence of heat inactivated human and bovine serum (Fig. 3.6 and 3.7). Interestingly, the anti-staphylococcal activity of AMPs was not inhibited but rather improved by serum, as in the case of BMAP27(1-18), although serum *per se* did not exhibit any adverse effect towards bacteria (Fig. 3.7c). One possible explanation could be that bacteria grown in the presence of serum become more susceptible to AMPs. Recently, other authors reported similar results. Benincasa et al. reported slightly decreased MIC values of the proline-rich Bac7(1-35) against *Salmonella typhimurium* in the presence of human serum, and even lower values in the presence of human plasma, whereas mouse serum and plasma strongly inhibited the peptide [280]. There are however important differences between this study and the present thesis concerning the antimicrobial peptide (i.e. PR-rich *vs* α-helical), the bacterial species (Gram-negative *vs* Gram-positive), and serum, that in our case was heat inactivated. In addition, we observed a potentiating effect by both human and bovine serum (Fig. 3.6a, b and c), whereas Benincasa et al. registered remarkable differences between human and murine blood components [280]. We found opposite results when the same experiments were performed by pre-incubating the AMPs under study with serum before adding the bacterial inoculum (Fig 3.6d, e and f). In this case, human serum inhibited the AMPs to various extents, depending on the peptide. It may be of note that the least inhibited was BMAP27(1-18) that is the more cationic and less hydrophobic among the three selected AMPs. To better understand the structural determinants involved in the serum-mediated peptide inhibition, the activity of BMAP27(1-18) was further investigated in comparison with the all-D enantiomer in the presence of human and bovine serum, either heat inactivated or not (Fig. 3.13). Inserting D-amino acid or other unnatural residues into the sequence of antimicrobial peptides is one of the approaches to improve their stability and avoid proteolytic degradation [281]. Several authors exploited the potential of partial or total D-amino acid substitution to improve the stability of cationic AMPs towards serum, and in particular human serum [282-285]. Despite different methods were used in these studies to assess the stability of D-substituted peptides, the common result was the improvement of peptide stability towards human serum and proteases [283-285]. Moreover, the inactivation of serum proteases restored also L-peptide activity [283, 285]. Our findings are only partially in line with these data. Indeed, we found that D-

BMAP27(1-18) is more resistant to serum inhibition than the L-peptide (Fig. 3.13c and d). However, unexpectedly, heat inactivation of serum did not restore the partial inhibition of D-BMAP27(1-18) (Fig. 3.13d and f). In the light of these results, we speculate that the inhibition exerted by human serum does not rely only on simple enzymatic degradation, but involves also aspecific interactions between the AMPs and unknown serum components. Moreover, these interactions can become even stronger upon heat inactivation, likely due to hydrophobic interactions with denatured serum proteins. In this respect, it is worth to note that in our serum stability experiments (see above), we were not able to reproducibly recover BMAP27(1-18) after incubation in heat inactivated serum and TCA precipitation, presumably for its precipitation together with serum proteins. Hong et al. found similar results, with heat-inactivated serum being capable to partially inhibit the activity of the all-D-enantiomer of their membrane-active peptide [286]. They speculated that certain serum components could interact with the peptides and inhibit their binding to pathogen membrane. Moreover, in the early '90s Peck-Miller et al. reported that the low-density lipoprotein in heat inactivated serum interacted with the α-helices of membrane-active peptides, decreasing their activity [287]. The involvement of lipoproteins has been also reported for LL-37 [247].

If used as coatings for orthopaedic prostheses, candidate AMP would come into contact with synovial fluid (SF), and in particular with its main component, hyaluronic acid (HA), which is an anionic, non-sulfated glycosaminoglycan. Being HA negatively charged, one could speculate that it may interact with cationic AMPs, sequestrating them and impeding their electrostatic interaction with bacterial membranes, therefore inhibiting their antimicrobial activity. Despite these considerations, when the selected AMPs were tested in the presence of two different concentrations of HA (i.e. 0.5 mg/mL and 3 mg/mL, concentrations found in arthroprosthetic patients and healthy subjects, respectively [251, 252]), no inhibition of their antimicrobial activity was observed (Fig. 3.8). This finding is in contrast with several references demonstrating inhibition of AMPs by polysaccharides [288-292]. In these latter works, cationic AMPs were incubated with different polysaccharides from pathogenic bacteria. In general, AMP activity was inhibited, but this inhibition only partially correlated with the charge of the polysaccharide. In fact, in these studies also charge density resulted important. Moreover, it has been speculated that also non-electrostatic interactions between the apolar surface of AMPs and hydrophobic patches of polysaccharide chains could take place. Therefore, the interaction between AMPs and negatively charged polysaccharides is not determined merely by the opposite charges of the two interactors, but also by their respective conformations. Therefore, it is possible that a different propensity of the AMPs to assume their secondary structure and the spatial distribution of HA led us to obtain different, promising results. Indeed, it has been demonstrated that peptides sharing a common structural scaffold can have distinct structuring and aggregational properties [293]. Peptides such as LL-37 self-assemble under physiological conditions, whereas the *rhesus* orthologue RL-37, despite 70% sequence homology with LL-37, does not fold or oligomerize in aqueous solution [293, 294]. As a consequence, the propensity of peptides such as LL-37 to oligomerize renders them more prone to sequestration by medium components [57, 293]. On the contrary, peptides like BMAPs, similar to RL-37 fold only in the presence of an anisotropic environment [100]. As a result, their antimicrobial activity appears less environmentally sensitive [295].

Besides hyaluronic acid, synovial fluid (SF) from inflamed joints contains many other components, and therefore it would be important also to evaluate the antimicrobial activity of the peptides in the presence of whole synovial fluid collected from orthopaedic patients. However, samples from patients were difficult to obtain and what is even harder to control the processing phases. Indeed, synovial fluid has to be processed as soon as possible after withdrawal to remove the cellular components that lyse and release their content during freezing and thawing of SF samples. Hence, it was possible to perform only a limited number of experiments. Moreover, we found that the majority of our SF samples inhibited *per se* bacterial growth (Fig. 3.11b, d and f). This observation is consistent with that of Gruber et al., which demonstrated the capability of SF collected from patients to inhibit the growth of gram positive bacteria such ad *S. aureus*, *S. epidermidis* and *S. pyogenes* [296]. We found that candidate peptides were not inhibited by SF, and their antimicrobial activity seemed to be improved (Fig 3.11a, c and e). This could be due to synergy between the tested AMPs and other antimicrobial peptidic components which are demonstrated to be present in SF [297, 298].

A remarkable finding of this study was the antimicrobial efficacy of the peptides immobilized on solid support (Fig. 3.15). The original goal of the project, i.e. covalent tethering of the most promising AMPs to a titanium surface, has been reformulated due to unforeseen technical problems in the coupling procedures. Therefore, the experiments have been carried out with biotinylated peptides bound to streptavidin-functionalized resin beads, by exploiting the extremely high affinity of the streptavidin-biotin interaction, which approaches that of a covalent bond [299]. Streptavidin-biotin technology is widely used in protein chemistry for a wide range of applications including protein and peptide immobilization on various supports [300-302]. Hilpert et al. used biotinylated AMPs, bound to streptavidin-coated microtiter plates, to demonstrate that immobilized short AMPs were able to kill different microorganisms, and this ability was independent of the support to which they were tethered [211]. In the present thesis, a commercially available streptavidin-agarose resin, designed for affinity protein purification, was used. The choice was based on the high loading capacity of the resin, as stated by the manufacturer (>300 nmol/ml settled resin), and on the possibility to perform extensive washings by simple centrifugation steps. Furthermore, coupling of biotinylated peptides to streptavidin-coated resin beads was carried out in aqueous solution, at physiological pH, at room temperature or at 4°C, thus avoiding extreme laboratory conditions that are frequently used in covalent procedures [303].

In the first set of experiments, the selected peptide candidate BMAP27(1-18) was compared to the full length BMAP27. Both peptides have been immobilized through the biotinylated N-terminus. An additional analogue of BMAP27(1-18), bearing a short spacer between the biotin and the N-terminus, was coupled to the resin. Extensive washes performed after the coupling step were successful in removing unbound peptide molecules from resin supernatants based on both the absence of antimicrobial activity and the UV absorbance levels reaching the baseline. All three resin-bound AMPs proved bactericidal against representative Gram-positive species, without differences between the full length BMAP27 and the shorter analogue BMAP27(1-18) (Fig. 3.15). The attenuated antimicrobial effect of immobilized biot-PEG-B27(1-18) could depend on lower activity of this derivative or lower resin loading, or both. The comparison of the antimicrobial activities of the biotinylated AMPs in solution revealed almost identical MIC values against *S. epidermidis* and only slightly higher values against *S. aureus* in the case of the biot-PEG-B27(1-18) (Tab. 3.3). On the other hand, to quantitatively assess resin loading it would be necessary to label the peptides with a fluorophore, because the detection of a fluorescent signal upon coupling would be the simplest and most sensitive method (see beyond).

Interestingly, none of the immobilized AMPs was toxic to osteoblast cells (Tab. 3.4), in keeping with a previous observation reporting absence of toxic effects for surface-bound BMAP27 [256], and consistent with the report of Hilpert et al., which observed that tethering of AMPs greatly reduced the hemolytic effect of their soluble counterparts [211]. These results, although qualitative, are promising and encourage further efforts to characterize the antimicrobial properties of the immobilized AMPs more in depth.

In the next set of experiments, the peptide candidate BMAP27(1-18) was chemically modified in order to obtain a fluorescent analogue without gross perturbation of its structure. Our concern was that the addition of a large fluorophore at either end of a relatively short peptide sequence could impact on peptide secondary structure and, in turn, on its biological activity. In this regard, the substitution of Phe residues with Phe_{CN} seemed non-invasive [294]. The emission quantum yield of Phe_{CN} is five times greater than that of Phe, and the polarity of the cyano group is intermediate between an amide and a methyl group, thus allowing the placement of this side chain in a hydrophilic, as well as in a hydrophobic environment [304]. Accordingly, Xhindoli et al. did not observe any effect on the secondary structure nor impairment of the antibacterial activity of the human cathelicidin LL-37 upon replacement of Phe by Phe_{CN} [294]. The four Phe residues of BMAP27(1-18) have been systematically substituted with Trp, Leu, Ile, and D-Phe by Lee et al. in a structure-activity-relationship (SAR) study aimed at reducing the cytotoxic and haemolytic effects of the parent peptide BMAP27 [259]. In the cited work, these analogues showed different hydrophobicity, as deduced by their RP-HPLC retention times. Nevertheless, they underwent a similar random coil to α-helical transition in the presence of LPS and displayed almost equal MIC values against Gram-positive and Gram-negative bacteria. In our hands, the replacement of Phe with Phe_{CN} produced a less hydrophobic derivative, which in anisotropic environment underwent the typical conformational transition of membrane-active α-helical AMPs and displayed highly similar MIC values against two staphylococcal reference strains with respect to the parent peptide (Fig. 3.17, Tab. 3.5). Unexpectedly, major differences in the behaviour of the Phe_{CN} derivative with respect to BMAP27(1-18) were observed in the presence of human serum, as upon preincubation with serum BMAP27(1-18)Phe_{CN} was inhibited to a significantly greater extent (Fig. 3.18). This finding would suggest the existence of soluble and heat stable serum components that are able to bind and sequester the peptide. A similar serum mediated inhibition was observed also in the case of pegylated Phe_{CN} derivatives, though at correspondingly higher peptide concentrations (Fig. 3.22). What is interesting to point out in this regard is the distinct inhibitory effect of human serum on the non-pegylated Phe_{CN} derivative (Fig. 3.18), whereas the activity of the pegylated peptide seems almost equally inhibited by both human and bovine serum (Fig. 3.22). This finding would suggest that pegylation protects the peptide from the action/sequestration by a species-specific component of the human serum. In this respect it is worth to recall that the covalent attachment of large PEG moieties (20-30 kDa) to peptide and protein-based drugs is used to improve stability and pharmacokinetics by protecting them from proteolysis, inhibiting aggregation, and reducing their immunogenicity [305]. A few cases of pegylation of AMPs are reported in the literature [280, 306-309]. The experimental conditions used in these publications are however highly variable with respect to the peptide, the site of attachment and the size of PEG moiety, and the target microorganism. The most resembling conditions respect to our approach are those used by the Matsuzaki research group. They modified two structurally different AMPs, i.e. the β-hairpin tachyplesin [308] and the α-helical magainin [307], by covalent attachment of PEG_{5000} at their N-terminus. Both pegylated AMPs showed MIC values against reference Gram-positive and Gram-negative bacteria increased to various extents. Interestingly, when the activity was assayed in the presence of normal human serum, as in the case of PEG_{5000}-tachyplesin, the increase of the MIC was less evident, suggesting that pegylation reduced the interaction of tachyplesin with serum components [308].

