Evaluation of free or anchored antimicrobial peptides as candidates for the prevention of orthopaedic device-related infections.

Evaluation of free or anchored antimicrobial peptides as candidates for the prevention of orthopaedic device-related infections.

Journal:	*Journal of Peptide Science*
Manuscript ID	PSC-17-0052.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	D'Este, Francesca; University of Udine, Department of Medicine, piazzale Kolbe 4 Oro, Debora; University of Udine, Department of Medicine, piazzale Kolbe 4 Boix Lemonche, Gerard; University of Udine, Department of Medicine, piazzale Kolbe 4 Tossi, Alessandro; University of Trieste, Department of Life Sciences, via Giorgieri 5 Skerlavaj, Barbara; University of Udine, Department of Medicine
Keywords:	α-helical antimicrobial peptide, prosthetic joint pathogens, Staphylococcus, human serum, hyaluronic acid, peptide immobilization

Evaluation of free or anchored antimicrobial peptides as candidates for the prevention of orthopaedic device-related infections.

Francesca D'Este[1§], Debora Oro[1§], Gerard Boix-Lemonche[1], Alessandro Tossi[2], Barbara Skerlavaj[1*].

[1]Department of Medicine, University of Udine, P.le Kolbe 4, 33100 Udine, Italy.
[2]Department of Life Sciences, University of Trieste, Via Giorgieri 5, 34127 Trieste, Italy.
[§]These authors contributed equally to this work.

*Corresponding author.
Mailing address: Department of Medicine, University of Udine, P.le Kolbe 4, 33100 Udine, Italy.
Phone: +39-0432-494390;
Fax: +39-0432-494301;
E-mail: barbara.skerlavaj@uniud.it
ORCID: 0000-0003-0437-802X

Short title: α-helical AMPs for the prevention of orthopaedic infections.

Abstract

The prevention of implant-associated infection, one the most feared complications in orthopaedic surgery, remains a major clinical challenge and urges development of effective methods to prevent bacterial colonization of implanted devices. Alpha-helical antimicrobial peptides (AMPs) may be promising candidates in this respect due to their potent and broad-spectrum antimicrobial activity, their low tendency to elicit resistance and possible retention of efficacy in the immobilized state.

The aim of this study was to evaluate the potential of five different helical AMPs, the cathelicidins BMAP-27 and BMAP-28, their (1-18) fragments and the rationally designed, artificial P19(9/G7) peptide, for the prevention of orthopaedic implant infections. Peptides were effective at micromolar concentrations against 22 *Staphylococcus* and *Streptococcus* isolates from orthopaedic infections, while only BMAP-28 and to a lesser extent BMAP-27 were active against *Enterococcus faecalis*. Peptides in solution showed activities comparable to those of cefazolin and linezolid, on a molar basis, and also a variable capacity to neutralize bacterial lipopolysaccharide, while devoid of adverse effects on MG-63 osteoblast cells at concentrations corresponding to the MIC. The (1-18) BMAP fragments and P19(9/G7) were selected for further examination, based on better selectivity indices, and showed effectiveness in the presence of hyaluronic acid and in synovial fluid, while human serum affected their activity to variable extents, with BMAP-27(1-18) best retaining activity. This peptide was immobilized on streptavidin-resin beads and retained activity against reference *S. epidermidis* and *S. aureus* strains, with negligible toxicity towards osteoblasts, underlining its potential for the development of infection-resistant biomaterials for orthopaedic application.

Keywords: α-helical antimicrobial peptide, prosthetic joint pathogens, *Staphylococcus*, human serum, hyaluronic acid, peptide immobilization.

Abbreviations: ALP, alkaline phosphatase; AMP, antimicrobial peptide; BHI, Brain-Heart Infusion; CFU, colony-forming unit; CLSI, Clinical and Laboratory Standards Institute; DEG, diethylene glycol; DMEM, Dulbecco's Modified Eagle's Medium; DMF, N,N-dimethylformamide; DXM, dexamethasone; FBS, foetal bovine serum; HA, hyaluronic acid; HBSS, Hanks' Balanced Salt Solution; HPLC, high performance liquid chromatography; HS, human serum; HSA, human serum albumin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MBC, minimum bactericidal concentration; MH, Mueller-Hinton; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MRSE, methicillin-resistant *Staphylococcus*

epidermidis; NO, nitric oxide; OM, osteogenic medium; PBS, phosphate buffered saline; RPMI, Roswell Park Memorial Institute; SF, synovial fluid; SD, standard deviation; TFA, trifluoroacetic acid.

Introduction

Implant-associated infection is a major challenge in orthopaedic surgery. Although it occurs in only a small proportion of cases, infection of prosthetic joints is a feared and devastating complication of arthroplasty procedures and is associated with substantial morbidity and a huge economic burden [1-3]. The incidence of infection can be much higher following trauma surgery, such as fracture fixation [4]. More than half of infections are caused by Gram-positive cocci, the most common being coagulase-negative *Staphylococcus* spp. and *Staphylococcus aureus*, followed by *Streptococcus* and *Enterococcus* spp. A relevant proportion of these is polymicrobial, often involving methicillin-resistant *S. aureus* (MRSA). Gram-negative bacilli, anaerobes and fungi are less frequent causative agents [1, 3]. Treatment of infections associated with orthopaedic devices is challenging as bacteria tend to grow in biofilms, which reduces antibiotic efficacy, added to which is the growing incidence of antibiotic resistance also in orthopaedic settings [1, 3, 5]. The prevention of initial bacterial colonization of the implant surface thus remains a priority.

In this respect, natural antimicrobial peptides (AMPs) may represent a valuable reservoir of lead compounds for the development of novel anti-infective agents. AMPs comprise structurally diverse molecules endowed with potent microbicidal properties, acting as relevant components of the immune system in a wide range of living organisms [6]. Unlike conventional antibiotics, which act on specific bacterial targets, the mode of action of AMPs in most cases relies on their capacity to selectively perturb bacterial membranes, owing to their cationic and amphipathic nature [6, 7]. This mechanism accounts for their broad spectrum of activity and efficacy also against antibiotic-resistant strains, as well as for their low propensity to promote bacterial resistance [7]. Additionally, in several instances AMPs have exhibited immunomodulatory properties due to their ability to interact with host cells and modulate their functions, without damaging them [8, 9]. Several natural or artificial AMPs are currently under preclinical or clinical development as novel anti-infectives [6, 9, 10]. Moreover, an emerging approach focuses on exploiting these molecules for the fabrication of infection-resistant biomaterials for medical use [10-12].

Among the diverse structural classes of AMPs, linear peptides adopting an α-helical conformation appear to possess the most robust antimicrobial activities, with lower susceptibility to medium conditons [13, 14]. In addition to broad-spectrum activity, they can bind and neutralize proinflammatory bacterial components such as lipopolysaccharide (LPS) [9, 15]. Given their abundance in nature and their relatively easy chemical production with respect to other structural classes, native peptide sequences have been exploited as templates for numerous synthetic helical AMPs with optimized structural parameters to enhance antimicrobial potency and selectivity [16, 17]. As their killing mechanism does not require internalization by target bacteria, α-helical AMPs

may retain activity upon immobilization, which may render them suitable for the development of antimicrobial surfaces [18-20].

In the present study five cationic α-helical AMPs, *i.e.*, two bovine members of the cathelicidin AMP family [21], two shorter derivatives [22] and a rationally designed artificial AMP [17], have been evaluated for potential applications in the prevention of orthopaedic device-related infections. Antimicrobial efficacy of peptide candidates has been assayed against bacterial isolates from clinical cases of orthopaedic infection, in comparison with two conventional antibiotics. The peptides were also evaluated for LPS-neutralizing capacity and for potential effects on osteoblast viability and differentiation. Based on their more favourable selectivity indices the study was then narrowed to the three shorter peptide candidates, that were examined for antimicrobial efficacy under conditions more closely resembling those encountered *in vivo* in arthroplasty settings. The peptide with the best activity overall was then tethered to resin beads via streptavidin-biotin technology to obtain a proof-of-concept demonstration of antimicrobial efficacy upon immobilization.

Materials and methods

Media and reagents

Derivatized polyethylene glycol–polystyrene (PEG-PS) resins, coupling reagents for peptide synthesis and 9-fluorenylmethoxy carbonyl (Fmoc)-amino acids were purchased from Applied Biosystems/Thermo Fisher Scientific (Waltham, MA, USA), Novabiochem (Laufelfingel, Switzerland) and ChemImpex (Wood Dale, IL, USA). Peptide synthesis-grade N,N-dimethylformamide (DMF), dichloromethane, piperidine and HPLC-grade acetonitrile were from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid (TFA), trifluoroethanol and N-methylmorpholine were from Acros Chimica (Beerse, Belgium). The biotinylating reagents D(+)-Biotin and O-(N-Biotinyl-3-aminopropyl)-O'-(N-glutaryl-3-aminopropyl)-diethyleneglycol (N-Biotinyl-NH-PEG_2-COOH) were obtained from Calbiochem (La Jolla, CA, USA) and Novabiochem, respectively. High-performance Streptavidin-Sepharose™ resin was purchased from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, UK).

Dehydrated media for microbiological assays were obtained from Difco laboratories (Detroit, MI, USA) and Oxoid/Thermo Fisher Scientific. Cefazolin sodium salt, linezolid, lipopolysaccharide (LPS) from *E. coli* O111:B4, dexamethasone (DXM), hyaluronic acid sodium salt (HA) and normal human serum (HS) were from Sigma-Aldrich (St. Louis, MO, USA). Media and supplements for cell culture were from Sigma-Aldrich with the exception of foetal bovine serum (FBS) (Euroclone, Pero, Italy). Unless otherwise specified, FBS and HS were inactivated at 56°C for 30 min prior to use. Synovial fluid (SF) samples were obtained with informed consent at Udine University Hospital (Udine, Italy); samples were centrifuged, aliquoted and stored at -80°C.

The Griess and PrestoBlue® reagents were from Molecular Probes/Thermo Fisher Scientific and Invitrogen/Thermo Fisher Scientific, respectively. The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit was from Promega (Madison, WI, USA). All other reagents, including p-Nitrophenyl Phosphate Liquid Substrate System, were from Sigma-Aldrich. Buffers were prepared in double glass-distilled water.

Peptide synthesis and characterization

Peptides (Table 1) were synthesized in the solid phase using the Fmoc-chemistry. Difficult coupling steps were handled as described previously [14, 23]. BMAP-27 and BMAP-27(1-18) were biotinylated 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 1H-hydroxybenzotriazole (HOBt) in DMF containing 0.6 N N-methylmorpholine for 4

h at room temperature. BMAP-27(1-18) was alternatively biotinylated with N-Biotinyl-NH-PEG_2-COOH using the same procedure. The biotinylated peptides are referred to as biot-B27, biot-B27(1-18) and biot-DEG-B27(1-18) in the following sections. After cleavage and deprotection, the peptides were HPLC-purified 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) for BMAP-27 and BMAP-27(1-18), and at 280 nm (Tyr and Trp residues) for BMAP-28, BMAP-28(1-18) and P19(9/G7) [14, 23].

