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Keywords:	cyclolipopeptide, Staphylococcus, polymyxin

NOVEL SYNTHETIC POLYMYXINS KILL GRAM-POSITIVE BACTERIA

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Running title: Synthetic Polymyxins Kill Gram-Positives

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Introduction

Among the world's most dangerous superbugs are multidrug resistant (MDR) bacteria of the so-called ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) group[1,2]. The proportion of these bacteria in common urinary, pulmonary, skin and bloodstream infections has increased recently notably in recent years. Moreover, patients with vascular prostheses, indwelling catheters, dialysis shunts, etc., are particularly vulnerable to the virtually untreatable infections resulting from ESKAPE colonization and subsequent biofilm formation.

S. aureus expresses toxins, enzymes, adhesins and several surface proteins which together promote the survival of this bacterium on tissues and other substrates and protect it from elimination by the immune system. Previously, *S. aureus* infections were treated with penicillin, but the The indiscriminate use of this antibiotic penicillin has increased the percentage of *S. aureus* penicillin-resistant strains to nearly 100% in some countries[3]. The same is true for several other antibiotics.

Overexpression of penicillin-binding protein 2A (PBP2a), the most abundant PBP in MRSA strains with low affinity for β-lactams, enables these bacteria to survive in the presence of high concentrations of β-lactams, including methicillin[4]. Vancomycin, the antimicrobial usually chosen for the treatment of MRSA infections, is becoming increasingly ineffective, as Isolates with intermediate or full resistance to vancomycin emerge[5]. Instead, antimicrobial peptides (AMPs) such as daptomycin, an anionic lipopeptide consisting of a peptide core and a lipid tail, have become very important drugs in the treatment of gram-positive infections. Nonetheless, strains resistant to AMPs have been recently identified.

Amphipathic polymyxins are cationic non-ribosomal antimicrobial polypeptides (CAMPs) produced by *Paenibacillus polymyxa* and available as antimicrobials since 1949. Their use was were abandoned because of their nephrotoxicity and neurotoxicity[6]. However, colistin and

polymyxin B have recently been rescued to treat infections caused by MDR gram-negatives, mainly *Pseudomonas aeruginosa* and *Acinetobacter baumanii*[7]. While polymyxins interact with the negatively charged outer membrane of gram-negative bacteria[8], their mechanisms of action may additionally include alterations in ribosome binding, respiration, cell division, cell structure and the production of reactive oxygen species[9]. Gram-positives ~~bacteria, by contrast,~~ lack ~~an~~ outer membrane and are thus naturally resistant to polymyxins.

~~A theoretically unlimited number of molecules with antimicrobial activity can be synthesized and/or modified in the laboratory; but the~~ The synthesis of new molecules less toxic and with greater activity than conventional antibiotics is challenging. One strategy is to make small changes in known molecules, such as polymyxin, to generate derivatives effective against gram-positive bacteria. ~~The~~ Our aim ~~of this study~~ was to explore the antimicrobial activity of two novel synthetic cyclolipopeptide analogues of polymyxin (CAMP113 and CAMP207) against *S. aureus*[10] and other gram-positive bacteria

Material and Methods

Bacterial strains and growth conditions. ~~The Clinical *S.taphylococcus aureus*~~ Strains ~~isolated at Laboratori de Referencia de Catalunya (Prat de Llobregat, Barcelona, Spain) and used in this study~~ are reported as supplementary data. ~~*S. aureus* ATCC 29213 and ATCC MRSA 700698 served as a control strains in susceptibility tests~~. Muller-Hinton (MH, Becton, Dickinson and Company, ~~San Agustin de Guadalix,~~ Madrid, Spain) ~~was used~~ to determine minimum inhibitory concentrations (MICs) and minimal biofilm eradication concentrations (MBECs), tryptic soy agar (TSA) ~~was used~~ for ~~the determination of~~ colony countings ~~in time kill assays.~~ , tryptic soy broth (TSB) with 0.25% glucose for biofilms ~~were obtained in tryptic soy broth supplemented with 0.25% glucose (TSB-G). All bacteria were routinely cultured in~~ and TSB ~~(all media~~ were from Scharlau Microbiology, Sentmenat, Spain~~)~~. Colistin sulfate was supplied by Zhejiang Shenghua Biok Biology Co., Ltd., (Shanghai, China). ~~Vancomycin, linezolid,~~

~~erythromycin, tetracycline and ciprofloxacin~~ other antibiotics were ~~obtained~~ from Sigma-Aldrich (Madrid, Spain). The cyclopolypeptides CAMP207 and CAMP113 were synthesized in our laboratory as described ~~below~~.