As a preliminary approach to study the mode of action of the immobilized AMPs, the antimicrobial activities of the N- and C-terminally coupled variants of the Phe_{CN} derivative were compared. To this aim, it was necessary to estimate the level of resin loading by taking advantage of the fluorescence emitted by the immobilized BMAP27(1-18)Phe_{CN}. However, we realized that quantification of the immobilized peptides by using calibration curves constructed previously with the soluble Phe_{CN} derivative could have some limitations, as fluorescence quenching by the resin and/or by the peptide itself cannot be excluded. Nevertheless, a rough estimation of peptide concentration, provided the term “concentration” in this case was correct, placed the resin-associated peptide in the order of magnitude of micromolar (approximately 5-7 μM). This estimate was obviously very similar for both the N- and the C-terminally coupled peptides. Despite very similar fluorescence intensities associated to both resins (Tab. 3.7), the C-terminally bound analogue seemed somehow less effective against *S. epidermidis* (Fig. 3.23). Contrariwise, in solution the C-terminally PEGylated Phe_{CN} derivative showed almost identical antimicrobial activity as the N-terminally PEGylated Phe_{CN} analogue (Tab. 3.6). Thus, provided resin loading was comparable, one could speculate that peptide immobilization through the C-terminus could have some negative repercussions on the antimicrobial activity. It should be noted, however, that direct comparisons between immobilized AMPs and their soluble counterparts are not entirely appropriate. The comparison of antimicrobial activities of different AMPs in solution is based on the comparison of their effective concentrations, i.e. their MIC values. It is obvious that the same concept does not apply to immobilized AMPs. In this context, a “surface concentration” would be difficult to define exactly, given the wide selection of different coupling procedures and supports. Even in the case of covalently bound peptides, standardized methods to determine unequivocally the density of peptide molecules on the surface are lacking. Moreover, the activity of surface-bound AMPs seems not always related to the activity of the soluble counterparts. In this regard, Hilpert et al. reported cases of both inactive and active soluble AMPs that gained and loose activity upon immobilization, respectively [211].

Collectively, the results obtained in the present thesis provide useful information about the antimicrobial properties of selected peptide candidates at a pre-clinical level. The AMPs have been investigated for their ability to kill relevant Gram-positive clinical isolates, also including antibiotic-resistant strains, in comparison with two conventional antibiotics. They have been also characterized for their ability to neutralize LPS- and LTA-induced responses in the mouse RAW 264.7 macrophage cell line. Short-term and long-term toxic effects on host cells have been characterized on the osteoblast-like MG-63 cell line. The peptides have been also incubated with these cells for longer time-periods to evaluate possible effects on osteogenic differentiation. These experiments led to the identification of three AMPs endowed with potent activity against Gram-positive bacteria and devoid of toxic or other negative effects on host cells. Importantly, the study then narrowed on the characterization of the antimicrobial activity in conditions more close to those encountered in the context of arthroplasty. The three selected AMPs were investigated against a reference strain of *S. epidermidis* in the presence of human and bovine serum, hyaluronic acid and samples of synovial fluid collected from patients. This phase of the study permitted the identification of the most active peptide candidate to be immobilized on solid support. BMAP27(1-18) was selected based on the observation that the antimicrobial activity of this peptide was less affected by the presence of human serum. In addition, a fluorescent analogue of BMAP27(1-18) was synthesized and characterized for quantification purposes related to surface-immobilization. Concerning peptide grafting on resin, despite the many limitations, the approach based on the streptavidin-biotin technology allowed the achievement of the proof-of-concept that the immobilized BMAP27(1-18) maintained antimicrobial activity against representative *staphylococcus* species. The results obtained are promising and encourage further efforts in approaching the chemical issues in the development of peptide-based materials resistant to infection for biomedical devices.

5. Material and methods

5.1 Peptide synthesis and characterization

5.1.1 Chemicals for peptide synthesis and other reagents

Derivatized PEG-PS resins, coupling reagents for peptide synthesis and 9-fluorenylmethoxy carbonyl (Fmoc)-amino acids were purchased from Applied Biosystems, Novabiochem and ChemImpex. The biotinylating reagents D(+)-biotin, O-(N-Biotinyl-3-aminopropyl)-O'-(N-glutaryl-3-aminopropyl)-diethyleneglycol (N-biotinyl-NH-PEG_2-COOH), and EZ-LINK® Maleimide-PEG_2-biotin were from Calbiochem, Novabiochem and ThermoFisher Scientific, respectively. Peptide synthesis-grade N,N-dimethylformamide (DMF), dichloromethane, piperidine and HPLC-grade acetonitrile were from Biosolve. Trifluoroacetic acid, trifluoroethanol and N-methylmorpholine were from Acros Chimica. The antibiotics cefazolin and linezolid were purchased from Sigma-Aldrich, resuspended in water (cefazolin) or in dimethyl sulfoxide (linezolid) and maintained at -20°C until use.

5.1.2 Peptide synthesis and biotinylation

The names and amino acid sequences of the synthesized peptides are reported in Table 3.1 of the Results section (page 38). All peptides were obtained by standard solid-phase synthesis using Fmoc chemistry, as previously reported [100]. To obtain C-terminal amidation, the PAL-PEG-PS resin with a substitution of 0.16-0.2 meq/g was used. Difficult coupling steps were carried out as described [100]. For the synthesis of the fluorescent BMAP27(1-18)Phe_{CN} derivative, the four Phe residues in the sequence of BMAP27(1-18) were replaced by 4-cyano-phenylalanine (Phe_{CN}) by using Fmoc-4–cyano-L-phenylalanine (ChemPep Inc). In addition, for immobilization and pegylation purposes, a Cys residue was introduced either at the C-terminus or at the N-terminus of the sequence by using Fmoc-S-trityl-L-Cys-pentafluorophenyl ester (OPfp) in the presence of equimolar 1H-hydroxybenzotriazole (HOBt). The all-D enantiomer of BMAP27(1-18) was a generous gift of prof. M. Scocchi from the Department of Life Science, University of Trieste.

BMAP27 and BMAP27(1-18) were biotinylated off-line by coupling 5 equivalents of D(+)-biotin to the N-terminus of resin-bound peptides in the presence of equimolar 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro-borate (TBTU) and HOBt in DMF containing 0.6 N N-methylmorpholine for 4 h at room temperature. In addition, BMAP27(1-18) was alternatively biotinylated with N-biotinyl-NH-PEG_2-COOH by using exactly the same procedure. In the following sections, these biotinylated peptides are referred to as biot-B27, biot-B27(1-18) and biot-PEG-B27(1-18). The completion of the reaction was monitored by Kaiser test [310] after washing with DMF, methanol, glacial acetic acid and diethyl ether of the resin-bound peptides. After deprotection and cleavage from the resin, the peptides were purified by reverse phase high performance liquid chromatography (RP-HPLC) on a C18 Delta-Pak column (Waters). Peptide identity was confirmed by mass spectrometry using a Q-STAR hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex) equipped with an electrospray ion source.

5.1.3 Peptide pegylation in solution

The two cysteinyl analogues of BMAP27(1-18) Phe_{CN}, carrying the cysteine residue at the N – or the C –terminus, were pegylated by incubation with PEG_{3000}-Maleimide (Sigma Aldrich) at 1:2 molar ratio in 0.1 M sodium phosphate buffer, pH 7.4 for 1 h at room temperature. The pegylated products were separated from the unpegylated peptides or peptide dimers by reverse-phase chromatography with the HPLC apparatus. The two resulting peptides are referred to as BMAP27(1-18)Phe_{CN}-PEG (pegylated at C-terminus) and PEG-BMAP27(1-18)Phe_{CN} (pegylated at N-terminus) in the following sections.

5.1.4 Determination of peptide concentration

Peptide concentrations were determined by measuring the UV absorbance at 257 and 280 nm in aqueous solution based on the molar extinction coefficients of specific amino acid residues as follows:

- BMAP27 and BMAP27(1-18), 195.1 cm^{-1} M^{-1} at 257 nm for Phe [311];
- BMAP28 and BMAP28(1-18), 5559 cm^{-1} M^{-1} at 280 nm for Trp [311] and 1280 cm^{-1} M^{-1} at 280 nm for Tyr [312];

- P19(9/G7), 1280 cm^{-1} M^{-1} at 280 nm for Tyr [312];
- BMAP27(1-18)Phe_{CN}, 850 cm^{-1} M^{-1} at 280 nm for Phe_{CN} [231, 304, 313].

In addition, a calibration curve of the latter peptide was constructed by taking advantage of its fluorescence. Briefly, a stock solution with a known concentration of BMAP27(1-18)Phe_{CN} was serially diluted in 100 µl of chloride-free aqueous buffer in a black 96 well plate and the emitted fluorescence was measured at 300 nm upon excitation at 240 nm with a microplate reader (Perkin Elmer).

5.1.5 Reverse-phase chromatography

Peptide purification was carried out with semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) with a linear aqueous acetonitrile gradient containing 0.05% trifluoroacetic acid (TFA) on a C18 Delta-Pak column (Waters) with the System Gold apparatus (Beckman). The same system equipped with the Symmetry C18 column (Waters) was used for analytical assays.

5.1.6 Circular Dichroism

Circular dichroism (CD) spectra were recorded at 20 µM peptide concentration in 10 mM sodium phosphate buffer, pH 7.4, in the absence and in the presence of 50% trifluoroethanol, using a Jasco J-600 spectropolarimeter and 2-mm-path length quartz cells. All spectra are the mean of at least two trials, each with the accumulation of three scans. Helical content was estimated from the ellipticity at 222 nm according to Chen et al. [314].

5.1.7 Quantitation of sulfhydryl groups

The determination of free sulfhydryl groups was performed by using the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), called the Ellman's reagent according to Ellman, G.L. [315]. The DTNB reacts quantitatively with reduced thiols yielding a coloured product. The determination was performed by measuring the absorbance at 412 nm of known solutions of cysteinyl-peptides incubated for 15 min with a fixed proportion of the reagent, by comparison to a standard curve composed of known concentrations of cysteine.

5.1.8 Peptide stability in the presence of human serum

Blood samples were collected from healthy donors and allowed to clot spontaneously to obtain normal human serum after centrifugation at 300 g. Some aliquots of serum were inactivated by heating at 56°C for 30 min. Peptides were diluted at a final concentration of 0.5 μg/μL in the presence of 25% of the selected serum in aqueous solution. Samples were then incubated at 37°C and 20 μL of solution were then collected at 0, 15, 30, 60, and 180 minutes and precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 2% (wt/v). After 10 min on ice, the samples were centrifuged and analysed on the Symmetry C18 column (Waters) with a linear aqueous acetonitrile gradient containing 0.05% TFA.

5.2 Peptide immobilization on solid support

The high performance streptavidin-Sepharose® resin (GE Healthcare Life Sciences), designed for the purification of biotinylated molecules, was used as support for peptide immobilization. The resin is composed of highly cross-linked agarose beads with a mean diameter of 34 μm and is supposed to have high binding capacity, as stated by the manufacturer. Handling of the resin occured at room temperature or at 4°C, in aqueous solution, with physiological salt concentrations, and at physiological pH values.