Peptide immobilization on Streptavidin-Sepharose resin

250 µL of 1 mM solutions of the biotinylated peptides [*i.e.*, biot-B17, biot-B27(1-18) and biot-DEG-B27(1-18)] in PBS were mixed with an equal volume of high performance Streptavidin-Sepharose resin (previously washed free of the storage buffer) resuspended in PBS, and allowed to react overnight at 4°C under gentle agitation. One resin sample was coupled in parallel with D(+)-Biotin for use as a reference control. Samples were then allowed to re-equilibrate at room temperature, centrifuged and washed extensively with PBS until the absorbance at 257 nm of the resin supernatants reached the baseline level. Resin samples were then resuspended in PBS and either tested immediately, or stored at 4°C for up to 1 month before use.

The amount of immobilized peptide was estimated after peptide elution from a resin aliquot by 15 min treatment with 0.05% (v/v) TFA and lyophilization. The lyophilized eluates were redissolved in water and assayed using the Bradford reagent with reference to standard curves generated by serial dilutions of the corresponding soluble peptides.

Bacteria and culture conditions

The reference strains were *Staphylococcus epidermidis* ATCC 12228 and ATCC 35984, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. Clinical bacterial isolates were collected from hip revision and other orthopaedic surgery at the Valdoltra Orthopaedic Hospital (Ankaran, Slovenia) and the Udine University Hospital (Udine, Italy), and included 8 strains of *Staphylococcus epidermidis*, 8 strains of *Staphylococcus aureus*, 1 strain each of *Staphylococcus capitis*, *Staphylococcus hominis* and *Staphylococcus caprae*, 2 strains of *Streptococcus agalactiae*, 1 group G *Streptococcus* and 2 isolates of *Enterococcus faecalis.* Bacteria were maintained on Mueller-Hinton (MH) agar plates. One isolate of *S. epidermidis* from the Udine hospital was methicillin-resistant as assessed

according to the guidelines and interpretative tables of the Clinical and Laboratory Standards Institute (CLSI). Bacteria were cultured in either liquid Brain Hearth Infusion (BHI) (*Staphylococcus*, *Streptococcus* and *Enterococcus* strains) or MH broth (*E. coli* and *P. aeruginosa*) for 18 h, 1:50-diluted in fresh medium and allowed to grow in an orbital shaker at 37°C. Mid-log phase bacteria were harvested after 10 min centrifugation at 1000 × g and resuspended in MH broth to an optimal density before use in antimicrobial assays. Bacterial density was assessed by turbidity at 600 nm, with reference to previously determined standards. For biofilm experiments, the bacterial inoculum was prepared essentially according to Pompilio *et al.* [24], by direct suspension of colonies grown overnight on MH plates in liquid medium.

Antimicrobial assays

The minimum inhibitory concentration (MIC) of the peptides in solution was determined by a broth microdilution assay in 96-well microtiter plates, using MH broth with logarithmic-phase microorganisms at 2.5×10^5 CFU/mL, as previously reported [14], following CLSI guidelines. The clinically used antibiotics cefazolin and linezolid were tested in parallel for comparison. The minimum bactericidal concentration (MBC) was determined by plating aliquots from wells showing no visible growth on solid medium to allow colony counts.

Assays for inhibition of biofilm formation

50 µL of a *S. epidermidis* ATCC 35984 suspension (2×10^6 CFU/mL in MH broth), prepared as described above, was dispensed into flat-bottomed 96-well microtiter plates containing 50 µL in MH of each test agent, at 2× the final dose. Plates were then incubated for 24 h at 37°C, followed by removal of non-adherent bacteria by aspiration and rinsing with PBS. Fresh MH broth was then added and adherent cells were quantified using the PrestoBlue cell viability reagent according to the manufacturer's specifications.

Bacterial growth kinetics analysis

Serial dilutions of peptides, at 2× the final concentration, were prepared in 50 µL PBS alone or in PBS supplemented either with 1 or 6 mg/mL hyaluronic acid (HA) (leading to 0.5 and 3 mg/mL final HA concentrations, respectively), or with 50% human serum (HS, 25% final concentration), or with 40% synovial fluid (SF, 20% final concentration) in U-bottom 96-well plates. 50 µL of adjusted suspensions of *S. epidermidis* ATCC 35984 or *S. aureus* ATCC 25923 in MH broth were added to each well to achieve the final density of 1×10^7 CFU/mL. Plates were then sealed with optically-clear plastic films to avoid evaporation, and bacterial growth was monitored at 600 nm for

6 h at 37°C, with 10-sec shaking steps at 15-min intervals. Bacteria grown in each medium in the absence of peptides served as growth controls. In some experiments peptide dilutions in the selected media were pre-incubated for 3 h at 37°C prior to the addition of bacteria. Data are reported either as growth curves, or as the calculated percent growth inhibition at 6 h with respect to bacteria incubated in corresponding media in the absence of peptides.

Antibacterial activity of immobilized peptides

10 µL aliquots of peptide-functionalized Streptavidin-Sepharose resins (5% v/v final resin concentration), or the corresponding supernatants, were diluted in PBS to a final volume of 100 µL in microcentrifuge tubes and supplemented with 100 µL of a 2×10^7 CFU/mL suspension of *S. epidermidis* ATCC 35984 or *S. aureus* ATCC 25923 in MH broth. Bacterial viability was assessed after 1 h incubation at 37°C on a rotating wheel by the PrestoBlue assay and CFU counts.

Cell culture and stimulation

The murine macrophage-like RAW 264.7 and the human osteoblast-like MG-63 cell lines were obtained from ATCC (Manassas, VA, USA) and maintained in RPMI-1640 and DMEM medium, respectively, in a humidified incubator at 37°C and 5% CO_2 atmosphere. Both media were supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (complete media).

To analyze the effects on LPS-induced nitric oxide (NO) production, RAW 264.7 cells were seeded in 24-well plates at a density of 9×10^5 cells /well, cultured overnight and treated for 24 h with 100 ng/mL LPS in the absence and presence of peptides at the indicated concentrations, in complete RPMI.

MG-63 cells used for cell viability and cytotoxicity assays were seeded in 96-well plates at a density of 7500/well and grown for 24 h in complete DMEM before being incubated with increasing peptide concentrations in complete medium, or, alternatively, in DMEM supplemented with 10% (v/v) HS, or in HBSS supplemented with 10% HS in the absence and presence of 0.5 mg/mL HA. Peptide-functionalized resins were tested at 5% (v/v) in complete medium. Incubation times were 1 h and 24 h for lactate dehydrogenase (LDH) release and cell viability assays, respectively.

For osteoblast differentiation experiments, MG-63 cells were seeded in 24-well plates at a density of 3×10^5 /well in complete medium, grown to 80% confluence and incubated for 7 days in serum-free DMEM supplemented with 10 mM β-glycerophosphate and 50 µg/mL L-ascorbic acid (osteogenic medium, OM) in the absence or presence of 2 µM peptides, of 100 nM dexamethasone

(DXM, positive control) or a combination of peptides and DXM. During the incubation period medium was replaced twice with fresh OM supplemented with the stimuli.

Nitric oxide (NO) determination

The amount of NO released by LPS- and peptide-treated RAW 264.7 cells was estimated by spectrophotometric quantification of the stable NO metabolite nitrite in the cell culture supernatants using the Griess reagent. Data were expressed as percent of nitrite produced in response to LPS in the absence of peptides.

Cytotoxicity and cell viability assays

Membrane damage to MG-63 cells was evaluated as leakage of the cytosolic enzyme lactate dehydrogenase (LDH). LDH activity was quantified in both cell-free supernatants and cell lysates using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit. Data were calculated as percentage of total cellular LDH activity. Cell viability was assessed using the resazurin-based PrestoBlue metabolic dye according to the manufacturer's instructions.

Measurement of alkaline phosphatase (ALP) activity

MG-63 cells, incubated in OM with peptides and DXM as described above, were harvested by trypsinization, resuspended in water and sonicated. Lysate proteins were quantified by the Bradford assay. ALP activity was assessed spectrophotometrically from the transformation of p-nitrophenyl phosphate to p-nitrophenol at 37°C. Briefly, 10 µg protein aliquots of cell lysates were diluted in water in microtiter plates to a final volume of 50 µL, mixed with 150 µL of p-Nitrophenyl Phosphate Liquid Substrate System and incubated for 80 min at 37°C. Absorbance was read at 405 nm and compared to a p-nitrophenol standard curve. Data were calculated as nmoles/mg/min and expressed as fold-increase with respect to basal ALP activity, or as percent of ALP activity induced by DXM alone.

Results

Design and selection of candidate peptides

The peptides used in this study (Table 1) were selected on the basis of their reported broad antimicrobial activity spectra that covered Gram-positive cocci [17, 22, 23, 25]. BMAP-27 and BMAP-28 are amphipathic α-helical bovine cathelicidins that were previously shown to exert potent bactericidal activity based on rapid membrane permeabilization [22, 25]. The two shorter derivatives, BMAP-27(1-18) and BMAP-28(1-18) (hereafter referred to as B27(1-18) and B28(1-18)), were synthetically more accessible and showed improved selectivity with respect to prokaryotic cells [22]. The peptide P19(9/G7) (hereafter P19) was rationally designed based on a consensus sequence derived from a panel of over 100 of natural helical AMPs of invertebrate, anuran and mammalian origin [17, 23]. This peptide is based on an amidated, 18-residue sequence, like the BMAP fragments, with an added C-terminal Tyr residue to improve quantification accuracy.

Antimicrobial activity against orthopaedic pathogens

The MIC and MBC values of the peptides towards representative reference strains of Gram-positive *S. epidermidis* and *S. aureus* and Gram-negative *E. coli* and *P. aeruginosa* are reported in Table 2. Based on the frequent presence of Gram-positive microorganisms among the causative agents of orthopaedic device-related infections [1, 3], the peptides were assayed against a panel of Gram-positive clinical isolates from such infections, including *Staphylococcus*, *Streptococcus* and *Enterococcus* species (Table 3). All peptides proved effective at low micromolar concentrations against most strains, with MBC values very close to the MICs, with the exception of *E. faecalis*, which was susceptible only to BMAP-28 and to a lesser extent to BMAP-27. On a molar basis, their potency was comparable to cefazolin and linezolid, two conventional antibiotics used in orthopaedic practice [26, 27]. Considering *S. aureus* and *S. epidermidis* species, susceptibility of clinical isolates and corresponding reference strains to the AMPs was similar. Among the peptides, the rationally designed P19 was overall somewhat less effective against *S. aureus*, while its activity against the other *Staphylococcus* strains was comparable to that of the BMAP peptides. In general, peptides were highly active against *S. epidermidis*, whereas somewhat higher MIC values were observed against *S. aureus* isolates. In addition, all peptides prevented the formation of *S. epidermidis* ATCC 35984 biofilm at their MIC values, as shown in Figure 1a. Cefazolin and linezolid, tested in parallel, resulted in complete biofilm inhibition at 16 μM (4-fold MIC) and 6 μM (MIC), respectively (Figure 1b and data not shown).

Neutralization of bacterial lipopolysaccharide

Peptides were next evaluated for their ability to neutralize the effects of bacterial lipopolysaccharide (LPS), an activity common to several amphipathic helical AMPs [15] that could be advantageous in the context of orthopaedic biomaterials [28-30]. This was tested by inhibition of LPS-induced nitric oxide (NO) release from RAW 264.7 macrophage-like cells co-stimulated with peptides and LPS. BMAP-27 and -28 completely abolished the NO response at concentrations as low as 0.5 - 1 μM (Fig. 2), consistent with previous reports on their direct LPS-binding properties [31-33]. Among the three shorter peptides, B28(1-18) virtually abolished the LPS-induced response at 2 μM, while B27(1-18) and P19 respectively caused 80% and 66% inhibition at 8 μM (Fig. 2). These results could not be ascribed to cytotoxic effects on the RAW cells, as cell viability was not impaired under the experimental conditions used (resazurin-based metabolic assays, data not shown).