Peptide synthesis and purification. Peptides CAMP113 and CAMP207 were synthesized manually following standard Fmoc/tBu [ref] ~~procedures and using DIPCDI/HOBt activation on a Rink amide resin. After the sequence was assembled, the peptides were cleaved from the resin by acidolysis for 90 min using trifluoroacetic acid (TFA)/triisopropylsilane/water (95:3:2, v/v). TFA was removed with a stream of nitrogen gas. The oily residue was treated with dry diethyl ether, and the precipitated peptide was isolated by centrifugation. The homogeneity of the crude peptide was assessed by analytical HPLC on Nucleosil C18 reverse phase columns (4 mm × 250 mm, 5 μm particle diameter, 120Å pore size). Elution was carried out at a flow rate of 1 mL min^{-1} in solutions of 0.045% TFA and acetonitrile containing 0.036% TFA, with UV detection at 220 nm. The peptides were cyclized in an aqueous solution of 5% dimethylsulfoxide for 24 h and lyophilized twice. The peptide was subsequently purified by preparative HPLC on a Waters DeltaPrep 3000 system with a Phenomenex C18 [10-13] column (250 mm× 10 mm, 5 μm particle diameter), elution with H_2O/acetonitrile/0.1% TFA gradient mixtures and UV detection at 220 nm. Final purity was >99% according to analytical HPLC.~~ ~~The peptides were~~ and characterized by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry using a PerSeptive Biosystems Voyager-DE instrument. MALDI-TOF MS: m/z (C55H96N16O12S2): 1237.9 [M + H]+, 1259.7 [M + Na]+, 1275.7 [M + K]+, 1219.9 [M − H2O]+ (Figure 1)[10]

MIC Determination. ~~A~~ Broth microdilution ~~method~~ was used ~~to determine the MICs. The results were~~ and interpreted according to the European Committee on Antimicrobial Susceptibility Testing EUCAST guidelines [14].

Time-kill curves and growth curves. Killing-curves assays were performed obtained with a starting inoculuma of 5 × 10^5 colony-forming units (CFU)/mL. Bacterial strains were tested against CAMP113 and CAMP207 at concentrations above and below the MICs. Antimicrobials were added to 10 mL of exponentially growing bacterial cultures incubated at 37°C with shaking. Samples were retrieved aseptically at 1, 2, 4 and 6 h, diluted in Ringers ¼ and plated on TSA for colony counting. The response of microbial strains to a single antimicrobial was determined based on a logarithmic decrease in viable bacteria. The effect of CAMPs on the growth of clinical *S. aureus* was assayed with a starting inoculuma of 10^6 and 10^8 CFU/mL respectively.

MBEC Determination. A modification of the procedure described by Moskowitz et al. [15] was used to determine MBECs as previously described[11].

Toxicity of peptides on human hepatocytes. Colistin, CAMP113 and CAMP207 and colistin sulfate were assayed for *in vitro* toxicity against human hepatocytes using the (HepG2) cell line. as previously described [ref]. In brief, the cells were seeded at 1.5 × 10^4/well in a 96-well cell plate (WWR Int, LLC, Radnor, PA) in RPMI (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum. After incubation of the plates for 24 h at 37°C and 5% CO_2, the cells were treated for 24 h with different concentrations of antimicrobials (500, 250, 100, 50, 5 mg/L), followed by incubation for 24 h with 200 µL of 70 µM RPMI containing resazurin sodium salt (Sigma Aldrich MO, USA). The fluorescence resulting from the conversion by viable cells of resazurin to fluorescent resorufin was then measured in an ELISA microplate reader (FLUOstar OPTIMA, BMG LABTECH, Germany) at an emission wavelength of 590 nm and an excitation wavelength of 530 nm.