Two slightly different procedures were used: a) peptides have been previously biotinylated at the N-terminus and afterwards coupled to resin beads in one step and b) resin beads were first decorated with a biotinylated small molecule carrying a maleimide moiety (EZ-LINK® Maleimide-PEG$_2$-biotin), and afterwards the cysteinylated peptides were added in a second step. This latter procedure has been used only with the cysteinylated Phe$_{CN}$ derivatives, with the aim to compare their activity upon immobilization through the N- or the C-terminus. Prior to any peptide immobilization, resin aliquots of 250 μL were transferred in Eppendorf tubes and thoroughly washed three times with 1 mL of sterile PBS, by 5 min centrifugation at 300 g at room temperature, to remove the ethanol present in the storage buffer.

5.2.1 a) One step functionalisation with biotinylated peptides

The three biotinylated peptides, i.e. biot-B27, biot-B27(1-18) and biot-PEG-B27(1-18), were dissolved in sterile PBS at 1 mM peptide concentration. 250 µL of each peptide solution were added to the same volume of resin samples and allowed to react overnight at 4°C under agitation. For reference, one resin sample was coupled in parallel with biotin. Samples were then allowed to re-equilibrate at room temperature, centrifuged, and washed five times with 1 mL PBS as previously described. The absorbance at 257 nm of resin supernatants was monitored throughout the incorporation procedure. The washing steps ceased when the UV absorbance reached the baseline level.

5.2.2 b) Two step functionalisation with cysteinyl Phe_{CN} derivatives

The EZ-LINK® Maleimide-PEG_2-biotin reagent was dissolved in sterile PBS at 1 mM concentration. 250 µL of this solution were added to the same volume of resin samples and allowed to react for 2 h at room temperature under agitation. Samples were then washed four times as described above. The biotinyl-PEG_2-maleimide functionalized resins were then incubated with either 250 µL of 1 mM cysteine (for reference), or 1 mM BMAP27(1-18)Phe_{CN}-Cys, or 2.4 mM Cys-BMAP27(1-18)Phe_{CN}, and allowed to react overnight at 4°C under agitation. Peptide concentrations of the two cysteinyl-AMPs have been adjusted in order to maintain a comparable concentration of free thiol groups, as assessed by the Ellman's test (see above). At the end of the incubation, resin samples were allowed to re-equilibrate at room temperature, centrifuged, and washed five times with 1 ml PBS as previously described. The absorbance at 280 nm of resin supernatants was monitored throughout the incorporation procedure. The washing steps ceased when the UV absorbance reached the baseline level.

An estimate of resin loading was performed at the end of the incorporation procedure by measuring the fluorescence associated to resin samples. To this aim, 100 µL of each resin sample were washed with 1 mL of 0.1 M chloride-free phosphate buffer, pH 7.4, resuspended in 100 µL of the same buffer, and transferred into the wells of a 96-well black microtiter plate. The fluorescence was measured with a microplate reader (Perkin Elmer) at the emission wavelength of 300 nm, and excitation set at 240 nm. Peptide concentrations were estimated referring to the calibration curve made with serial dilutions of the soluble Phe_{CN}

analogue, as described previously. The resin functionalised with cysteine alone was used as reference.

5.3 Antimicrobial assays

5.3.1 Bacterial strains

Bacterial organisms used in the study included reference strains obtained from the American Type Culture Collection (ATCC) and clinical isolates from orthopaedic patients, collected and characterized at the Microbiology Unit of the University Hospital of Udine (Italy) and at the Valdoltra Orthopedic Hospital (Ankaran, Slovenia) during a 6-months period in 2012. Specifically, the microorganisms were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and ATCC 35984, and 27 clinical isolates: 8 *Staphylococcus aureus*, 8 *Staphylococcus epidermidis* (one methicillin-resistant *Staphylococcus epidermidis*, MRSE), 2 *Enterococcus faecalis*, 2 *Streptococcus agalactiae*, one *Staphylococcus hominis*, one *Staphylococcus capitis*, one *Staphylococcus caprae*, one Group G *Streptococcus*. Bacteria were cultured on Brain Hearth Infusion (BHI) agar plates (Oxoid) at 37°C for 24-48 h and maintained at 4°C.

5.3.2 Antimicrobial activity in standard conditions

Bacteria were cultured overnight in Mueller-Hinton (MH) broth (Difco) and 1:25 diluted in fresh BHI medium and allowed to grow in a shaker at 37°C. Mid-log phase bacteria were harvested by 10 min centrifugation at 1000g and resuspended in MH broth. Bacterial density was assessed by turbidity at 600 nm, with reference to previously determined standards, and diluted to obtain a $5 \cdot 10^5$ CFU/mL inoculum. Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method [258]. Briefly, two-fold serial dilutions of each test agent, in MH liquid medium were prepared in 96-well microtiter plates to a final volume of 50 µl. A total of 50 µl of the adjusted inoculum was added to each well to achieve the final concentration of 2.5×10^5 CFU/mL. Samples were then incubated at 37°C for 24 h. The MIC was taken as the lowest concentration of the test agent that resulted in complete inhibition of visible growth after 24 h incubation. Minimum bactericidal concentrations (MBCs) were determined by transferring 50 µl aliquots of each sample treated with concentrations higher than the MIC into BHI agar plates, and incubating plates at 37°C for 24 h.

5.3.3 Antimicrobial activity in the presence of serum

Inoculum was prepared in MH as described above with the difference that bacterial suspension was diluted to obtain a $2 \cdot 10^7$ CFU/mL inoculum. Two-fold serial dilutions of each peptide were prepared in PBS alone, PBS containing 50% normal human serum (HS) or 50% fetal bovine serum (FBS) (25% final serum concentration), in 96-well, flat bottom, microtiter plates, in a final volume of 50 µl. When required, sera were inactivated by heating at 56°C for 30 min. In the case of pre-incubation, the dilutions were prepared and incubated at 37°C 3 h prior to the addition of bacteria. For co-incubation, peptide dilutions were prepared immediately before the addition of bacteria. A total of 50 µl of the adjusted inoculum was added to each well to achieve the final concentration of $1 \cdot 10^7$ CFU/mL. Samples where then incubated at 37°C for 24 h in a Perkin Elmer microplate reader, and bacterial growth was monitored by reading the optical density at 600 nm every 30 min, with shaking immediately before readings.

5.3.4 Antimicrobial activity in the presence of hyaluronic acid

Inoculum was prepared in MH as described above with the difference that bacterial suspension was diluted to $5 \cdot 10^7$ CFU/mL. Two-fold serial dilutions of each peptide, in 37.5% MH liquid medium (50% final concentration) supplemented with 0.625 mg/mL or 3.75 mg/mL hyaluronic acid (HA) in PBS (0.5 and 3 mg/mL final concentration, respectively) or with PBS alone, were prepared in 96-well, flat bottom, microtiter plates to a final volume of 80 µl. A total of 20 µl of the adjusted inoculum was added to each well to achieve the final concentration of $1 \cdot 10^7$ CFU/mL. Samples where then incubated at 37°C for 24 h in a Perkin Elmer microplate reader, and bacterial growth was monitored by reading the optical density at 600 nm every 30 min, with shaking immediately before readings.

5.3.5 Antimicrobial activity in the presence of synovial fluid

Synovial fluid (SF) samples have been collected from patients at the Orthopaedic Unit of the University Hospital of Udine and at the Valdoltra Orthopaedic Hospital.

Whenever possible, SF samples were centrifuged immediately after withdrawal, aliquoted and stored at -80°C. Inoculum was prepared in MH as described above with a suspension of $5 \cdot 10^7$ CFU/mL. Two-fold serial dilutions of each peptide, in the presence or absence of 25% or 62.5% SF (20% and 50% final concentration, respectively) in PBS were prepared in 96-well, flat bottom, microtiter plates to a final volume of 80 µl. A total of 20 µl of the adjusted inoculum was added to each well to achieve the final concentration of $1 \cdot 10^7$ CFU/mL. Samples where then incubated at 37°C for 24 h and optical density at 600 nm was red every 30 minutes for the first 6 h and then at 24 h.

5.3.6 Checkerboard experiments

Inoculum was prepared as described above. Bacterial suspension was diluted to obtain a $5 \cdot 10^5$ CFU/mL inoculum. A total of 50 µl of Mueller-Hinton broth was distributed into each well of a 96 wells microtiter plate. The first test agent of the combination was serially diluted up to zero along the rows, while the second molecule was diluted up to zero along the columns. A total of 50 µl of the adjusted inoculum was added to each well to achieve the final concentration of $2.5 \cdot 10^5$ CFU/mL and the plates were incubated at 37°C for 24 h. The resulting checkerboard contains each combination of two antibiotics, with wells containing the highest concentration of each antibiotic at opposite corners. The fractionary inhibitory concentration (FIC indexes) were calculated as follows: FIC index = $FIC_A + FIC_B$, where FIC_A is the MIC of drug A in the combination/MIC of drug A alone, and FIC_B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the FIC index is ≤0.5, indifferent when FIC index is >0.5 to <4, and antagonistic when FIC index is ≥4 [316].

5.3.7 Inhibition of biofilm formation

Inoculum was prepared according to Pompilio et al. [265], with some modifications. Briefly, *S. epidermidis* ATCC 35984 cells were seeded on a MH agar plate and incubated overnight at 37°C. Bacteria were then harvested with PBS and diluted in MH at a density of $1 \cdot 10^7$ CFU/ml. This suspension was dispensed at 100 µl/well into 96-well polystyrene microtiter plates, and incubated at 37°C for 24 h in the absence and presence of each test agent at MIC and 2 x MIC dose. At the end of the incubation time non-adherent cells were removed by aspiration and adherent cells were rinsed once with PBS and quantified by PrestoBlue® (Sigma Aldrich) according to the manufacturers' instructions.

5.3.8 Antimicrobial activity of functionalised resins

Inoculum was prepared in MH liquid medium as described above at a density of $2 \cdot 10^7$ CFU/mL inoculum. 10 µL or 50 µL of functionalised resins or their supernatants were diluted to 100µL in PBS in Eppendorf vials, and then 100 µL of the adjusted inoculum was added to each well to achieve the final concentration of $1 \cdot 10^7$ CFU/mL (5% or 25% resin and supernatant final concentration in 50% MH, respectively). Samples were incubated 1 h at 37°C under agitation. Bacterial viability was evaluated by the PrestoBlue® metabolic assay and CFU counts. To this aim, samples were serially diluted with PBS, plated on BHI agar plates and incubated overnight at 37°C to allow colony detection.

5.4 Effects on host cells

5.4.1 Cell cultures

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC) and maintained in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (complete medium) in a humidified 37 °C incubator in 5% CO_2 atmosphere.
The human osteoblast-like cell line MG-63 was obtained from ATCC and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (complete medium) in a humidified 37 °C incubator in 5% CO_2 atmosphere.
Cells were subcultured when reaching 80% confluence.

5.4.2 LPS/LTA neutralization assays

RAW 264.7 cells were plated in 24-well dishes at $9 \cdot 10^5$ cells/well in complete medium cultured for 24 h. Medium was aspirated from cells and replaced with fresh complete medium additioned with 100 ng/mL LPS from *E. coli* O111:B4 or 5 μg/mL LTA from *S. aureus*, in the presence of different concentrations of the selected AMPs or polymyxin B. Following 24 h incubation, the nitrite concentration in the culture media was measured with Griess reagent (Molecular Probes) according to the manufacturers' instructions.

5.4.3 Cytotoxicity and cell viability assays

Cells were seeded in 96-well plates at a density of 7500 cells/well, cultured for 24 h in complete medium and then incubated with increasing peptide concentrations in complete medium, or, alternatively, in DMEM supplemented with 10% (v/v) h.i. HS, or in HBSS supplemented with 10% h.i. HS in the absence and presence of 500 μg/ml HA. The release of the cytosolic enzyme lactate dehydrogenase (LDH) was determined after 1 h incubation by spectrophotometric quantification of LDH activity in cell-free supernatants and cell lysates using the CytoTox 96™ non-radioactive cytotoxicity assay (Promega). Data were calculated as percentage of total cellular LDH activity. Cell viability was assessed using the PrestoBlue® metabolic dye after 24 h cell exposure to the peptides under standard culture conditions (complete medium) according to the manufacturers' instructions.