Effects on osteoblast viability and differentiation

To assess their safety for orthopaedic applications, the effects of peptides on osteoblast cells were investigated using the human osteosarcoma-derived MG-63 cell line, as a well-established *in vitro* osteoblast model [34, 35]. Potential short- and long-term effects were assessed in terms of membrane damage, cell viability and cell differentiation. Figure 3a shows the percentage of lactate dehydrogenase (LDH) release by cells incubated with the peptides for 1 h in standard cell culture medium, as an indicator of impaired plasma membrane integrity. None of the peptides caused detectable LDH release at concentrations in each peptide's MIC range (cf. Fig. 3a and Table 3). The shorter BMAP derivatives were safe up to 10 μM, while the parent peptides caused 38-54% LDH release at this concentration. P19 did not cause detectable effects up to at least 20 μM (Fig. 3a). Remarkably, the toxicity of full-length and truncated BMAPs was clearly reduced when using human rather than standard foetal bovine serum in the cell culture medium (Fig. 3c), indicating that the range of safe concentrations under conditions relevant to *in vivo* settings could actually be wider. To mimic the joint environment, peptides were assayed in Hanks' Balanced Salt Solution (HBSS) supplemented with human serum (HS) and hyaluronic acid (HA) at 0.5 mg/mL, a concentration reflecting those found in periprosthetic synovial fluid [36]. Under these conditions, peptides were safe up to 10 μM with the exception of BMAP-27, which caused approximately 40% and 68% LDH release at 10 and 20 μM, respectively (Fig. 3d). The three shorter peptides, and in particular P19 and B27(1-18), showed lower overall toxicity. This was also confirmed in terms of cell viability after 24 h incubation with 1.25 - 10 μM peptides in standard culture medium, which indicated the impairment of cell viability only on treatment with the full-length BMAPs at the

highest tested concentration (Fig. 3b). Long-term effects on osteoblast cells were also addressed by measuring alkaline phosphatase (ALP) activity as a differentiation marker for these cells [37]. Peptides were evaluated in comparison with dexamethasone (DXM) as a reference osteogenic stimulus [37]. MG-63 cells incubated for 7 days with 2 μM peptides in osteogenic medium showed comparable ALP activity as control cells except after treatment with BMAP-27, which caused an approximately 50% reduction in activity (Fig. 3e). A 2.7-fold increase in ALP activity was detected in DXM-treated cells (Fig. 3e). The three shorter peptides were further investigated in combination with the latter stimulus, showing no significant effects on the DXM-induced response (Fig. 3f), suggesting they would not be detrimental to the osseointegration processes.

Antimicrobial activity in the presence of hyaluronic acid, synovial fluid and blood serum

Given their compatibility with osteoblast cells, the (1-18) BMAP derivatives and P19 were subjected to further investigation. Their antimicrobial efficacy against *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923 was assayed in the presence of HA (as it could interact electrostatically with the cationic peptides due to its negative charge), used at 0.5 and 3 mg/mL to mimic periprosthetic and normal synovial fluid concentrations, respectively [36, 38]. Figure 4 shows growth kinetics analyses in the presence of increasing peptide concentrations, expressed as percent growth inhibition after 6 h incubation in each media. HA *per se* did not significantly affect bacterial growth (data not shown). Remarkably, all of the peptides retained full activity against both *Staphylococcus* species under these conditions. Furthermore, full peptide efficacy was retained even after 3 h pre-incubation of peptides with HA (6 mg/mL) before the addition of the bacterial suspension (data not shown), suggesting that peptides should retain efficacy in the joint environment, where HA is a relevant component. This was further supported by growth kinetics analysis of *S. aureus* ATCC 25923 incubated with the peptides in the presence of a clinical sample of synovial fluid (SF, 20%) (Fig. 5). A limited number of SF samples were available, showing a rather variable intrinsic inhibition of bacterial growth (data not shown); the one that was chosen for the reported experiment allowed an appreciable *S. aureus* growth within the 6 h experimental time frame. Peptides, tested at 2 μM in its presence instead completely prevented bacterial growth (Fig. 5).

The applicative potential of the peptides was next assessed by antimicrobial assays performed in the presence of human serum (HS), based on reported evidence of inhibitory effects of serum components on AMP activity [14, 39-41]. Figure 6 shows the percentage of growth inhibition of *S. epidermidis* and *S. aureus* incubated for 6 h with increasing peptide concentrations in the absence and presence of commercial pooled human serum at 25%. HS *per se* did not impair

growth of either strain (data not shown), while affecting the peptides' inhibitory activity to variable extents. Complete inhibition of *S. epidermidis* growth in the presence of HS was achieved at the same B27(1-18) concentrations as those effective in its absence, and only slightly higher B28(1-18) and P19 concentrations (Fig. 6a, b). In the case of *S. aureus*, B27(1-18) and P19 showed a two-fold increase and B28(1-18) a four-fold increase in the concentrations necessary to obtain complete bacterial inhibition (Fig. 6d, e). Notably, when peptides were pre-incubated with 50% HS for 3 h prior to addition of the bacterial suspension, all of the peptides were similarly affected, with the concentration required for complete inhibition of *S. aureus* growth increasing to 64 μM (Fig. 6f). With respect to *S. epidermidis* growth inhibition under these conditions, B27(1-18) clearly retained the best activity [complete inhibition at 2-4 μM *vs* >8 μM for B28(1-18) and P19] (Fig. 6c). When B27(1-18) efficacy against *S. epidermidis* was tested in the presence of heat-inactivated HS (as in the experiments described thus far) or in the presence of HS not subjected to heat inactivation, results were overlapping (data not shown).

Antimicrobial efficacy in the immobilized state

Considering the overall results obtained thus far, the functional analysis of peptide candidates narrowed the choice to B27(1-18) for further evaluation of efficacy upon immobilization. Its parental BMAP-27 was previously reported to retain activity when covalently linked to a solid surface [19], so it was tested in parallel for comparison. Peptides were N-terminally biotinylated and conjugated to a commercial agarose resin functionalized with streptavidin. B27(1-18) was also derivatized with a biotinylated diethylene glycol (DEG) spacer to favour peptide mobility. In solution, the biotinylated derivatives showed comparable MIC values to the corresponding non-biotinylated peptides against reference *S. epidermidis* and *S. aureus* strains (data not shown), indicating that this modification did not alter activity. Peptide-functionalized resin samples were incubated with *S. epidermidis* ATCC 35984 and *S. aureus* ATCC 25923 suspensions for 1 h under agitation. Antibacterial effects were evaluated by the resazurin-based metabolic assay and colony counts. As reported in Figure 7a and c, total bacterial inactivation was achieved upon exposure to BMAP-27 and B27(1-18)-derivatized resins, while with the DEG-B27(1-18)-functionalized sample 65% and 33% inhibition of *S. epidermidis* and *S. aureus* was respectively observed. Consistent with this, CFU counts revealed complete killing in the case of immobilized BMAP-27 and B27(1-18), and a partial effect with DEG-B27(1-18) (Fig. 7b, d). It is important to note that the control resin, derivatized with biotin only, and the supernatants from peptide-derivatized resin samples did not affect bacterial viability (Fig. 7a, c). To verify the stability of the functionalized resins, their activity against *S. epidermidis* ATCC 35984 was also measured after 1

month storage at 4°C and, as shown in Figure 7e and f, it was fully retained, indicating that the peptide-resin coupling is quite stable. This was also supported by the lack of activity of supernatants from 1 month-old resin samples (Fig. 7e). Finally, LDH release assays on MG-63 cells incubated for 1 h with 5% resin suspensions did not reveal any impairment of cell membrane integrity (Table 4) supporting compatibility of functionalized resins with osteoblast cells.

Discussion

Surface modifications to render implants refractory to bacterial colonization are among the main strategies currently pursued to prevent orthopaedic implant-associated infection [42, 43]. The antimicrobial peptides of innate immunity are receiving increasing attention as novel anti-infective agents also in the field of medical devices [8-11]. The aim of the present study was to identify a peptide candidate active against relevant orthopaedic implant pathogens under conditions resembling *in vivo* settings, and suitable to be immobilized onto solid supports in view of future development of peptide-based infection-resistant biomaterials for orthopaedic applications.

Five α-helical peptide candidates were compared for their *in vitro* efficacy against clinically relevant Gram-positive bacterial strains isolated from orthopaedic infections. In keeping with previous reports, the BMAP peptides displayed similar low micromolar MIC values against most of the tested strains under standard conditions [14, 25, 44-46]. The comparable, or only slightly decreased potency of the (1-18) BMAP fragments confirms that antimicrobial activity is mainly mediated by the N-terminal segment predicted to adopt the amphipathic helical conformation [22, 25, 33]. In this regard, peptide modifications on parental or truncated BMAPs not subverting the overall helical fold were shown to be irrelevant to peptide activity [33, 39, 47, 48], supporting a relatively non-specific mode of peptide interaction with bacterial membranes that accounts for broad-spectrum permeabilizing effects.

While showing comparable efficacy to the BMAPs on other tested bacterial species, P19 displayed a clearly decreased activity against *S. aureus* strains. This suggests a partially different mode of action. Indeed, peptides of the P19 series have been proposed to act on *Staphylococcus* strains by a mechanism dependent on interference with functional processes at membrane level rather than on direct permeabilization [49].

With the exception of BMAP-28, the peptides under investigation showed lower efficacy against the clinical isolates of *E. faecalis* with P19 being the least effective. Concerning the BMAPs and derivatives, despite the limited number of strains tested, the observed MICs and the differences in potency are on the whole in line with a previous study assaying their activity against ten *E. faecalis* isolates, including vancomycin-resistant strains [25].

In general, the peptides under study showed a comparable efficacy to conventional antibiotics on a molar basis, and moreover proved effective against a methicillin-resistant clinical isolate of *S. epidermidis* (MRSE). This adds to previous reports on the efficacy of BMAPs and their derivatives, as well as of P19, on antibiotic-resistant Gram-positives, including MRSA [23, 25, 44, 50]. Moreover, peptides prevented *S. epidermidis* biofilm formation at microbicidal concentrations, with an all-or-nothing effect overall, likely due to rapid killing of bacteria before their

deposition/attachment to the surface of microplate wells. At variance with the AMPs, both cefazolin and linezolid displayed a gradual inhibition of biofilm formation, consistent with a mode of action not directly targeting the bacterial membrane [51, 52]. Furthermore, while linezolid was effective at preventing biofilm formation also at sub-MIC concentrations, cefazolin showed complete inhibition only at 4-fold its MIC. Comparing the efficacy of the peptides to cefazolin, the antibiotic of choice in perioperative orthopaedic prophylaxis [26], the selected AMPs appear to be suitable candidates for the prevention of bacterial colonization of implanted devices.