5.4.4 Osteoblast differentiation

MG-63 cells were seeded at a concentration of $3 \cdot 10^5$/well in 24 wells tissue culture plates in complete DMEM. When cells reached 80% confluence media was removed and substituted with serum free DMEM additioned with 10 nm β-glycerophosphate and 50 μg/mL ascorbic acid (osteogenic medium – OM) in the presence or absence of 100 nM dexamethasone and 2 μM peptide. Cells were incubated at 37°C, 5% CO_2, for 7 days, replacing media two times. Cells were then harvested by trypsinization, centrifuged at 200 g for 5 minutes, resuspended in 100 μL of milli Q water, and lysed by sonication with a Hielscher UP50H sonicator at 50% intensity two times for 10 seconds each, on ice. Total lysate proteins were quantified by Bradford assay [317]. Samples for alkaline phosphatase (ALP) quantification were prepared by diluting 10 μg of proteins to a total volume of 50 μl in a 96-wells, flat bottom, microtiter plate and then 150 μL of the p-Nitrophenyl Phosphate Liquid Substrate System reagent (Sigma-Aldrich) were added to each sample. Samples were then incubated for 80 minutes at 37°C. ALP activity was determined by quantification of p-nitrophenol produced, by measuring the absorbance of the samples at 405 nm, referring to a standard curve, and expressed as nmoles·mg^{-1}·minute^{-1}.

5.5 Statistical analysis

Data are presented as the mean ± SD. Statistical evaluation was performed using the GraphPad Prism version 5.01 software (GraphPad Software) by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. The level of significance was set at 0.05.

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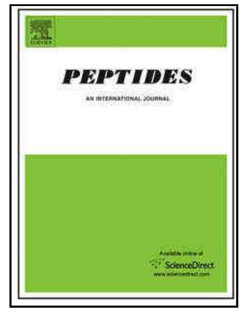
8. Annex – Scientific publication



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Antifungal activity of cathelicidin peptides against planktonic and biofilm cultures of *Candida* species isolated from vaginal infections

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Running title: Anti-*Candida* biofilm activity of cathelicidin peptides

Abbreviations: AMB, amphotericin B; CFU, colony forming unit; CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FLC, fluconazole; ITC, itraconazole; MCZ, miconazole; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; PBS, phosphate buffered saline; SE, silicone elastomer; VSF, vaginal simulated fluid; VVC vulvovaginal candidiasis; XTT, [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide].

Highlights

- BMAP-28 was effective against vaginal isolates of *Candida* spp.
- BMAP-28 killed 48-h old *Candida* biofilms by membrane permeabilization
- LL-37 inhibited adhesion of *Candida* cells to polystyrene and medical grade silicone
- Pre-coating of silicone with LL-37 and BMAP-28 prevented *C. albicans* adhesion

Abstract

Vulvovaginal candidiasis (VVC) is a frequent gynecological condition caused by *C. albicans* and a few *non-albicans Candida* spp. It has a significant impact on the quality of life of the affected women also due to a considerable incidence of recurrent infections that are difficult to treat. The formation of fungal biofilm may contribute to the problematic management of recurrent VVC due to the intrinsic resistance of sessile cells to the currently available antifungals. Thus, alternative approaches for the prevention and control of biofilm-related infections are urgently needed. In this regard, the cationic antimicrobial peptides (AMPs) of the innate immunity are potential candidates for the development of novel antimicrobials as many of them display activity against biofilm formed by various microbial species. In the present study, we investigated the *in vitro* antifungal activities of the cathelicidin peptides LL-37 and BMAP-28 against pathogenic *Candida* spp. also including *C. albicans*, isolated from vaginal infections, and against *C. albicans* SC5314 as a reference strain. The antimicrobial activity was evaluated against planktonic and biofilm-grown *Candida* cells by using microdilution susceptibility and XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assays and, in the case of established biofilms, also by CFU enumeration and fluorescence microscopy. BMAP-28 was effective against planktonically grown yeasts in standard medium (MIC range, 2-32 µM), and against isolates of *C. albicans* and *C. krusei* in synthetic vaginal simulated fluid (MIC range 8-32 µM, depending on the pH of the medium). Established 48-h old biofilms formed by *C. albicans* SC5314 and *C. albicans* and *C. krusei* isolates were 70-90% inhibited within 24 hour incubation with 16 µM BMAP-28. As shown by propidium dye uptake and CFU enumeration, BMAP-28 at 32 µM killed sessile *C. albicans* SC5314 by membrane permeabilization with a faster killing kinetics compared to 32 µM miconazole (80-85% reduced biofilm viability in 90 min vs 48 hours). In addition, BMAP-28 at 16 µM prevented *Candida* biofilm formation on polystyrene and medical grade silicone surfaces by causing a >90% reduction in the viability of planktonic cells in 30 min. LL-37 was overall less effective than BMAP-28 against planktonic *Candida* spp. (MIC range 4 - ≥64 µM), and was ineffective against established *Candida* biofilms. However, LL-37 at 64 μM prevented *Candida* biofilm development by inhibiting cell adhesion to polystyrene and silicone surfaces. Finally**,** *Candida* adhesion was strongly inhibited when silicone was pre-coated with a layer of BMAP-28 or LL-37, encouraging further studies for the development of peptide-based antimicrobial coatings.

Keywords:

Cathelicidin; alfa-helical antimicrobial peptide; *Candida albicans*; biofilm; *Candida* isolates; vulvovaginal candidiasis.

1. Introduction

Mucosal infections caused by *Candida* spp., particularly *Candida albicans*, are very common, and vulvovaginal candidiasis (VVC) is the second most frequent gynecological condition after bacterial vaginosis being diagnosed in a considerable part of women with vaginitis [1]. Results from a recent internet panel survey involving 6000 women in the US and Europe indicate an overall rate of VVC ranging from 29% to 49%, with four or more recurrences over a 12-month period in more than one fifth of the cases [23]. VVC results from overgrowth of various *Candida* species, usually present in vagina as commensals, in otherwise healthy women [68]. The infection is mainly caused by *C. albicans* (85-95% of cases) and a few non-albicans *Candida* spp [68]. The prevalence of the latter species varies widely in different parts of the world and among them, *C. glabrata* is the most common [1]. Several studies report higher percentages of non-*C.albicans* species from women with recurrent VVC, HIV-infected or post-menopausal women, and women with uncontrolled diabetes [1]. Particularly, VVC is not a life-threatening condition, yet it has a significant impact on the quality of life of the affected women [1]. The standard treatment of uncomplicated VVC is principally based on oral or topically applied azoles, that are effective in more than 90% of *C. albicans* infections [1]. On the contrary, treatment of complicated VVC such as recurrent VVC and that caused by non-*C.albicans* species is still challenging mainly due to reduced azole susceptibility of non-*C.albicans* species and to persistence of *C.albicans* on the external vulva in patients with recurrent VVC after the cessation of treatment [1, 6].

C. albicans is a polymorphic yeast which associates with natural surfaces, e.g. epithelial or endothelial cells, as well as with abiotic surfaces, such as central venous and urinary catheters, forming biofilms [40]. The biofilm forming ability is considered as a virulence factor since the sessile lifestyle provides protection against the host immune system and results in fungal resistance to antimycotics [1, 46, 60]. This is particularly worrisome in hospital settings, considering that *Candida* spp. are able to form biofilms on virtually any implanted medical device in the human host and that *C. albicans* is the most common fungal organism associated with biofilm-related infections [12, 72].

Candida biofilms may contribute to the pathogenesis of superficial as well as systemic *Candida* infections. In this respect, *C. albicans* strains isolated both from blood and mucosa showed similar *in vitro* biofilm formation capacity, without significant difference between invasive and non invasive isolates, despite high intra-species variability [31, 71]. Concerning gynecological infections, *C. albicans* and other *Candida* spp., isolated from VVC patients, displayed *in vitro* the ability to adhere both to the plastic of microtiter plates and to the surface of intrauterine contraceptive devices, producing biofilm to different extents [11, 56, 57]. In an experimental mouse model of *Candida* vaginitis, Noverr and colleagues reported the presence of sessile *C. albicans* attached to the vaginal mucosa and encased in a typical biofilm architecture with abundant extracellular matrix, which became evident after 24-48 h of infection [30]. They further demonstrated in a recent *in vivo* study that key transcriptional regulators controlling the yeast-to-hypha switch are implicated in the immunopathogenesis of vaginal candidiasis and in the transition from asymptomatic colonization to symptomatic infection [58]. In addition, Auler et al. isolated *C. albicans* from intrauterine devices that have been removed from patients with recurrent VVC, thus indicating the presence of biofilm on the contraceptive device as a risk factor for recurrence [4]. Recurrent VVC is considered a multifactorial disease and the formation of biofilm may contribute to the problematic management of this condition due to the intrinsic resistance of sessile cells to commonly used antifungal drugs [60].

Alternative approaches for the prevention and control of biofilm-related infections are urgently needed. In the last two decades, the cationic antimicrobial peptides (AMPs) of the innate immunity have received increasing attention as potential candidates for the development of novel antimicrobials [22, 29, 87]. Considerable interest in these molecules is based on their potent broad-spectrum antimicrobial activity, low propensity to induce resistance, and ability to modulate the host immune system [64, 84, 85]. These natural peptides are part of the first line defense in a wide range of organisms including mammals [87]. Apart from being produced by professional phagocytes, they are found on the skin and on the epithelia lining the respiratory, the gastrointestinal and genitourinary tract where they provide protection against infection [50, 64]. One of the best characterized AMPs in humans is the cathelicidin LL-37 [78, 86], a linear cationic alfa-helical peptide that has been identified also in the squamous epithelium of the cervix and vagina [26] and in cervicovaginal secretions. Increased levels of LL-37, among a few other AMPs, were found in vaginal fluids of women affected by multiple ongoing vaginal infections including candidiasis [43]. Collectively, these data would suggest a role for this peptide in antimicrobial defence of the vaginal epithelium. Recently, Lan and colleagues have shown that LL-37 inhibits the adhesion of *C. albicans* to plastic surfaces and epithelial cells by interfering with yeast carbohydrate and protein cell-wall components [10, 77]. As microbial adhesion represents a crucial step in biofilm formation and the ability of LL-37 to inhibit the development of bacterial biofilm is well characterized [36, 54], we asked ourselves whether LL-37 was also active against biofilm formed by *C. albicans*. We were further interested in comparison between LL-37 and the bovine ortholog BMAP-28, a cathelicidin peptide [85] highly active against a broad spectrum of pathogens including yeasts and filamentous fungi [7, 8, 74, 76]. BMAP-28 was recently shown to be active against bacterial biofilm formed by cystic fibrosis isolates [59], but its activity against fungal biofilm has not been studied so far. Although both are linear helical peptides, the differences in their structuring and aggregational properties [48] could affect their antifungal specificities.

In the present study, we investigated the antifungal activity of LL-37 and BMAP-28 against pathogenic *C. albicans* and non-albicans *Candida* spp. with reduced azole-susceptibility, isolated from vaginal infections. XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assays have been used to evaluate the ability of these peptides to inactivate yeast cells in the planktonic state, to prevent yeast adhesion to microtiter plates and medical grade silicone surfaces, and to eradicate established biofilms. Biofilm killing was further confirmed by colony counts. The effects of the AMPs on fungal membrane permeability in target biofilms were addressed by measuring propidium dye uptake by fluorescence microscopy. The anti-biofilm activity of the AMPs under study was evaluated in comparison with that of the polyene amphotericin B (AMB) and the imidazole miconazole (MCZ).

2. Materials and methods

2.1 Antimicrobial peptides and drugs

Peptides (BMAP-28, GGLRSLGRKILRAWKKYGPIIVPIIRI-NH_2; LL-37 LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) were chemically synthesized according to standard methods [75] and confirmed by mass spectrometry using a Q-STAR hybrid quadrupole time-of-flight mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with an electrospray ion source. Peptide concentrations were determined in aqueous solution by measuring the absorbance at 257 nm (Phe residues) and 280 nm (Tyr and Trp residues) for LL-37 and BMAP-28, respectively [75].

Miconazole and amphotericin B were purchased from Sigma-Aldrich, resuspended in dimethyl sulfoxide (miconazole) or in water (amphotericin B) and maintained at -20°C until use as reference antifungals.