A distinctive feature of cationic AMPs is their capacity to neutralize proinflammatory bacterial components such as lipopolysaccharide (LPS, endotoxin) [15]. This property may be advantageous for applications in the field of orthopaedic biomaterials since endotoxin contamination may be responsible for adverse tissue reactions and can negatively affect the osseointegration process [28-30, 53]. Indeed, the murine cathelicidin CRAMP, another α-helical cathelicidin that is paralogous to BMAPs, has been shown to inhibit LPS- and flagellin-induced osteoclastogenesis by direct neutralization of these bacterial components, and accordingly has been proposed to act as a protector of bone resorption induced by bacterial infection in mice [54]. In the present study, in comparison to the complete LPS-neutralizing activity of the full length BMAPs, the three shorter peptides displayed a lower but appreciable effect. With respect to (1-18) BMAP peptides, this residual but still appreciable LPS-neutralizing effect, as already reported by Lee *et al*. [33], is in line with that also reported for the rabbit paralog CAP18, and sheep BMAP ortholog SMAP-29 [55]. These latter peptides have been proposed to bear LPS-binding sites, located both at the N- and C-terminal ends, that function cooperatively [55]. The lack of the equivalent to one of these sites in the (1-18) BMAP fragments could explain their somewhat lower LPS-binding capacity compared to the parental molecules. Nevertheless, the residual activity might still be relevant in an *in-vivo* setting, where LPS levels are expected to be significantly lower than those normally used in *in-vitro* assays [56].

One of the major drawbacks in the clinical development of AMPs are cytotoxic effects on host cells [8, 9], that offsets several beneficial activities on host cell functions, in addition to their direct microbicidal properties [8, 9]. In this respect the human cathelicidin LL-37, while exerting some toxic effects on osteoblasts [57], has been shown to promote bone regeneration *in vivo* by distinct mechanisms involving stimulation of other cell types [58-60]. In addition, LL-37 has been shown to inhibit osteoclastogenesis *in vitro* [61]. Moreover, the toxic effects of this peptide may be counteracted *in vivo* by endogenous factors [62, 63]. Another class of human AMPs, the beta-defensins, are reported to display stimulatory effects on osteoblast proliferation and differentiation [64]. In the present study, all candidate peptides proved safe to osteoblast cells in their MIC value

range, with BMAP fragments and P19 clearly displaying a wider selectivity index. This was observed both in standard media and in the presence of human serum and hyaluronic acid as relevant factors in view of an orthopaedic application. Our data confirm the improved selectivity of BMAP truncated analogs against prokaryotic *vs* mammalian cells with respect to the parent peptides [22, 33]. Moreover, these fragments as well as P19 did not affect osteoblast differentiation *per se*, nor impaired the effect of a recognized osteogenic stimulus [37], which bolsters their suitability for applications on orthopaedic devices.

Although in contrast with several reports on AMP activity being inhibited by anionic polysaccharides [65-68], in the present study the activity of B27(1-18), B28(1-18) and P19 against *S. epidermidis* and *S. aureus* was fully retained in the presence of HA at concentrations representative of those found in periprosthetic and normal synovial fluid [36, 38]. The potential for peptide efficacy in the skeletal joint environment was further supported by potent activity in the presence of a clinical synovial fluid sample, selected among a limited number of available samples due to its compatibility with *S. aureus* growth. Notably, expression of several AMPs was reported in both healthy and inflamed synovial membranes and joint fluid [69-71], and this may reasonably contribute to the inhibitory effects of joint fluid on microbial growth [72]. In an applicative perspective, the presence of endogenous AMPs could be advantageous since they may synergize with exogenously introduced AMPs.

Influence of blood components, *e.g.*, serum albumin and lipoproteins [41, 73, 74], on AMP activity represents a crucial issue to be addressed in view of potential therapeutic applications. In fact, both BMAP-27 and BMAP-28 have been shown in previous studies to retain antimicrobial properties in the presence of heat-inactivated bovine serum, although at higher peptide concentrations [14, 39]. In our growth kinetics assays, the AMPs were effective against both *Staphylococcus* species in serum coincubation experiments, at concentrations comparable or only slightly higher than those in serum-free medium (see Fig. 6b, e). Peptides showed a more evident decrease in potency upon pre-incubation with serum (Fig. 6c, f), but it was interesting to note that while a decrease in activity against *S. aureus* was observed for all the tested AMPs, B27(1-18) was the least affected in the case of *S. epidermidis*. The inhibitory effect of serum on peptide activity likely depends on peptide sequestration by serum components, as supported by overlapping activity of B27(1-18) against *S. epidermidis* in the presence of active and heat-inactivated HS, suggesting a minimal contribution of enzymatic degradation. In this respect, the different behaviour of B27(1-18) towards two *Staphylococcus* species was rather unexpected, since peptide binding to serum proteins would be expected to comparably affect peptide activity regardless of the target microorganism. It is widely accepted that membrane-active AMPs exert their microbicidal action through both

electrostatic and hydrophobic interactions with bacterial membrane surfaces [6, 7], while binding to serum seems preponderantly of a hydrophobic nature [74-76]. As B27(1-18) is the least hydrophobic and most cationic of the three shorter AMPs (see Table 1), hydrophobic interactions with serum factors may be relatively weaker and electrostatic attractions with the bacterial surface play a more dominant role. One could thus reasonably speculate that in the presence of a sufficiently anionic bacterial surface, peptide-bacteria interaction would be favoured even in the presence of serum.

According to published reports *S. epidermidis* has a more anionic surface than *S. aureus* [77, 78], which might explain the generally higher activity observed for the three short AMPs against *S. epidermidis* strains as compared to *S. aureus* (Table 2 and 3). Moreover, for the same reason, *S. epidermidis* could be less prone to shielding by serum, and this is particularly evident for the less hydrophobic and more cationic B27(1-18). The competition of serum and bacterial surface for this AMP is evidently finely balanced, as the less anionic *S. aureus* has a more serum-sensitive susceptibility to this peptide. A similar hypothesis has been proposed by Huang *et al.* [73], to explain the different susceptibilities of selected bacterial species to a cationic α-helical AMP in the presence of human serum albumin (HSA). Notably, in this latter study *S. epidermidis* was the only microorganism whose susceptibility to the peptide was not affected by HSA [73], again indicating competitive binding to AMPs of this structural class.

Due to its better performance under *in vivo*-mimicking conditions, B27(1-18) was the best candidate for further studies with immobilized peptide. Like the parent BMAP-27, reported to maintain activity on surface tethering [19], B27(1-18) was bactericidal to both *S. epidermidis* and *S. aureus* when bound to resin beads. The streptavidin/biotin-based anchoring method, that approaches the strength of a covalent bond [79], was selected as being a facile and straightforward procedure, suitable for obtaining a proof-of-concept demonstration of peptide efficacy on immobilization [80]. In our study, peptides were tethered via the N-terminus as this facilitated the biotinylation procedure, also given the reported efficacy of several N-terminally immobilized α-helical AMPs [11, 18, 20]. In our hands, immobilization of biotinylated peptides on streptavidin-functionalized resin beads led to a highly stable conjugate, as bactericidal activity from free peptide was not detected in the resin supernatants, even after a month of storage.

Comparison between full length surface-linked BMAP-27, surface-linked B27(1-18) and also a derivative of the latter with a diethylene glycole spacer [DEG-B27(1-18)] indicated similar activity of BMAP-27 and B27(1-18) on both tested strains, whereas DEG-B27(1-18) showed a somewhat lower efficacy. This however may be due to a lower peptide-loading on the resin, as assessed by peptide quantification upon elution from resin samples. For the DEG-modified peptide,

resin loading was assessed to be only 25% that of unmodified B27(1-18) (data not shown). In fact, a five-fold increase in DEG-B27(1-18) resin (from 5 to 25%) led to complete inhibition of *S. epidermidis* and an almost 80% reduction of *S. aureus* viability (data not shown). The reason for including a flexible spacer is that it could be relevant to the efficacy of surface-immobilized AMPs [11, 12], but in our system activity appeared to be retained also in its absence, in keeping with the findings of Rapsch *et al.* on directly-immobilized α-helical AMPs, including BMAP-27 [19]. In that study, immobilized BMAP-27 was significantly less cytotoxic than the soluble counterpart, and was proposed to act via bacterial membrane depolarization [19]. Notably, in our study also, none of the anchored peptides induced appreciable membrane damage to osteoblast cells. In view of a potential application of these peptides in the prevention of orthopaedic device-related infections, surface-immobilized full-length BMAP-27 and the synthetically more accessible B27(1-18) fragment appear to have equivalent antimicrobial efficacy and host-cell compatibility, so that issues related to chemical synthesis procedures and production costs would obviously play in favour of the shorter analog.

Conclusions

Collectively, the results of this study highlight the potential of the α-helical peptide B27(1-18) for the prevention of orthopaedic implant-related infections. It effectively killed bacterial species responsible for orthopaedic infections and prevented staphylococcal biofilm formation. Moreover it attenuated an LPS-induced inflammatory response and was devoid of adverse effects on osteoblast cells. Its antimicrobial efficacy was comparable to that of cefazolin, widely used in orthopaedic perioperative prophylaxis, and was retained in the presence of relevant biological fluids and components. The proof-of-concept demonstration of its efficacy upon immobilization, obtained with B27(1-18)-functionalized resin beads, encourages further efforts aimed at tethering this peptide onto supports relevant for orthopaedic medical devices, such as titanium or ceramic, in view of the development of peptide-based infection-resistant biomaterials for prostheses.

Acknowledgements

This study was carried out within Trans2Care (Transregional Network for Innovation and Technology Transfer to Improve Health Care) project financed by European Regional Development Fund – the Cross-Border Cooperation Italy-Slovenia Programme 2007–2013, with the financial support of departmental research funds (University of Udine). The authors are grateful to Dr. A. Arzese (Microbiology Unit, Udine University Hospital and Department of Medicine, University of Udine, Udine, Italy) and Dr. M. Kavcic (Institute of Public Health Koper, Koper, Slovenia) for providing clinical bacterial isolates, and to Prof. A. Causero (Clinic of Orthopaedics, Udine University Hospital and Department of Medicine, University of Udine, Udine, Italy) for synovial fluid samples.