2.2 Silicone disks production

Silicone elastomer (SE) sheets were purchased from G.F. Electromedics (Firenze, Italy). Flat circular disks, 0.5 cm in diameter, were obtained by cutting with a cork borer, rinsed extensively with distilled water and autoclaved following the manufacturer's instructions. Sterilized disks were transferred to 96-well plates immediately before use.

2.3 Fungal isolates and culture conditions

Yeast strains tested in this study included *Candida albicans* SC5314 reference strain and twenty-seven clinical isolates from patients with vaginitis. All clinical strains were obtained by vaginal swabs from symptomatic out-patients attending the S.M. Misericordia Udine University Hospital, Microbiology unit within a six month period (January to June 2013). Specimens were streaked onto Sabouraud agar plates (Biomerieux, France) and incubated at 37°C for 24 to 48 h, according to a standard diagnostic protocol [67]. Identification of suspected colonies was performed by subculturing on chromogenic agar (ChromID Candida, MEUS S.r.l., PD, Italy) and by biochemical tests (API ID 32 C - Biomerieux). Susceptibility to amphotericin B, fluconazole, itraconazole, ketoconazole, 5-fluorocytosine and voriconazole was performed by using Sensititre™ YeastOne™ MIC IVD Plate (Trek Diagnostic Systems, UK), a ready-to-use broth microdiluition assay controlled by a fully automatic, bench-top incubating and reading system (Sensititre ARIS®, Trek Diagnostic Systems, UK) based on EUCAST guidelines [13, 21]. Yeast cells were cultured on Sabouraud dextrose agar plates (Oxoid, UK) at 30°C for 24 to 48 h, maintained at 4°C and subcultured on Sabouraud dextrose agar plates prior to use.

2.4 Planktonic antifungal susceptibility testing

Yeast cells were resuspended in Sabouraud liquid medium or in vaginal simulated fluid (VSF) adjusted to pH 4.2, 5.5 or 6.5. The composition (g/l) of VSF was as follows: NaCl, 3.51; KOH, 1.40; $Ca(OH)_2$, 0.222; bovine serum albumin, 0.018; lactic acid, 2.00; acetic acid, 1.00; glycerol, 0.16; urea, 0.4; glucose, 5.0 [55]. Cell density was assessed by measuring turbidity at 600 nm and was adjusted to obtain the proper inoculum size. Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method [76]. Briefly, two-fold serial dilutions of each test agent, either in Sabouraud liquid medium or in VSF, were prepared in 96-well microtiter plates to a final volume of 50 µl. A total of 50 µl of the adjusted inoculum was added to each well to achieve the final concentration of $5x10^4$ cells/ml (or $1x10^7$ cells/ml, when MICs against biofilm-forming concentrations of *C. albicans* SC5314 were to be determined). Samples were then incubated at 30°C for 48 h. The MIC was taken as the lowest concentration of the test agent that resulted in complete inhibition of visible growth after 48 h incubation. Minimum fungicidal concentration (MFC) were determined by transferring 50 µl aliquots of each sample treated with concentrations higher than the MIC into Sabouraud dextrose agar plates, and incubating plates at 30°C for 24 h.

2.5 Assessment of biofilm formation

Candida biofilms were formed as described by Ramage G. et al [61] with some modifications. Briefly, overnight cultures of *C. albicans* SC5314 were adjusted with Sabouraud-dextrose broth to give a 1×10^7 CFU/ml suspension and then incubated at 100 µl/well into flat bottom 96 well polystyrene microtiter plates (Sarstedt), or into SE disk-containing 96 well microtiter plates, at 37°C for 48 h. At the end of the incubation time non-adherent cells were removed and wells were gently rinsed with PBS. Biofilms were quantified using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay as a direct measure of the metabolic activity of adherent cells. The XTT assay was adapted from [61]. Briefly, XTT was prepared at 2 g/liter in PBS and filter sterilized through a 0.22-µm-pore-size filter. Menadione (Sigma-Aldrich) was added to 20 µM final concentration. A 100-µl aliquot of XTT-menadione solution was added to each biofilm-containing well. Microtiter plates were incubated in the dark for 30 min at 37°C. Changes in the absorbance of XTT were measured spectrophotometrically at 490 nm using a microtiter plate reader (BioTek Instruments Inc.). The percentage of viable cells was calculated as: (OD_{490} of treated cells/OD_{490} of untreated cells) × 100.

2.6 Biofilm formation inhibition assay

Planktonic *C. albicans* SC5314 cells at 1×10^7 CFU/ml in Sabouraud liquid medium were dispensed at 100 µl/well into 96-well polystyrene microtiter plates or in SE disk-containing 96-well polystyrene microtiter plates, and incubated at 37°C for 30 min or 48 h in the absence and presence of each test agent at MIC and half-MIC dose for 1×10^7 CFU/ml *C. albicans* SC5314. Alternatively, SE disks were pre-coated with peptides by incubating SE disk-containing wells for 1 h at 37°C with 100 µl Sabouraud liquid medium in the absence and presence of 8 µM, 16 µM and 32 µM BMAP-28 and of 16 µM, 32 µM and 64 µM LL-37. Unbound peptide was removed from the wells by aspiration after 1 h incubation, wells were carefully rinsed with PBS, *C. albicans* SC5314 cells were dispensed onto peptide-coated SE disks at 1×10^7 CFU/ml in peptide-free Sabouraud liquid medium, and incubated at 37°C for 48 h. At the end of the incubation times, adherent cells were quantified by XTT after removing non-adherent cells by aspiration and rinsing with PBS. Total (i.e., adherent and non adherent) cell numbers were determined in replica samples run in parallel, following microtiter plate centrifugation at 1300×g to sediment non-adherent cells.

2.7 Antifungal susceptibility testing of Candida biofilms

Mature (i.e., 48-h-old) *C. albicans* SC5314 biofilms were incubated for 48 h at 37°C in Sabouraud liquid medium, in the absence and presence of doubling concentrations of BMAP-28, LL-37, MCZ and AMB. At the end of the incubation period, non-adherent cells were removed by aspiration, wells were carefully rinsed with PBS and the metabolic activity of adherent cells was quantified using the XTT assay. Decrease in biofilm cell viability was assessed as a decrease in absorbance at 490 nm, compared with untreated biofilms. Sessile minimum inhibitory concentrations (SMICs) were determined at 50% and 90% biofilm cell inhibition. For time-kill studies, *C. albicans* SC5314 biofilms were exposed to 16 and 32 µM BMAP-28 and 16 and 32 µM MCZ at 37°C, for 15 and 90 min and for 3, 6, 24 and 48 h. At the end of the incubation times, samples were processed as above for quantification of metabolic activity of adherent cells by XTT. For selected time points the survival of sessile *Candida* cells was evaluated in parallel by CFU enumeration. In this case, after the last washing step, 200 µl PBS were added to the wells and *Candida* biofilms were detached by vigorous pipetting and scraping with the micropipette tip. Detached biofilm samples were then transferred, together with the pipette tip, to tubes containing 3 ml PBS, vortexed for 30" and serially diluted. Aliquots were plated on solid Sabouraud medium and colony counts were performed following 48 h incubation at 30°C. Percent biofilm killing was calculated as: 100 – [(CFU of treated /CFU of untreated samples) x 100].

2.8 Fluorescence microscopy

Mature (48-h-old) *C. albicans* SC5314 biofilms in 96-well microtiter plates were incubated for 90 min with BMAP-28 or MCZ at 16 and 32 µM in Sabouraud medium. The medium was then replaced with fresh medium containing 1 µg/ml propidium iodide and 25 µg/ml concanavalin A Alexa Fluor® 488 conjugate (Life Technologies) and plates were incubated for 40 min at 37°C. Stained biofilms were examined under an inverted Leica DMI6000RB epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) placed in a climate chamber at 37°C and images were captured with a Micromax charge-coupled device camera (Roper Scientific).

2.9 Statistical analysis

Statistical differences among groups of data were analyzed by one-way ANOVA followed by the Bonferroni post test, using GraphPad Prism version 5.0. In all comparisons, $P < 0.05$ was considered significant.

3. Results

3.1 Antifungal activity against clinical isolates of S. cerevisiae and Candida spp

The antifungal activity of the α-helical cathelicidins BMAP-28 and LL-37 was tested *in vitro* against wild-type *C. albicans* SC5314 as a reference strain and against 27 yeast strains obtained from vaginal exudates of patients with vaginal yeast infection. The isolates included 3 strains of *Saccharomyces cerevisiae* and 24 *Candida* spp. strains half of which were non-albicans strains (Table 1). All *S. cerevisiae* and *C.albicans* strains were azole-susceptible. Apart from one azole-susceptible *C. parapsilosis*, the *non-albicans Candida* isolates were resistant (R) or susceptible dose-dependent (SDD) to azoles, and were identified to the species level as follows: *C. glabrata* (6/7 SDD and 1/7 R to ITC; 2/7 SDD to FLC), *C. krusei* (3/3 SDD to ITC; 2/3 SDD and 1/3 R to FLC), *C. norvegensis* (1 isolate SDD to ITC and to FLC).

BMAP-28 and LL-37 revealed comparable potencies against *S. cerevisiae* (Table I). However their activities against *Candida* spp. strains varied considerably. BMAP-28 was effective in the low micromolar concentration range against *C. albicans* SC5314 and against all *Candida* spp. isolates but *C. glabrata* CG-2, CG-5, CG-6 and CG-7 (Table I). LL-37 did not inhibit the growth of *C. albicans* SC5314 at up to 64 µM and was inactive or poorly active against most *Candida* spp. isolates (Table 1).

The antifungal effects of BMAP-28 and LL-37 on representative isolates of *C. albicans* and *C. krusei* were also investigated in synthetic vaginal simulated fluid (VSF) [55] at pH values ranging from 4.2 to 6.5 to mimic the physical and chemical properties of vaginal secretions. *C. glabrata, C. parapsilosis* and *C. norvegensis* were not tested in VSF because of poor cell growth in this medium. As shown in Table 2, the antifungal effects of BMAP-28 against *C. albicans* and *C. krusei* were substantially preserved in VSF although, lowering the pH from 6.5 to 4.2 resulted in 2- to 3-fold increase in MIC values. LL-37 was ineffective up to 64 µM against all *Candida* spp. strains tested (not shown).

The activity of BMAP-28 against susceptible *Candida* spp. isolates was further examined in terms of minimum fungicidal concentration (MFC), to ascertain whether the antifungal susceptibility testing correlated with killing capacity. For all *Candida* spp. isolates, the MFC values of BMAP-28 were equal to MIC values (not shown), consistent with powerful candidicidal activity.

3.2 Activity against pre-formed Candida biofilm

Evidence from a mouse model of vaginal infection indicates that *Candida* cells have the ability to adhere to vaginal epithelium and produce biofilm [30]. Since this mode of growth in *Candida* confers increased resistance to antifungal agents [46, 72], we were interested in examining the activity of BMAP-28 and LL-37 against sessile *Candida* cells relative to that of conventional anti *Candida* agents such as amphotericin B (AMB) and miconazole (MCZ). When tested against planktonic *C. albicans* SC5314, AMB and MCZ exhibited MICs of respectively 0.27 µM and 2 µM. The anti-biofilm activity was quantified using the XTT reduction assay and, in selected experiments, by parallel CFU determination. As reported in Table 3, BMAP-28 effectively reduced the viability of mature *C. albicans* SC5314 biofilms grown on the surface of microtiter plates, with $SMIC_{50}$ and $SMIC_{90}$ of 8 µM and 16 µM following 48 h biofilm treatment. MCZ was somewhat less efficient than BMAP-28, and LL-37 and AMB were ineffective at concentrations up to 32 µM (Table 3).

Time-kill studies conducted with 16 and 32 µM BMAP-28 indicated an initial rapid drop in biofilm cell viability as assessed by XTT reduction, with approximately 55% and 25% live cells after 90 min treatment, and virtually complete killing after 48 h treatment (Figure 1A). Compared with BMAP-28, MCZ at 16 and 32 µM exhibited a significantly slower killing kinetics (Figure 1A) although most biofilm cells were inactivated by this drug after 48 h (Figure 1A). In order to verify that sessile *Candida* cells were killed by BMAP-28, colony forming units (CFUs) were quantified in parallel samples treated with 16 and 32 µM BMAP-28 or MCZ for 90 min and 48 hours. The results are reported in Figure 1B as percent killing. A good correlation was observed between biofilm viability, as assessed by the metabolic dye XTT (Figure 1A), and the sessile cell survival, as determined by CFU counts after mechanical dislodgement of biofilm embedded yeasts and plating on solid medium (Figure 1B). Overall, the decrease in CFU counts was even more pronounced as compared to reduction in metabolic activity, likely due to the intrinsic delay of the CFU read-out compared to the XTT absorbance measurement (48 h *vs*. 30 min, respectively).

The ability of BMAP-28 to rapidly kill *Candida* cells within the biofilm structure was further assessed by fluorescent microscopy examination of 48-h-old *C. albicans* SC5314 biofilms stained with PI to reveal cell membrane damage, following 90 min incubation in the absence and presence of BMAP-28 or MCZ at 16 and 32 µM. Whereas the staining pattern in biofilms exposed to BMAP-28 (representative image in Figure 2 panel B), suggested extensive cell permeabilization, virtually no PI-positive cells were detected in untreated (Figure 2 A) and in MCZ-treated (not shown) biofilms.

To confirm and extend our investigation of the anti-*Candida*-biofilm activity of BMAP-28, representative clinical vaginal isolates of *C. albicans*, *C. krusei* and *C. glabrata* were grown in sessile mode for 48 h and treated for 24 h with 16 µM BMAP-28. The killing activity of the peptide against *C. albicans* and *C. krusei* biofilms was comparable to or higher than against *C. albicans* SC5314 (Figure 3). However the peptide at 16 µM was virtually ineffective against *C. glabrata* CG-4 biofilm (Figure 3) although, under planktonic growth conditions this isolate was susceptible to BMAP-28.

3.3 Effect on biofilm formation

Having examined the activity against preformed biofilms, we asked whether the compounds under study have the capacity to block the initial step in the formation of *Candida* biofilm so as to prevent biofilm production. To answer this question, planktonic *C. albicans* SC5314 cells were seeded into microtiter plates at $1x10^7$ CFU/ml for biofilm formation and each test compound was added to the culture medium immediately afterwards, at concentrations corresponding to the MIC exhibited against $1x10^7$ CFU/ml *C. albicans* SC5314 cells (i.e. 16 µM, 64 µM, 32 µM and 2 µM for BMAP-28, LL-37, MCZ and AMB), and to one-half MIC. Plates were incubated at 37° C for 30 min or 48 h, and adherent vs total viable cell numbers were determined at the end of each incubation period using the XTT assay.

As can be observed in Figure 4 A, both BMAP-28 and LL-37 produced a marked decrease in the number of adherent viable cells compared with untreated control samples, already at the early time point. BMAP-28 also caused a significant decrease in total (i.e., adherent and non-adherent) cell numbers (Figure 4 C), suggesting it prevented establishment of *Candida* biofilms by rapidly killing planktonic cells. At the 30 min time point, LL-37 at MIC caused an approximately 80% reduction in adherent cell numbers (Figure 4 A). However unlike BMAP-28, LL-37 hardly affected total cell numbers even at the late time point (Figure 4 D). These results suggest that LL-37 acted primarily by inhibiting cell adhesion to the plastic surface rather than by killing planktonic cells. AMB was highly effective in inhibiting biofilm formation at the late time point by affecting total cell numbers, and MCZ exhibited similar inhibitory effects on both, total and adherent cells (Figure 4 B and D).

BMAP-28 and LL-37 were further tested for ability to prevent *C. albicans* SC5314 biofilm formation on the surface of medical grade silicone elastomer (SE) disks - a material which has found widespread use in medical device manufacturing industries - placed into microtiter wells. Peptides were added to the cell culture medium immediately after seeding cells onto uncoated SE disks (Figure 5 A) or, alternatively, cells were dispensed on peptide-coated SE disks and incubated in peptide-free Sabouraud medium (Figure 5 B). Cell viability was determined following 48 h cell incubation at 37° C.

When added to the medium immediately after cell seeding, BMAP-28 at 16 μM effectively reduced the number of adherent and total *Candida* cells, whereas LL-37 up to 64 μM only showed anti-adhesive activity (Figure 5 A). A dose-dependent decrease in the number of viable adherent cells was detected when cells were seeded on BMAP-28 or LL-37 coated disks. Under this condition, neither peptide affected the viability of total *Candida* cells (Figure 5 B).

4. Discussion

Given the growing threat of drug resistance to the most common treatments, there is a great demand for novel anti *Candida* agents effective also against biofilm-grown *Candida* spp. In this respect, antimicrobial peptides appear promising candidates as they demonstrate activity also against biofilm-grown fungi [5]. In the present study, we investigated the *in vitro* antifungal activity of the alfa-helical cathelicidins BMAP-28 and LL-37 against clinical isolates of *C. albicans* and other *non-albicans* species from patients with vaginal candidiasis.

We show that BMAP-28 exhibited good fungicidal activity against most planktonic *Candida* spp. isolates and retained substantial activity in vaginal simulated fluid. The increase in the MIC values observed in this latter condition may be ascribed to the presence of elevated calcium concentrations (i.e. 3 mM). The antagonistic effect of this cation on the candidacidal activity of cationic AMPs is well documented [9, 42, 82] and has been recently confirmed also in VSF in the case of hepcidin-20 [19]. The antifungal activity of BMAP-28 reported here is consistent with previous studies indicating ability of this peptide to inactivate *in vitro* a variety of pathogenic fungi grown in planktonic form [7, 76], also including a collection of *Candida* species isolated from clinical samples. In this respect, we show that BMAP-28 is effective against various azole-resistant *non-albicans Candida* spp. isolates. Our finding possibly reflects the distinctive nature of the interaction of this peptide with the yeast membranes. Unlike antifungal azoles, that act by interfering with the biosynthesis of membrane ergosterol, amphipathic AMPs such as BMAP-28 exert their antimicrobial effects via membrane binding and permeabilization [7, 76]. The latter mechanism is faster and somewhat less specific than the azole mechanism and thus, the development of microbial resistance to AMPs is generally less likely to arise. However, concerning the *non-albicans Candida* spp. isolates, BMAP-28 displayed an overall reduced activity towards the strains of *C. glabrata* with a highly variable MIC range (4 - >64 μM). This observation is in line with published reports on poor susceptibility of *C. glabrata* to various cationic AMPs [7, 32, 35], and may reflect the unique and distinctive features of the cell wall of this pathogen [16].

In the case of LL-37, a possible explanation for the poor antifungal activity observed in this study could be related to the many escape strategies developed by microbial pathogens, including *C. albicans*, to overcome the activity of AMPs [70]. As already shown for histatin 5, proteolytic degradation of LL-37 by fungal membrane anchored and secreted aspartyl proteases (SAPs) has been recently reported [62] and such fragmentation would cause loss of antimicrobial activity. In addition, a secreted glycodomain of the *C. albicans* membrane protein Msb2 has been shown to protect microorganisms by binding a wide range of AMPs, also including cathelicidins [70]. Nevertheless, in spite of its structural and evolutionary relatedness to LL-37, BMAP-28 was highly effective against most tested *Candida* strains. Indeed, a relevant number of studies has demonstrated that individual differences in size and sequence among AMPs sharing a common structural scaffold can significantly affect the antimicrobial specificities [48]. In the case of BMAP-28 and LL-37, these differences have resulted in distinct structuring and aggregational properties. BMAP-28 behaves as a random-coil monomer in physiological solution and adopts a helical conformation when interacting with membranes [76] while conversely, LL-37 has a salt-dependent ability to structure and aggregate in saline solution [34]. This distinct behaviour influences the mode of membrane interaction and membrane damage by the two peptides. Specifically, the aggregate-forming tendency of LL-37 would favour non-specific binding to medium components and microbial surfaces, with negative effects on its lytic capacity [83]. In fact, microbicidal activity based on fungal membrane permeabilization by full-length LL-37 was observed in diluted low salt media [20, 45, 53]. Conversely, the propensity of BMAP-28 to structure only at the membrane level would allow for efficient penetration across cell-wall components. This would explain the broader spectrum of activity and the antimicrobial efficacy of BMAP-28 in complex biological fluids, such as blood serum and mastitic milk [74], as well as its ability to also inactivate biofilm-grown *Candida* cells thanks to a rapid diffusion through the biofilm matrix.

Amphotericin B (AMB), a polyene antifungal drug showing a dual mode of action i.e., ergosterol binding and membrane permeabilization via channel formation [27], was effective against planktonic but not against biofilm-associated *C. albicans*. This is in line with prior reports indicating higher resistance of sessile, compared with planktonic *Candida* cells, to antifungal drugs also including AMB [46, 73]. Several factors have been proposed to explain the antimycotic drug tolerance of *C. albicans* biofilms, including expression of resistance genes such as those coding for efflux pumps [46, 60], and specific binding of antifungals by beta-1,3-glucan, a major extracellular matrix component, which prevents antifungals from reaching their targets [52, 81]. Furthermore, the low activity of AMB against mature biofilm could be explained by the decreased levels of ergosterol in sessile *C. albicans* [49], being ergosterol the primary membrane target for this antifungal agent [27]. It should be noted however that *Candida* biofilms are instead susceptible to lipid formulations of AMB [39]. The mechanisms behind the unique activities of liposomal and other lipid formulations of AMB are at present unknown [72].

Unlike AMB and LL-37, both BMAP-28 and miconazole (MCZ) effectively reduced the viability of the 48 h-old mature *Candida* biofilms. Their sessile inhibitory concentrations ($SMIC_{90}$) were however 8- and 16-fold increased, respectively, over their planktonic MIC values. MCZ is an imidazole agent with unique properties among azoles. Similar to the other azoles, it inhibits fungal growth by interfering with ergosterol biosynthesis [24]. In addition, MCZ displays fungicidal activity against planktonic *C. albicans* related to the induction of reactive oxygen species (ROS) [24]. High concentrations of MCZ have been reported to be fungicidal also against *Candida* spp. biofilms [79]. The underlying mechanism seems rather complex and is still the subject of active research [80]. In the present study, a significant decrease in metabolic activity (Fig. 1A) and a remarkable CFU reduction (Fig. 1B) of MCZ-treated biofilms was only evident at later time points (i.e. 48 hours), whereas the effect of BMAP-28 was apparent as early as after 90 min incubation, indicative of distinct modes of action for the two compounds (Fig. 1). Particularly, our fluorescence microscopy data show a rapid uptake of propidium iodide in BMAP-28-treated *Candida* biofilms (Fig. 2), clearly indicating permeabilization of the fungal membrane within 90 min incubation. On the other hand, propidium dye uptake was not observed in miconazole-treated samples. Our present results thus would suggest that, at variance with MCZ, the plasma membrane is a direct target of BMAP-28 in mature *Candida* biofilms. These data extend prior reports on the ability of BMAP-28 to interact with ergosterol containing zwitterionic large unilamellar vesicles and permeabilize yeast membranes of planktonic microorganisms [7, 76].

The capability of BMAP-28 to kill biofilms formed by clinical isolates of *C. albicans* and *C. krusei* is another relevant finding of this study. The ability to target biofilms formed by *non-albicans Candida* species is especially important and deserves further investigation in the perspective of developing novel peptide-based antifungal agents, as infection by these species is becoming increasingly prevalent [46].

An important observation reported in this study is the considerably reduced biofilm formation on abiotic surfaces in the presence of BMAP-28 and LL-37. The two peptides however showed distinct modes of action. The effect of BMAP-28 was mediated by rapid killing of planktonic cells, resulting in reduction of the number of adherent and total cells, whereas LL-37 inhibited cell adhesion without significantly affecting total cell viability. The latter finding is in line with studies by Tsai et al. and Chang et al., suggesting that LL-37 interferes with the process of fungal cell adhesion by interacting with yeast carbohydrate and protein cell-wall components [10, 77]. The antifungal drugs AMB and MCZ were used for comparison in our study and inhibited to different extents biofilm formation, by affecting total cell viability at the late time point, thus suggesting a mechanism mediated by planktonic cell killing.