References

[1] P. C. Matthews, A. R. Berendt, M. A. McNally, I. Byren, Diagnosis and management of prosthetic joint infection, *BMJ* **2009**, *338*, b1773.
[2] P. Sadoghi, M. Liebensteiner, M. Agreiter, A. Leithner, N. Bohler, G. Labek, Revision surgery after total joint arthroplasty: a complication-based analysis using worldwide arthroplasty registers, *J Arthroplasty* **2013**, *28*, 1329.
[3] A. J. Tande, R. Patel, Prosthetic joint infection, *Clin Microbiol Rev* **2014**, *27*, 302.
[4] W. J. Metsemakers, R. Kuehl, T. F. Moriarty, R. G. Richards, M. H. Verhofstad, O. Borens, S. Kates, M. Morgenstern, Infection after fracture fixation: Current surgical and microbiological concepts, *Injury* **2016**.
[5] ECDC. European Centre for Disease Prevention and Control. Surveillance of surgical site infections in Europe 2010–2011., Stockholm: ECDC; 2013.**2013**.
[6] R. E. Hancock, H. G. Sahl, Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, *Nat Biotechnol* **2006**, *24*, 1551.
[7] L. T. Nguyen, E. F. Haney, H. J. Vogel, The expanding scope of antimicrobial peptide structures and their modes of action, *Trends Biotechnol* **2011**, *29*, 464.
[8] S. C. Mansour, O. M. Pena, R. E. Hancock, Host defense peptides: front-line immunomodulators, *Trends Immunol* **2014**, *35*, 443.
[9] A. T. Yeung, S. L. Gellatly, R. E. Hancock, Multifunctional cationic host defence peptides and their clinical applications, *Cell Mol Life Sci* **2011**, *68*, 2161.
[10] G. Wang, B. Mishra, K. Lau, T. Lushnikova, R. Golla, X. Wang, Antimicrobial peptides in 2014, *Pharmaceuticals (Basel)* **2015**, *8*, 123.
[11] F. Costa, I. F. Carvalho, R. C. Montelaro, P. Gomes, M. C. Martins, Covalent immobilization of antimicrobial peptides (AMPs) onto biomaterial surfaces, *Acta Biomater* **2011**, *7*, 1431.
[12] S. A. Onaizi, S. S. Leong, Tethering antimicrobial peptides: current status and potential challenges, *Biotechnol Adv* **2011**, *29*, 67.
[13] I. Nagaoka, S. Hirota, S. Yomogida, A. Ohwada, M. Hirata, Synergistic actions of antibacterial neutrophil defensins and cathelicidins, *Inflamm Res* **2000**, *49*, 73.
[14] L. Tomasinsig, G. De Conti, B. Skerlavaj, R. Piccinini, M. Mazzilli, F. D'Este, A. Tossi, M. Zanetti, Broad-spectrum activity against bacterial mastitis pathogens and activation of mammary epithelial cells support a protective role of neutrophil cathelicidins in bovine mastitis, *Infect Immun* **2010**, *78*, 1781.
[15] Y. Rosenfeld, N. Papo, Y. Shai, Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action, *J Biol Chem* **2006**, *281*, 1636.
[16] Z. Y. Ong, N. Wiradharma, Y. Y. Yang, Strategies employed in the design and optimization of synthetic antimicrobial peptide amphiphiles with enhanced therapeutic potentials, *Adv Drug Deliv Rev* **2014**, *78*, 28.
[17] I. Zelezetsky, A. Tossi, Alpha-helical antimicrobial peptides--using a sequence template to guide structure-activity relationship studies, *Biochim Biophys Acta* **2006**, *1758*, 1436.
[18] M. Gabriel, K. Nazmi, E. C. Veerman, A. V. Nieuw Amerongen, A. Zentner, Preparation of LL-37-grafted titanium surfaces with bactericidal activity, *Bioconjug Chem* **2006**, *17*, 548.
[19] K. Rapsch, F. F. Bier, M. Tadros, M. von Nickisch-Rosenegk, Identification of antimicrobial peptides and immobilization strategy suitable for a covalent surface coating with biocompatible properties, *Bioconjug Chem* **2014**, *25*, 308.
[20] J. W. Soares, R. Kirby, L. A. Doherty, A. Meehan, S. Arcidiacono, Immobilization and orientation-dependent activity of a naturally occurring antimicrobial peptide, *J Pept Sci* **2015**, *21*, 669.
[21] M. Zanetti, Cathelicidins, multifunctional peptides of the innate immunity, *J Leukoc Biol* **2004**, *75*, 39.
[22] B. Skerlavaj, R. Gennaro, L. Bagella, L. Merluzzi, A. Risso, M. Zanetti, Biological characterization of two novel cathelicidin-derived peptides and identification of structural requirements for their antimicrobial and cell lytic activities, *J Biol Chem* **1996**, *271*, 28375.
[23] I. Zelezetsky, U. Pag, H. G. Sahl, A. Tossi, Tuning the biological properties of amphipathic alpha-helical antimicrobial peptides: rational use of minimal amino acid substitutions, *Peptides* **2005**, *26*, 2368.
[24] A. Pompilio, M. Scocchi, S. Pomponio, F. Guida, A. Di Primio, E. Fiscarelli, R. Gennaro, G. Di Bonaventura, Antibacterial and anti-biofilm effects of cathelicidin peptides against pathogens isolated from cystic fibrosis patients, *Peptides* **2011**, *32*, 1807.
[25] M. Benincasa, B. Skerlavaj, R. Gennaro, A. Pellegrini, M. Zanetti, In vitro and in vivo antimicrobial activity of two alpha-helical cathelicidin peptides and of their synthetic analogs, *Peptides* **2003**, *24*, 1723.

[26] D. W. Bratzler, E. P. Dellinger, K. M. Olsen, T. M. Perl, P. G. Auwaerter, M. K. Bolon, D. N. Fish, L. M. Napolitano, R. G. Sawyer, D. Slain, J. P. Steinberg, R. A. Weinstein, Clinical practice guidelines for antimicrobial prophylaxis in surgery, *Am J Health Syst Pharm* **2013**, *70*, 195.
[27] D. R. Osmon, E. F. Berbari, A. R. Berendt, D. Lew, W. Zimmerli, J. M. Steckelberg, N. Rao, A. Hanssen, W. R. Wilson, Diagnosis and management of prosthetic joint infection: clinical practice guidelines by the Infectious Diseases Society of America, *Clin Infect Dis* **2013**, *56*, e1.
[28] L. A. Bonsignore, J. R. Anderson, Z. Lee, V. M. Goldberg, E. M. Greenfield, Adherent lipopolysaccharide inhibits the osseointegration of orthopedic implants by impairing osteoblast differentiation, *Bone* **2013**, *52*, 93.
[29] L. A. Bonsignore, R. W. Colbrunn, J. M. Tatro, P. J. Messerschmitt, C. J. Hernandez, V. M. Goldberg, M. C. Stewart, E. M. Greenfield, Surface contaminants inhibit osseointegration in a novel murine model, *Bone* **2011**, *49*, 923.
[30] R. Lieder, P. H. Petersen, O. E. Sigurjonsson, Endotoxins-the invisible companion in biomaterials research, *Tissue Eng Part B Rev* **2013**, *19*, 391.
[31] F. D'Este, L. Tomasinsig, B. Skerlavaj, M. Zanetti, Modulation of cytokine gene expression by cathelicidin BMAP-28 in LPS-stimulated and -unstimulated macrophages, *Immunobiology* **2012**, *217*, 962.
[32] R. Ghiselli, O. Cirioni, A. Giacometti, F. Mocchegiani, F. Orlando, C. Bergnach, B. Skerlavaj, C. Silvestri, A. D. Vittoria, M. Zanetti, M. Rocchi, G. Scalise, V. Saba, Effects of the antimicrobial peptide BMAP-27 in a mouse model of obstructive jaundice stimulated by lipopolysaccharide, *Peptides* **2006**, *27*, 2592.
[33] E. K. Lee, Y. C. Kim, Y. H. Nan, S. Y. Shin, Cell selectivity, mechanism of action and LPS-neutralizing activity of bovine myeloid antimicrobial peptide-18 (BMAP-18) and its analogs, *Peptides* **2011**, *32*, 1123.
[34] X. Luo, J. Chen, W. X. Song, N. Tang, J. Luo, Z. L. Deng, K. A. Sharff, G. He, Y. Bi, B. C. He, E. Bennett, J. Huang, Q. Kang, W. Jiang, Y. Su, G. H. Zhu, H. Yin, Y. He, Y. Wang, J. S. Souris, L. Chen, G. W. Zuo, A. G. Montag, R. R. Reid, R. C. Haydon, H. H. Luu, T. C. He, Osteogenic BMPs promote tumor growth of human osteosarcomas that harbor differentiation defects, *Lab Invest* **2008**, *88*, 1264.
[35] C. Pautke, M. Schieker, T. Tischer, A. Kolk, P. Neth, W. Mutschler, S. Milz, Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts, *Anticancer Res* **2004**, *24*, 3743.
[36] L. E. Guenther, B. W. Pyle, T. R. Turgeon, E. R. Bohm, U. P. Wyss, T. A. Schmidt, J. M. Brandt, Biochemical analyses of human osteoarthritic and periprosthetic synovial fluid, *Proc Inst Mech Eng H* **2014**, *228*, 127.
[37] Y. Xiong, H. J. Yang, J. Feng, Z. L. Shi, L. D. Wu, Effects of alendronate on the proliferation and osteogenic differentiation of MG-63 cells, *J Int Med Res* **2009**, *37*, 407.
[38] D. Tercic, B. Bozic, The basis of the synovial fluid analysis, *Clin Chem Lab Med* **2001**, *39*, 1221.
[39] A. Ahmad, S. Azmi, R. M. Srivastava, S. Srivastava, B. K. Pandey, R. Saxena, V. K. Bajpai, J. K. Ghosh, Design of nontoxic analogues of cathelicidin-derived bovine antimicrobial peptide BMAP-27: the role of leucine as well as phenylalanine zipper sequences in determining its toxicity, *Biochemistry* **2009**, *48*, 10905.
[40] J. Johansson, G. H. Gudmundsson, M. E. Rottenberg, K. D. Berndt, B. Agerberth, Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37, *J Biol Chem* **1998**, *273*, 3718.
[41] Y. Wang, B. Agerberth, A. Lothgren, A. Almstedt, J. Johansson, Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37, *J Biol Chem* **1998**, *273*, 33115.
[42] D. Campoccia, L. Montanaro, C. R. Arciola, A review of the biomaterials technologies for infection-resistant surfaces, *Biomaterials* **2013**, *34*, 8533.
[43] J. J. Swartjes, P. K. Sharma, T. G. van Kooten, H. C. van der Mei, M. Mahmoudi, H. J. Busscher, E. T. Rochford, Current Developments in Antimicrobial Surface Coatings for Biomedical Applications, *Curr Med Chem* **2015**, *22*, 2116.
[44] S. Blodkamp, K. Kadlec, T. Gutsmann, H. Y. Naim, M. von Kockritz-Blickwede, S. Schwarz, In vitro activity of human and animal cathelicidins against livestock-associated methicillin-resistant Staphylococcus aureus, *Vet Microbiol* **2016**, *194*, 107.
[45] M. Mardirossian, A. Pompilio, V. Crocetta, S. De Nicola, F. Guida, M. Degasperi, R. Gennaro, G. Di Bonaventura, M. Scocchi, In vitro and in vivo evaluation of BMAP-derived peptides for the treatment of cystic fibrosis-related pulmonary infections, *Amino Acids* **2016**, *48*, 2253.