Finally, we show that BMAP-28 and LL-37 efficiently prevented the formation of biofilm on medical grade silicone. These results are in line with those obtained using polystyrene surfaces. Silicone elastomer was used in these assays because it is widely used for the manufacturing of medical devices such as stents, shunts and various types of catheters, due to its well recognized biocompatibility and biodurability. Microbial colonization of indwelling medical implants with subsequent biofilm formation is of great concern because it can lead to severe complications such as bloodstream infections and systemic inflammation [38, 60]. Thus, it is of utmost importance to prevent microbial adhesion to and biofilm formation on the surfaces of the implanted devices. One of the strategies that have been proposed to address this issue is the incorporation of antimicrobial drugs into biomaterials, particularly antifungal agents that are widely used in the clinical practice, including polyenes and azoles [12], as well as several antimicrobial peptides. These have been immobilized onto biomedical materials either by covalent binding or by physical entrapment [3]. Reduced biofilm formation in an *in vitro* biofilm model system was reported when *C. albicans* was grown on cyclodextrin-functionalized polyethylene and polypropylene loaded with miconazole [51] or on polydimethyl siloxane disks impregnated with miconazole [17]. Polydimethyl siloxane disks were also used to covalently immobilize synthetic histatin-like [18] and newly engineered cationic tryptophan-rich peptides derived from the C-terminus of a human beta-defensin 28 variant [44]. *C. albicans* adhesion to and biofilm formation on the obtained biomaterials was reduced to various extents depending on the peptide and the type of the linker used. Importantly, the latter study suggested that the membrane-active killing mechanism also applies to covalently immobilized peptides [44].

In the present study, biofilm formation was inhibited also when silicone disks were simply precoated with the peptides, suggesting that surface-adsorbed peptide molecules were able to hinder fungal adhesion. This finding was not unexpected for LL-37 as the anti-adhesive properties of this molecule against planktonic *C. albicans* have recently been recognized [77]. However in the case of BMAP-28, inhibition of fungal adhesion could not simply be explained by massive killing of planktonic cells, since total cell viability was not significantly affected. These results are intriguing and deserve further consideration in view of the increased attention received by the anti-adhesive properties of AMPs and of the numerous attempts to develop AMP-coated surfaces [3]. Thus, future studies will aim to ascertain whether BMAP-28 and LL-37 hold potential to be used as coating material for medical device surfaces.

An important issue to address in this context are possible effects of these AMPs on host cells, also including cytotoxicity. Both peptides have been shown to induce cytokine production in epithelial and immune cells of different mammalian species [14, 74, 86]. In the case of LL-37 both, canonical and unusual receptor-mediated pathways have been postulated to explain the underlying mechanisms [25, 75, 78]. Conversely, for the cytokine-inducing ability of BMAP-28, the exact molecular mechanism has not yet been defined [14]. It is becoming increasingly clear that the models developed for the AMP - microbial membrane interaction are not directly transposable to the interaction with host cells. This concept is further supported by the distinct effects of relevant components present in the physiological environment, such as divalent cations and serum proteins, on AMP activities towards microbial and host cells [84]. Concerning specifically the vaginal milieu, LL-37 was recently shown to exert selective inhibitory effects on sperm fertilizing ability in mice without apparent impairment to the female reproductive tract [69]. In addition, the increased vaginal expression of endogenously present AMPs during infection argues in favor of a protective role of these molecules in antimicrobial defence of the vaginal epithelium [43].

Concerning direct antimicrobial activity, the mode of action of membrane-active peptides is based on their contact with microbial surface molecules. Cationicity and amphiphilicity of the peptide structure rather than amino acid sequence specificity are key features for such interaction to occur [65, 87]. Linear peptide sequences could easily be modified for structure-activity-relationship (SAR) studies to obtain shorter/simpler analogs with equal or improved antimicrobial properties [88]. In this respect, it is worth remembering that naturally derived shorter LL-37 fragments and synthetic analogs derived from the sequence of the mouse ortholog CRAMP proved active against, respectively, planktonic and biofilm forms of *C. albicans* [15, 45].

In view of a therapeutic application of these peptides, the SAR-based approach appears valuable also to elucidate the structural properties required for biocompatibility. For example, a retro-inversed isomer of BMAP-28 was resistant to proteolytic degradation, was safe to host cells, and effectively prevented bacterial infection in a mouse model [37], while synthetic analogues with specific amino acid substitutions in the heptad repeat sequences of BMAP-28 displayed unmodified antibacterial activity and reduced toxicity to fibroblasts and red blood cells [2]. Synthetic truncated analogues with remarkably improved target cell selectivity were further obtained upon deletion of the hydrophobic C-terminal tail in the sequences of BMAP-28 and of the highly similar congener BMAP-27 [28, 41, 66]. It is quite obvious that shorter sequences would have the advantage to be simpler and cheaper to be synthesized. Moreover, such sequences could be more suitable for chemical grafting onto materials of medical interest. It is worth to note that immobilized peptides often show a significantly reduced cytotoxic potential when compared to their soluble counterparts [33, 47, 63]. In this respect, ongoing studies are aimed at the identification of shorter peptide analogs and derivatives most suitable for chemical immobilization on biomedical materials.

In conclusion, the present study confirmed and extended the antifungal properties of LL-37 and BMAP-28. Whereas the anti-adhesive properties of full-length LL-37 against *C. albicans* were essentially confirmed, we showed here for the first time that this peptide is not effective towards preformed *Candida* biofilms. Conversely, the efficacy of BMAP-28 against biofilms formed by *C. albicans* and *non-albicans* clinical isolates is the most relevant finding of this study. Compared to LL-37, BMAP-28 appeared to be less sensitive to medium components, showed a broader spectrum of susceptible microorganisms, was more effective in preventing fungal growth on polystyrene and silicone surfaces, and killed *Candida* cells in a 48-hours old biofilm by membrane permeabilization. These results encourage further studies on this peptide in view of its possible development for antifungal applications.

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Figure legends

Figure 1. Killing kinetics of BMAP-28 and MCZ against *C. albicans* SC5314 biofilm.

(A) *C. albicans* SC5314 biofilms were incubated in the presence of BMAP-28 (open squares) or MCZ (closed triangles) at 16 μM (continuos line) and 32 μM (dashed line), for the indicated time periods. At the end of each incubation period, biofilm cell viability was assessed using the XTT assay. Results are reported as percent viable cells relative to untreated samples and are the mean ± SD of three independent experiments performed in triplicate. (B) *C. albicans* SC5314 biofilms were incubated in the presence of 16 μM (open bars) and 32 μM (solid bars) BMAP-28 or 16 μM (light gray bars) and 32 μM (dark gray bars) MCZ for the indicated time periods. At the end of each incubation period, sessile cells were detached from the wells as described in the Materials and methods section, serially diluted in PBS, and plated to allow colony counts. Results are reported as percent killing relative to untreated samples and are the mean ± SD of three independent experiments performed in triplicate.

Figure 2. Fluorescence microscopy images of *C. albicans* biofilms.

Mature, 48-h-old *C. albicans* SC5314 biofilms were incubated for 90 min at 37°C in the absence (A) and presence (B) of 16 μM BMAP-28, and then stained with propidium iodide and concanavalin A Alexa Fluor® 488 conjugate. Magnification, X40. Boxed regions in (B) are presented at X2 magnification in B1-B4. Bars, 10 μm. Fluorescence microscopy was performed three times on two coverslips per condition.

Figure 3. Effect of BMAP-28 against biofilms formed by vaginal *Candida* isolates.

Biofilms formed by *C. albicans* SC5314 and by clinical isolates of *C. albicans* (CA), *C. krusei* (CK) and *C. glabrata* (CG) were incubated at 37 °C in the presence of 16 μM BMAP-28 for 24 h. Cell viability was assessed using the XTT assay. Results are reported as percent viable cells relative to untreated samples and are the mean ± SD of at least three independent experiments performed in triplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Figure 4. Inhibition of *C. albicans* biofilm formation on polystyrene plates.

C. albicans SC5314 cells were seeded at 1×10^7 cells /ml into 96-well polystyrene microtiter plates and incubated for 30 min (A, C) or 48 h (B, D) in the absence and presence of LL-37, BMAP-28, AMB or MCZ at concentrations corresponding to ½ MIC (open bars) and MIC (solid bars) against 1×10^7 planktonic cells /ml. Adherent (A, B) and total (C, D) *Candida* cell numbers were quantified by the XTT assay. Results are reported as the mean ± SD of at least three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 5. Inhibition of *C. albicans* biofilm formation on silicone surface.

C. albicans SC5314 cells were seeded onto SE disks and incubated in the absence and presence in the culture medium of 16 μM BMAP-28 or 64 μM LL-37 (A), or were deposited onto SE-disks preincubated with the indicated peptide concentrations and then rinsed to remove unbound peptide prior to cell seeding (B). Following incubation at 37°C for 48 h, the number of adherent (open bars) and total (solid bars) cells was quantified by the XTT assay. The results are reported as the mean ± SD of at least three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[b]MIC value at which ≥90% of *Candida* isolates are inhibited

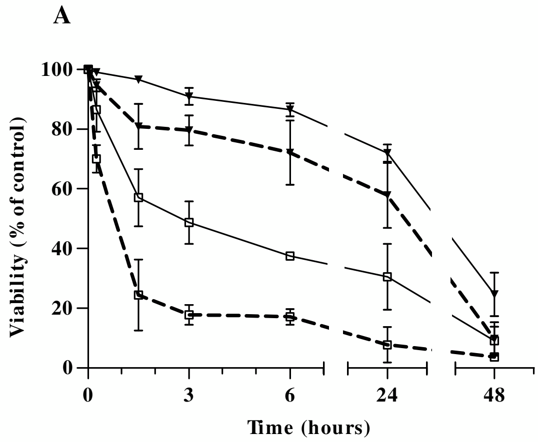
[c]Susceptible-dose dependent to Itraconazole (MIC 0.25 – 0.5 μg/ml)

[d]Resistant to Itraconazole (MIC > 1 μg/ml)

[e]Susceptible-dose dependent to Fluconazole (MIC 16 – 32 μg/ml)

[f]Resistant to Fluconazole (MIC > 64 μg/ml)

Figure 1.



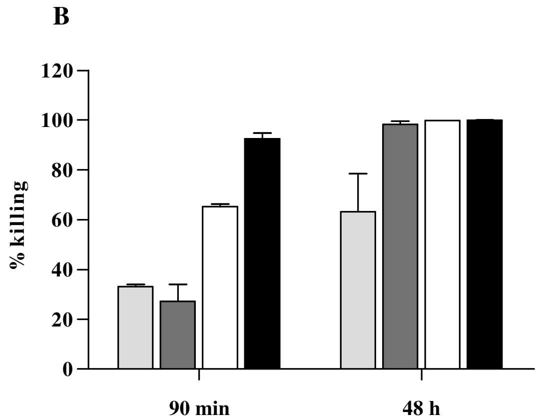


Figure 2.

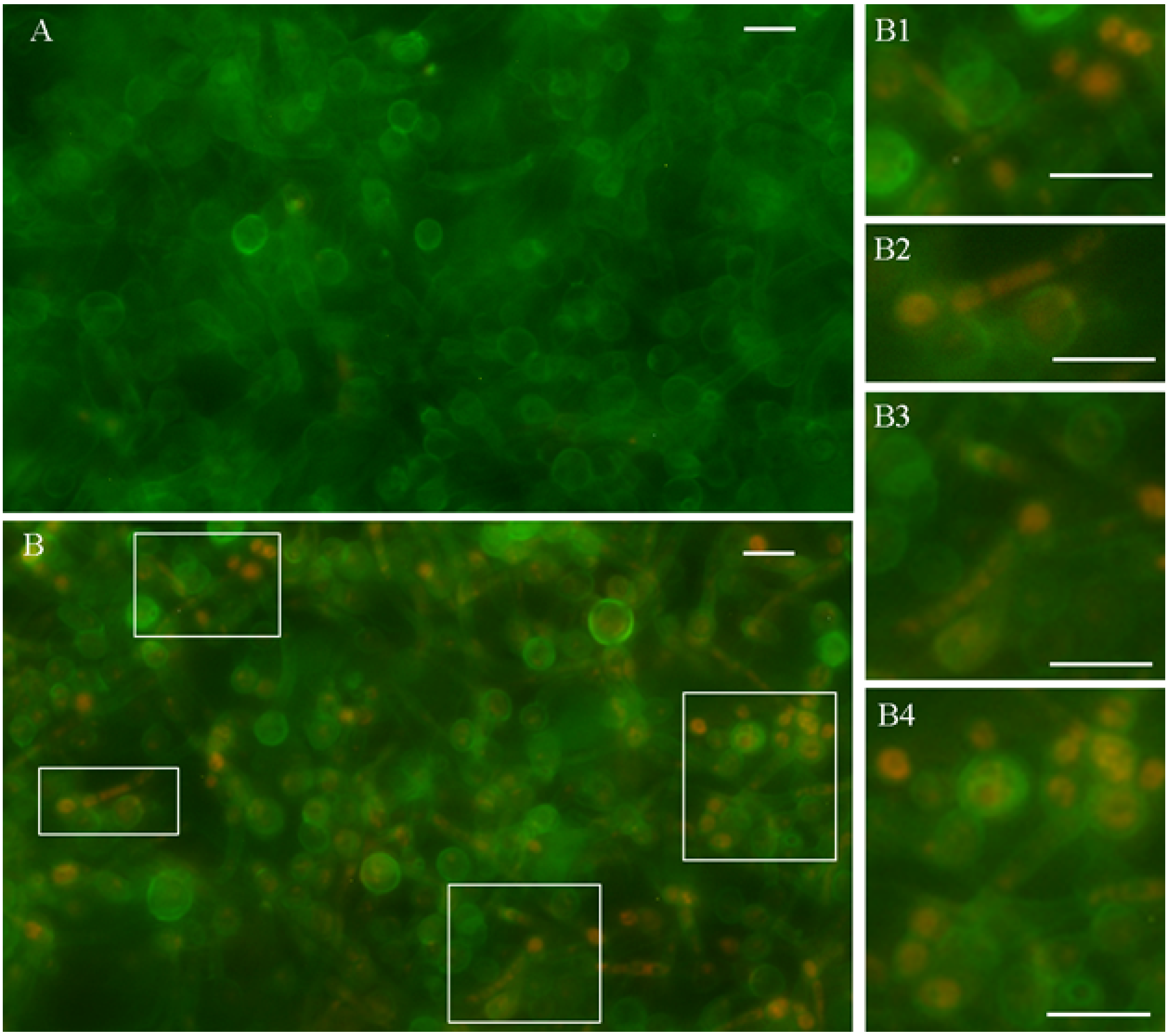
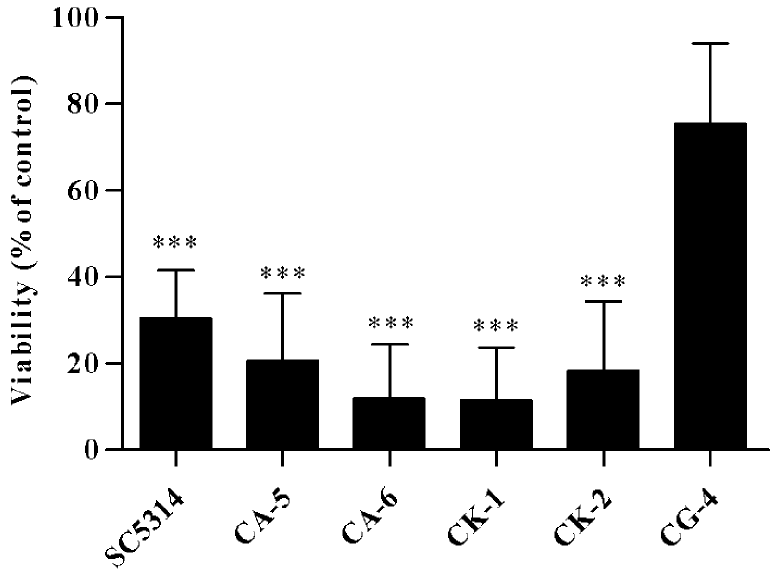


Figure 3.



Viability (% of control)
100
80
60
40
20
0
***
***
***
***
***
SC5314
CA-5
CA-6
CK-1
CK-2
CG-4

Figure 4.

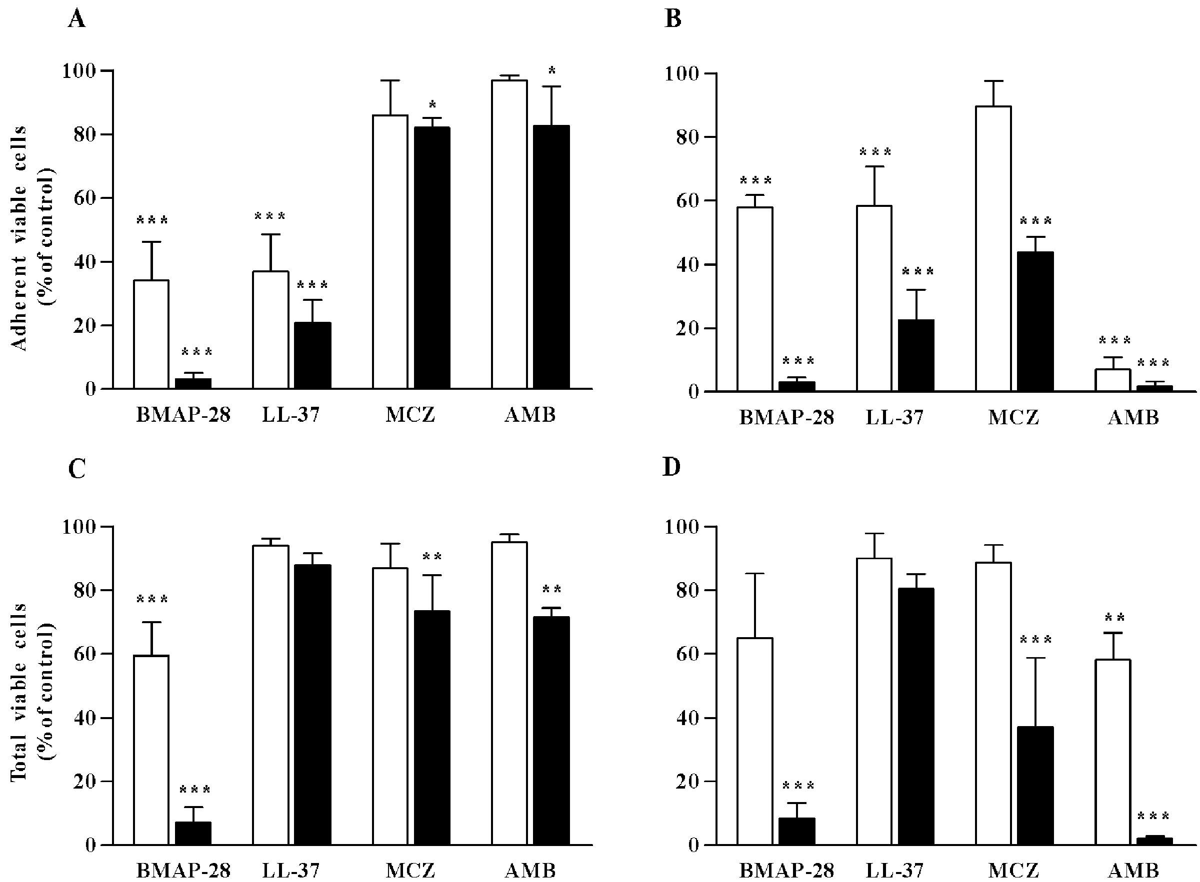


Figure 5.

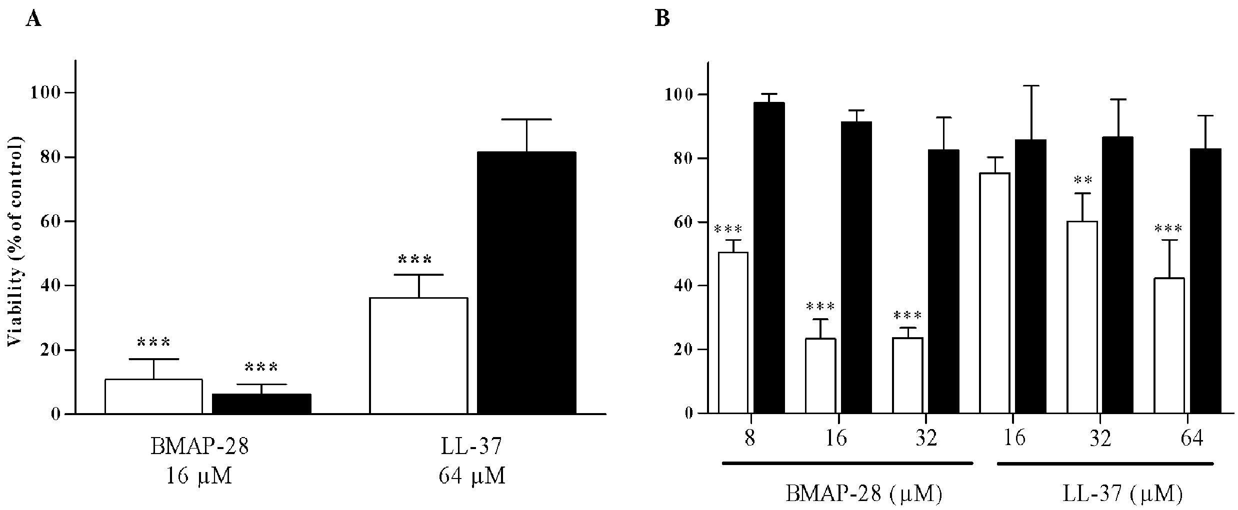


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Table 1. Antifungal activity of BMAP-28 and LL-37 against vaginal isolates of *Candida* spp and *S. cerevisiae.*

Organism	MIC (μM)	
	BMAP-28	LL-37
C. albicans SC5314	2	> 64
C. albicans CA-1	2	> 64
C. albicans CA-2	2	16
C. albicans CA-3	4	> 64
C. albicans CA-4	8	> 64
C. albicans CA-5	2	16
C. albicans CA-6	2	> 64
C. albicans CA-7	2	16
C. albicans CA-8	2	64
C. albicans CA-9	2	> 64
C. albicans CA-10	4	> 64
C. albicans CA-11	2	32
C. albicans CA-12	4	> 64
MIC_{50}[a]	2	> 64
MIC_{90}[b]	4	> 64
C. glabrata CG-1[c]	8	> 64
C. glabrata CG-2	32	> 64
C. glabrata CG-3[c]	8	> 64
C. glabrata CG-4[d,e]	4	> 64
C. glabrata CG-5[c]	32	> 64
C. glabrata CG-6[c,e]	32	> 64
C. glabrata CG-7[c]	> 64	> 64
MIC_{50}[a]	32	> 64
MIC_{90}[b]	32	> 64
C. krusei CK-1[c,e]	2	4
C. krusei CK-2[c,e]	4	32
C. krusei CK-3[c,f]	8	64
C. norvegensis[c,e]	8	16
C. parapsilosis	2	32
S. cerevisiae SC-1	2	2
S. cerevisiae SC-2	2	2
S. cerevisiae SC-3	2	2

[a]MIC value at which ≥50% of *Candida* isolates are inhibited

Table 2. Anti-*Candida* activity of BMAP-28 in vaginal simulated fluid.

	MIC (μM)	
	Sabouraud	VSF

Organism	pH 5.5	pH 6.5	pH 5.5	pH 4.2
C. albicans SC5314	2	8	16	32
C. albicans CA-5	4	8	16	32
C. albicans CA-6	2	16	16	32
C. krusei CK-1	2	16	32	32
C. krusei CK-2	4	16	16	32

Table 3. Activity of BMAP-28, LL-37, MCZ and AMB against *C. albicans* SC5314 biofilm.

Antifungal agent	$SMIC_{50}$ * (μM)	$SMIC_{90}$ * (μM)
BMAP-28	8	16
LL-37	> 32	> 32
MCZ	16	32
AMB	> 32	> 32

*$SMIC_{50}$ and $SMIC_{90}$ were defined as the lowest concentration of test compound resulting in at least 50% or 90% reduction in metabolic activity after 48 h treatment, compared with the untreated control.