[46] A. Pompilio, V. Crocetta, M. Scocchi, S. Pomponio, V. Di Vincenzo, M. Mardirossian, G. Gherardi, E. Fiscarelli, G. Dicuonzo, R. Gennaro, G. Di Bonaventura, Potential novel therapeutic strategies in cystic fibrosis: antimicrobial and anti-biofilm activity of natural and designed alpha-helical peptides against Staphylococcus aureus, Pseudomonas aeruginosa, and Stenotrophomonas maltophilia, *BMC Microbiol* **2012**, *12*, 145.
[47] A. Ahmad, N. Asthana, S. Azmi, R. M. Srivastava, B. K. Pandey, V. Yadav, J. K. Ghosh, Structure-function study of cathelicidin-derived bovine antimicrobial peptide BMAP-28: design of its cell-selective analogs by amino acid substitutions in the heptad repeat sequences, *Biochim Biophys Acta* **2009**, *1788*, 2411.
[48] S. Takagi, J. Nishimura, L. Bai, H. Miyagi, K. Kuroda, S. Hayashi, H. Yoneyama, T. Ando, H. Isogai, E. Isogai, Susceptibility difference between methicillin-susceptible and methicillin-resistant Staphylococcus aureus to a bovine myeloid antimicrobial peptide (BMAP-28), *Anim Sci J* **2014**, *85*, 174.
[49] U. Pag, M. Oedenkoven, V. Sass, Y. Shai, O. Shamova, N. Antcheva, A. Tossi, H. G. Sahl, Analysis of in vitro activities and modes of action of synthetic antimicrobial peptides derived from an alpha-helical 'sequence template', *J Antimicrob Chemother* **2008**, *61*, 341.
[50] S. Takagi, S. Hayashi, K. Takahashi, H. Isogai, L. Bai, H. Yoneyama, T. Ando, K. Ito, E. Isogai, Antimicrobial activity of a bovine myeloid antimicrobial peptide (BMAP-28) against methicillin-susceptible and methicillin-resistant Staphylococcus aureus, *Anim Sci J* **2012**, *83*, 482.
[51] K. L. Leach, S. J. Brickner, M. C. Noe, P. F. Miller, Linezolid, the first oxazolidinone antibacterial agent, *Ann N Y Acad Sci* **2011**, *1222*, 49.
[52] L. Zaffiri, J. Gardner, L. H. Toledo-Pereyra, History of antibiotics. From salvarsan to cephalosporins, *J Invest Surg* **2012**, *25*, 67.
[53] E. M. Greenfield, M. A. Beidelschies, J. M. Tatro, V. M. Goldberg, A. G. Hise, Bacterial pathogen-associated molecular patterns stimulate biological activity of orthopaedic wear particles by activating cognate Toll-like receptors, *J Biol Chem* **2010**, *285*, 32378.
[54] K. Horibe, Y. Nakamichi, S. Uehara, M. Nakamura, M. Koide, Y. Kobayashi, N. Takahashi, N. Udagawa, Roles of cathelicidin-related antimicrobial peptide in murine osteoclastogenesis, *Immunology* **2013**, *140*, 344.
[55] B. F. Tack, M. V. Sawai, W. R. Kearney, A. D. Robertson, M. A. Sherman, W. Wang, T. Hong, L. M. Boo, H. Wu, A. J. Waring, R. I. Lehrer, SMAP-29 has two LPS-binding sites and a central hinge, *Eur J Biochem* **2002**, *269*, 1181.
[56] S. M. Opal, P. J. Scannon, J. L. Vincent, M. White, S. F. Carroll, J. E. Palardy, N. A. Parejo, J. P. Pribble, J. H. Lemke, Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock, *J Infect Dis* **1999**, *180*, 1584.
[57] J. Sall, M. Carlsson, O. Gidlof, A. Holm, J. Humlen, J. Ohman, D. Svensson, B. O. Nilsson, D. Jonsson, The antimicrobial peptide LL-37 alters human osteoblast Ca2+ handling and induces Ca2+-independent apoptosis, *J Innate Immun* **2013**, *5*, 290.
[58] M. Kittaka, H. Shiba, M. Kajiya, T. Fujita, T. Iwata, K. Rathvisal, K. Ouhara, K. Takeda, H. Komatsuzawa, H. Kurihara, The antimicrobial peptide LL37 promotes bone regeneration in a rat calvarial bone defect, *Peptides* **2013**, *46*, 136.
[59] Z. Zhang, J. E. Shively, Generation of novel bone forming cells (monoosteophils) from the cathelicidin-derived peptide LL-37 treated monocytes, *PLoS One* **2010**, *5*, e13985.
[60] Z. Zhang, J. E. Shively, Acceleration of bone repair in NOD/SCID mice by human monoosteophils, novel LL-37-activated monocytes, *PLoS One* **2013**, *8*, e67649.
[61] C. Supanchart, S. Thawanaphong, A. Makeudom, J. G. Bolscher, K. Nazmi, U. Kornak, S. Krisanaprakornkit, The antimicrobial peptide, LL-37, inhibits in vitro osteoclastogenesis, *J Dent Res* **2012**, *91*, 1071.
[62] D. Svensson, J. Westman, C. Wickstrom, D. Jonsson, H. Herwald, B. O. Nilsson, Human endogenous peptide p33 inhibits detrimental effects of LL-37 on osteoblast viability, *J Periodontal Res* **2015**, *50*, 80.
[63] D. Svensson, L. Wilk, M. Morgelin, H. Herwald, B. O. Nilsson, LL-37-induced host cell cytotoxicity depends on cellular expression of the globular C1q receptor (p33), *Biochem J* **2016**, *473*, 87.
[64] D. Kraus, J. Deschner, A. Jager, M. Wenghoefer, S. Bayer, S. Jepsen, J. P. Allam, N. Novak, R. Meyer, J. Winter, Human beta-defensins differently affect proliferation, differentiation, and mineralization of osteoblast-like MG63 cells, *J Cell Physiol* **2012**, *227*, 994.

[65] M. Benincasa, M. Mattiuzzo, Y. Herasimenka, P. Cescutti, R. Rizzo, R. Gennaro, Activity of antimicrobial peptides in the presence of polysaccharides produced by pulmonary pathogens, *J Pept Sci* **2009**, *15*, 595.
[66] Y. Herasimenka, M. Benincasa, M. Mattiuzzo, P. Cescutti, R. Gennaro, R. Rizzo, Interaction of antimicrobial peptides with bacterial polysaccharides from lung pathogens, *Peptides* **2005**, *26*, 1127.
[67] A. Nelson, I. Berkestedt, A. Schmidtchen, L. Ljunggren, M. Bodelsson, Increased levels of glycosaminoglycans during septic shock: relation to mortality and the antibacterial actions of plasma, *Shock* **2008**, *30*, 623.
[68] M. Toppazzini, A. Coslovi, M. Boschelle, E. Marsich, M. Benincasa, R. Gennaro, S. Paoletti, Can the interaction between the antimicrobial peptide LL-37 and alginate be exploited for the formulation of new biomaterials with antimicrobial properties?, *Carbohyd Polym* **2011**, *83*, 578.
[69] H. Gollwitzer, Y. Dombrowski, P. M. Prodinger, M. Peric, B. Summer, A. Hapfelmeier, B. Saldamli, F. Pankow, R. von Eisenhart-Rothe, A. B. Imhoff, J. Schauber, P. Thomas, R. Burgkart, I. J. Banke, Antimicrobial peptides and proinflammatory cytokines in periprosthetic joint infection, *J Bone Joint Surg Am* **2013**, *95*, 644.
[70] F. Paulsen, T. Pufe, L. Conradi, D. Varoga, M. Tsokos, J. Papendieck, W. Petersen, Antimicrobial peptides are expressed and produced in healthy and inflamed human synovial membranes, *J Pathol* **2002**, *198*, 369.
[71] D. Varoga, T. Pufe, R. Mentlein, S. Kohrs, S. Grohmann, B. Tillmann, J. Hassenpflug, F. Paulsen, Expression and regulation of antimicrobial peptides in articular joints, *Ann Anat* **2005**, *187*, 499.
[72] B. F. Gruber, B. S. Miller, J. Onnen, R. D. Welling, E. M. Wojtys, Antibacterial properties of synovial fluid in the knee, *J Knee Surg* **2008**, *21*, 180.
[73] J. Huang, D. Hao, Y. Chen, Y. Xu, J. Tan, Y. Huang, F. Li, Inhibitory effects and mechanisms of physiological conditions on the activity of enantiomeric forms of an alpha-helical antibacterial peptide against bacteria, *Peptides* **2011**, *32*, 1488.
[74] K. A. Peck-Miller, R. P. Darveau, H. P. Fell, Identification of serum components that inhibit the tumoricidal activity of amphiphilic alpha helical peptides, *Cancer Chemother Pharmacol* **1993**, *32*, 109.
[75] C. D. Ciornei, T. Sigurdardottir, A. Schmidtchen, M. Bodelsson, Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37, *Antimicrobial agents and chemotherapy* **2005**, *49*, 2845.
[76] O. Sorensen, T. Bratt, A. H. Johnsen, M. T. Madsen, N. Borregaard, The human antibacterial cathelicidin, hCAP-18, is bound to lipoproteins in plasma, *J Biol Chem* **1999**, *274*, 22445.
[77] J. S. Dickson, M. Koohmaraie, Cell-Surface Charge Characteristics and Their Relationship to Bacterial Attachment to Meat Surfaces, *Appl Environ Microb* **1989**, *55*, 832.
[78] L. A. Rawlinson, J. P. O'Gara, D. S. Jones, D. J. Brayden, Resistance of Staphylococcus aureus to the cationic antimicrobial agent poly(2-(dimethylamino ethyl)methacrylate) (pDMAEMA) is influenced by cell-surface charge and hydrophobicity, *J Med Microbiol* **2011**, *60*, 968.
[79] C. M. Dundas, D. Demonte, S. Park, Streptavidin-biotin technology: improvements and innovations in chemical and biological applications, *Appl Microbiol Biotechnol* **2013**, *97*, 9343.
[80] K. Hilpert, M. Elliott, H. Jenssen, J. Kindrachuk, C. D. Fjell, J. Korner, D. F. Winkler, L. L. Weaver, P. Henklein, A. S. Ulrich, S. H. Chiang, S. W. Farmer, N. Pante, R. Volkmer, R. E. Hancock, Screening and characterization of surface-tethered cationic peptides for antimicrobial activity, *Chem Biol* **2009**, *16*, 58.

Table 1. Peptide sequences and molecular characteristics.

Peptide	Sequence[a]	MW	Length	q[b]	%H[c]
BMAP-27	GRFKRFRKKFKKLFKKLSPVIPLLHL	3225	26	+12	42
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRI	3074	27	+8	48
BMAP-27(1-18)	GRFKRFRKKFKKLFKKLS	2342	18	+11	33
BMAP-28(1-18)	GGLRSLGRKILRAWKKYG	2058	18	+7	39
P19(9/G7)	GLLKKIGKKAKKALKKLGY	2085	19	+9	42

[a] The C-terminus of all peptides is amidated

[b] q, net charge

[c] %H, percent hydrophobic residues (sum of A, F, I, L, Y and W residues divided by number of residues)

Table 2. Antimicrobial activities against reference strains.

Organism	BMAP-27	BMAP-28	B27(1-18)	B28(1-18)	P19
	MIC (MBC) (μM)[a,b]				
S. epidermidis ATCC 12228	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
S. epidermidis ATCC 35984	2 (2)	2 (2)	2 (2)	2 (2)	4 (4)
S. aureus ATCC 25923	2 (4)	2 (2)	4 (8)	2 (4)	16 (32)
E. coli ATCC 25922	1 (1)	2 (2)	2 (2)	1 (1)	2 (2)
P. aeruginosa ATCC 27853	2 (4)	4 (16)	2 (4)	2 (4)	2 (2)

[a] Determined in MH broth

[b] Data are means of at least three independent experiments

Table 3. Antimicrobial activities against bacterial isolates obtained from clinical cases of orthopaedic infections.

Organism (no. of isolates)	BMAP-27	BMAP-28	B27(1-18)	B28(1-18)	P19	cefazolin	linezolid
	MIC range (μM)[a,b]						
S. epidermidis (8)	0.5-2 (1[c])	1-2 (2[c])	0.5-4 (1[c])	1-2 (1[c])	0.5-32 (1[c])	0.5-16 (>16[c])	<0.4->95 (3[c])
S. aureus (8)	2-4	1-4	2-16	2-8	16->64	0.5-2	6
other *Staphylococcus* spp. (3)	0.5-1	1-2	0.5-1	0.5-1	0.5-1	0.5-4	3
E. faecalis (2)	8-32	4	>64	64	>128	>16	6
Streptococcus spp. (3)	1-2	0.5-4	1-2	1-2	0.5-2	0.25	1.5-3
	MBC range (μM)[a,b]						
S. epidermidis (8)	1-2 (1[c])	1-2 (2[c])	0.5-4 (1[c])	0.5-4 (1[c])	0.5-64 (0.5[c])	0.5-16 (>16[c])	n.d.
S. 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->16	n.d.
E. faecalis (2)	8->32	4	>64	>64	>128	>16	n.d.
Streptococcus spp. (3)	1-2	2-4	1-2	1-2	0.5-2	1	n.d.
	MIC_{50} (MIC_{90}) (μM)[a,b,d]						
S. epidermidis (8)	1 (1)	2 (2)	1 (1)	1 (1)	1 (2)	2 (8)	3 (6)
S. aureus (8)	2 (2)	2 (2)	4 (8)	4 (4)	16 (32)	1 (2)	6 (6)

[a] Determined in MH broth

[b] Data are means of at least three independent experiments

[c] Methicillin-resistant strain

[d] MIC_{50} and MIC_{90}: concentrations that inhibited 50% and 90%, respectively, of the strains

n.d., not determinable

Table 4. Effects of immobilized peptides on cell membrane integrity in osteoblast cells.

treatment[a]	% LDH release[b]
control	3.6 ± 0.8
resin-biotin	1.7 ± 2.1
resin-B27	4.9 ± 1.9
resin-B27(1-18)	3.0 ± 0.3
resin-DEG-B27(1-18)	2.7 ± 0.3

[a] 1 h exposure of MG-63 cells to 5% resins in complete medium

[b] Data were calculated as percentage of total cellular LDH activity, and are the means ± SD of three separate experiments

Figure captions

Figure 1. Inhibition of *S. epidermidis* biofilm formation. *S. epidermidis* ATCC 35984 (1×10^6 CFU/mL in MH broth) was seeded into flat-bottom 96-well polystyrene microtiter plates in the absence and presence of the indicated concentrations of peptides (a) or antibiotics (b). Adherent bacterial cells were quantified after 24 h incubation by the Prestoblue metabolic assay. Results are expressed as percent viability compared to untreated bacteria, and are the means ± SD of three independent experiments performed at least in triplicate.

Figure 2. Lipopolysaccharide (LPS) neutralization. RAW 264.7 cells were stimulated with 100 ng/mL *E. coli* O111:B4 LPS in complete RPMI, in the absence and presence of peptides at the indicated concentrations. Nitrite values were determined in the culture supernatants after 24 h incubation using the Griess reagent. Data were calculated as percent of nitrite produced in response to LPS in the absence of peptides, and are the means ± SD of three independent experiments.

Figure 3. Effects on osteoblast viability and differentiation. a, c, d) MG-63 osteoblast cells were incubated in the absence and presence of the indicated peptide concentrations under the following medium conditions: a) DMEM supplemented with 10% FBS (complete medium); c) DMEM supplemented with 10% human serum (HS); d) HBSS supplemented with 10% HS and 0.5 mg/mL hyaluronic acid (HA). LDH activity was measured in the culture supernatants after 1 h incubation and expressed as percentage of total cellular LDH activity. b) Cells were incubated with peptides for 24 h in complete medium. Cell viability was evaluated by the Prestoblue metabolic assay and is reported as percentage of untreated cells. e, f) Cells were incubated in osteogenic medium (OM) in the absence and presence of 2 μM peptides or 100 nM dexamethasone (DXM) (e), or a combination of peptides and DXM (f). Alkaline phosphatase (ALP) activity was measured in cell lysates after 7 days of incubation, and expressed as fold-increase with respect to basal ALP activity (e) or as percent of ALP activity induced by DXM alone (f). Data are the means ± SD of three independent experiments.

Figure 4. Peptide effects on bacterial growth in the presence of hyaluronic acid (HA). *S. epidermidis* ATCC 35984 (a-c) and *S. aureus* ATCC 25923 (d-f) (1×10^7 CFU/mL in 50% MH broth) were incubated with the indicated concentrations of B27(1-18) (a, d), B28(1-18) (b, e) or P19 (c, f) in the absence (circles, solid lines) and presence of 0.5 mg/mL (squares, dashed lines) and 3 mg/mL (triangles, dotted lines) HA. Bacterial growth was monitored for 6 h by optical density (OD)

at 600 nm. Data were calculated as percentage of growth inhibition at the 6 h time point with respect to bacteria incubated in corresponding media in the absence of peptides. The means ± SD of three independent experiments are reported.

Figure 5. Effects on *S. aureus* growth in the presence of synovial fluid (SF). *S. aureus* ATCC 25923 was incubated at 1×10^7 CFU/mL in 50% MH broth in the absence (closed symbols) and presence (open symbols) of 20% synovial fluid and of 2 μM of the indicated peptides. OD readings at 600 nm (OD_{600}) at 30 min intervals are reported.

Figure 6. Effects on bacterial growth in the presence of human serum (HS). *S. epidermidis* ATCC 35984 (a-c) and *S. aureus* ATCC 25923 (d-f) (1×10^7 CFU/mL) were grown in 50% MH broth in the absence (a, d) and presence (b, c, e, f) of 25% human serum (HS) and of peptides at the indicated concentrations. Bacteria were added immediately after the preparation of peptide dilutions in PBS (a, d) or HS-containing PBS (b, e) or, alternatively, were dispensed to the wells after 3 h pre-incubation of the peptides at 37°C in the presence of HS (c, f). Bacterial growth was monitored for 6 h by OD_{600} determinations at 30 min intervals. Data were calculated as percentage of growth inhibition at 6 h with respect to corresponding controls incubated in the absence of peptides, and are the means ± SD of three independent experiments.

Figure 7. Antimicrobial efficacy of biotinylated peptides immobilized on Streptavidin-Sepharose resin. Streptavidin-Sepharose resin beads functionalized with biotinylated derivatives of BMAP-27 and B27(1-18) as described in the method section were assayed for antimicrobial efficacy either immediately after completion of the coupling procedure (a-d) or upon 1 month storage at 4°C (e, f). *S. epidermidis* ATCC 35984 (a, b, e, f) and *S. aureus* ATCC 25923 (c, d) (1×10^7 CFU/mL in 50% MH broth) were incubated for 1 h with 5% of the indicated resins (closed bars) or their respective supernatants (open bars) under gentle agitation. a, c, e) Bacterial viability was determined by the Prestoblue metabolic assay and expressed as percent relative to bacteria incubated with the control resin functionalized with biotin only, or the corresponding supernatant. b, d, f) Bacterial killing in resin-treated samples was determined based on colony counts, and expressed relative to the control resin. The means ± SD of three independent experiments are reported.

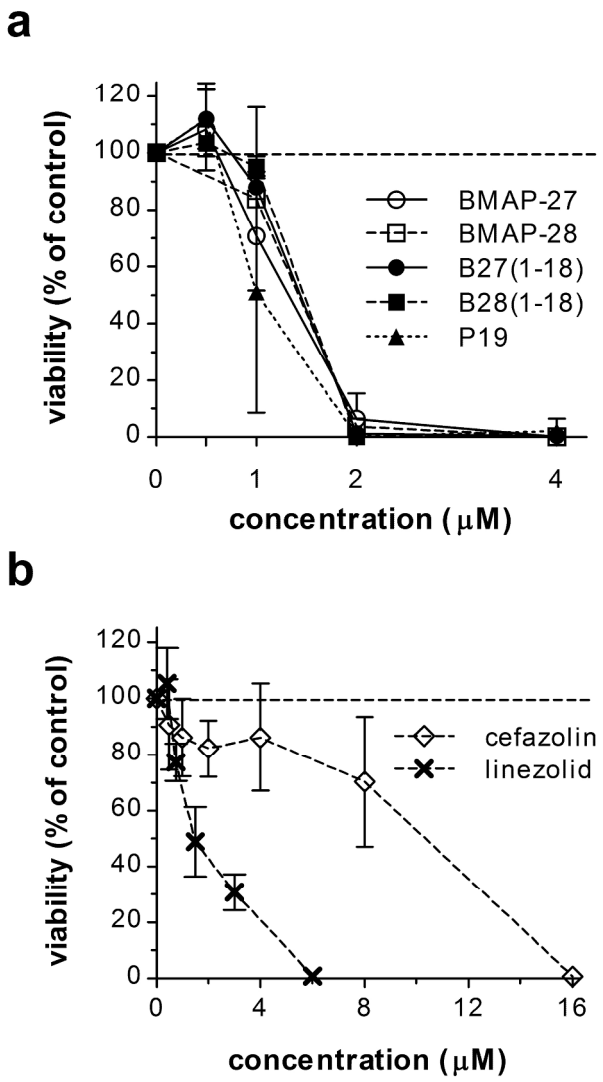

Figure 1. Inhibition of *S. epidermidis* biofilm formation.

108x169mm (600 x 600 DPI)

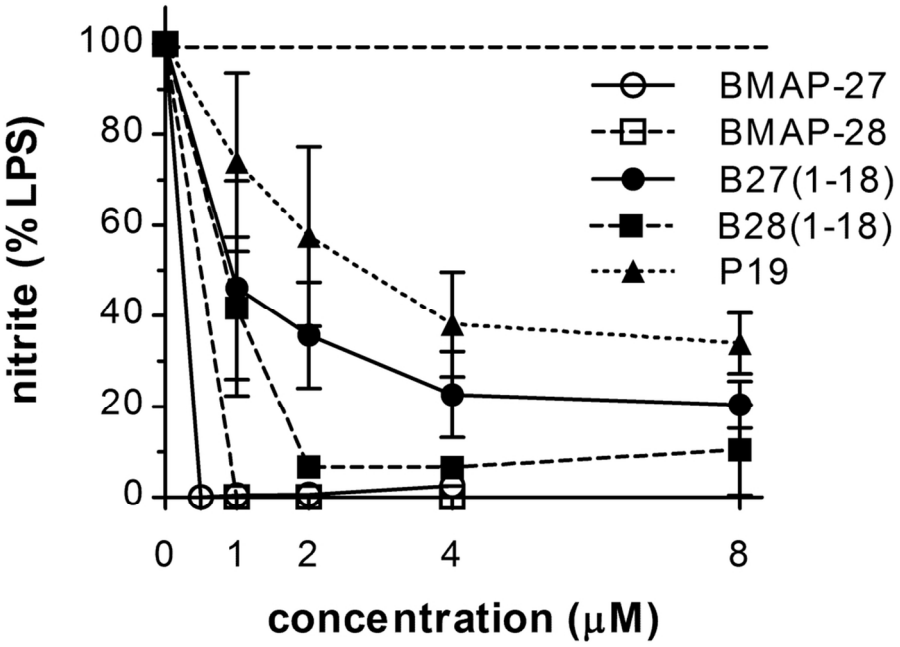

Figure 2. Lipopolysaccharide (LPS) neutralization.

52x40mm (600 x 600 DPI)

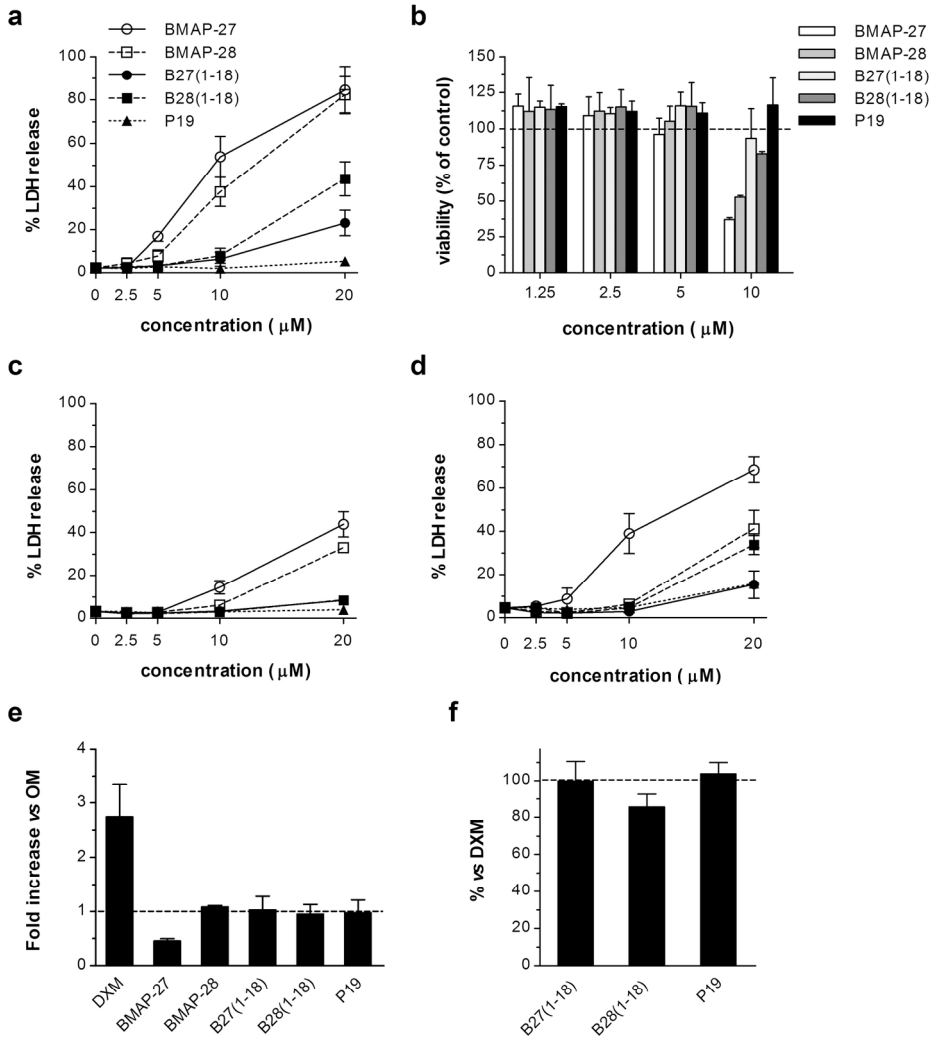

Figure 3. Effects on osteoblast viability and differentiation.

170x190mm (300 x 300 DPI)

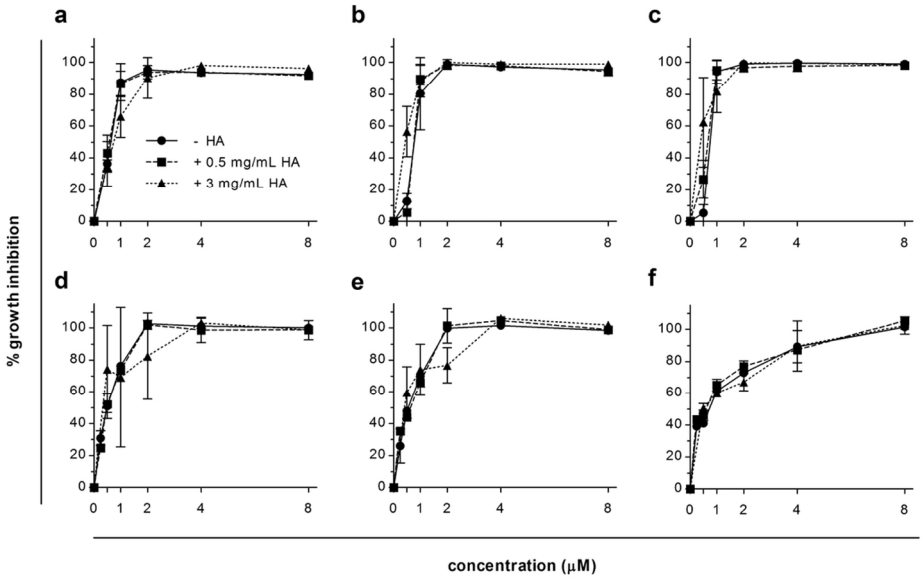

Figure 4. Peptide effects on bacterial growth in the presence of hyaluronic acid (HA).

109x67mm (300 x 300 DPI)

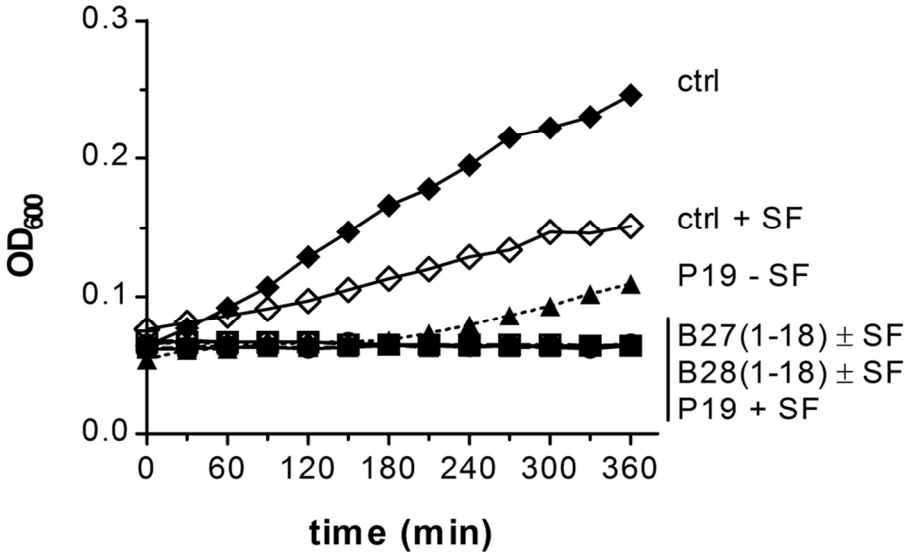

Figure 5. Effects on *S. aureus* growth in the presence of synovial fluid (SF).

51x33mm (600 x 600 DPI)

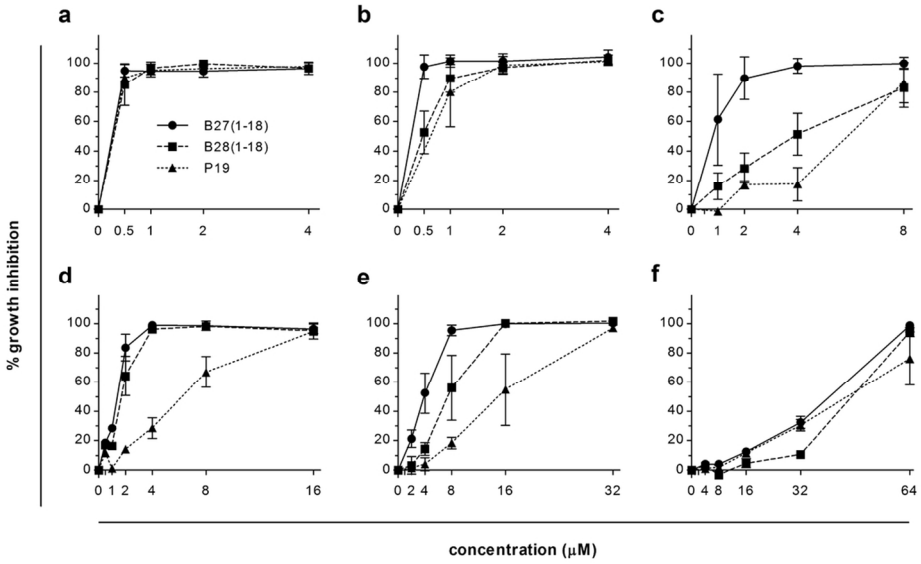

Figure 6. Effects on bacterial growth in the presence of human serum (HS).

107x64mm (300 x 300 DPI)

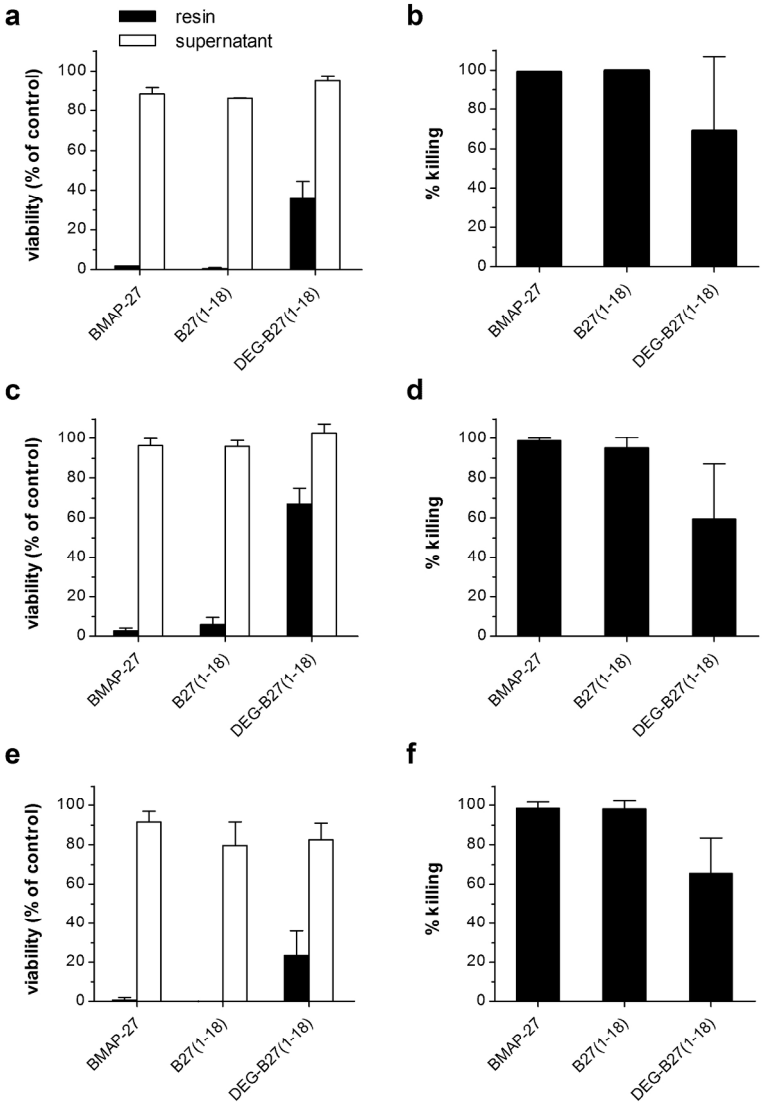

Figure 7. Antimicrobial efficacy of biotinylated peptides immobilized on Streptavidin-Sepharose resin.

176x256mm (300 x 300 DPI)