Confocal laser scanning microscopy imaging. Biofilms on an 8-well glass slide were washed once with Ringer ¼ to remove unfixed bacteria and then treated with antimicrobials at the selected concentration. After 4 h of incubation at (37°C), the biofilms were rinsed once with

Ringer ¼ and stained using the Live/Dead BacLight kit (TermoFisher, Eugene, Oregon, USA) ~~following the manufacturer´s guidelines~~. Fluorescence was observed using a Leica TCS-SL filter-free spectral confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with a 488-nm argon laser, 543-nm and 633-nm He/Ne lasers ~~(Scientific and Technological Centers, University of Barcelona, Spain)~~. All experiments were performed in duplicate. The images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The percentages of live and dead bacterial cells were calculated from the total cell number.

Results and Discussion

~~In this study, in~~ In an effort to produce new molecules with enhanced antimicrobial capacity, ~~we simplified colistin by adding a disulfide bond between two cysteine residues, which facilitated subsequent synthetic steps~~. ~~Concurrently, based on the scaffold of polymyxins, both~~ ~~Both,~~ Peptides CAMP113 and CAMP207 have a disulfide bond between two cysteine residues ~~were modified such that both,~~ contained a dodecanoyl chain instead of (S)-6-methyloctanoyl, ~~CAMP113 and CAMP207~~ have a Cys residue at position 4, Norleucine (NLe) at position 7 and (D)-Cys at position 11, instead ~~whereas polymyxin E has a~~ 2,4-Diaminobutyric Acid (Dab), ~~a residue at position 4,~~ Leu ~~at position 7~~ and Thr ~~at position 11~~ of polymyxin. CAMP113 was further modified with (D)-Phe and CAMP207 with (D)-(4F)-Phe instead of Leu at position 6. In addition, an Arg was substituted at position 8 of CAMP113 whereas colistin contains Dab (Figure 1).

Polymyxin action involves interaction with lipid A. ~~The mechanism of action of polymyxins is thought to involve their direct interaction with the lipid A component of lipopolysaccharide (LPS)[16]~~ and the displacement of ~~the divalent cations (~~Mg^{2+} and Ca^{2+}~~)~~ bridging adjacent lipopolysaccharide (LPS) molecules.[17] ~~The minimum inhibitory concentration (~~MIC~~s)~~ of both ~~CAMP113 and CAMP207~~ peptides is 4 mg/L. ~~The~~ Tested strains were ~~fully~~ resistant to colistin

(> 512 mg/L) and susceptible to vancomycin (1–0.5 mg/L), ~~with the exception of the vancomycin-resistant isolate~~ but *SasVR* (~~Table 1~~ supplementary data). T~~hus, t~~he activity of the two CAMPs was ~~128 times~~ high~~er~~ ~~than that of colistin~~ (Table 1) being MICs within the clinical use range. ~~However, the main limitation of~~ MIC values ~~as an indicator is that they~~ represent only the cumulative effect at 18 h, rather than the time course of activity~~. By contrast,~~ while time-kill kinetics or growth curves describe the antimicrobial action ~~of the test compound while~~ providing insights into the events during its first hours ~~of contact with the tested strains. Thus, we conducted~~ ~~Time-kill curves assays to~~ depicting the biological behaviour over time ~~of different clinical strains incubated with CAMP113 and CAMP207~~. ~~The results revealed that,~~ Despite their chemical similarities~~, the colistin- and polymyxin-derived CAMPs~~ ~~they differed in their actions,~~ ~~as described below, but~~ both peptides ~~were effective in killing~~ killed the bacteria in a concentration-dependent manner (Figure 2.) being ~~Moreover,~~ their behaviour ~~of each compound~~ ~~was~~ very similar irrespective of the strain tested.

Two types of responses were observed in the presence of CAMPs: either the bacterium was completely inhibited at all concentrations tested or inhibition was achieved only at high concentrations (Figure 2). Neither peptide was able to fully eradicate the bacteria solely as a function of its concentration; rather, the activities of the two CAMPs strongly depended on the bacterial strain. For example, CAMP113 fully eradicated strain SaS06 (2A) in 1 h at 16 mg/L, in 2 h at 8 mg/L, but a few individuals survived at 4 mg/L whereas strain SaS16 was completely eradicated at this latter concentration (Figure 2B). At a concentration of 8 mg/L or higher, CAMP113 produced the rapid death of strain SaS16 (2C), during the first hour whereas at an equal concentration of CAMP207 (Figure 2D) 2 h were required. At 4 mg/L, both peptides required 4 h to fully kill strain SaS16. In tests of strain SaS18, complete eradication was achieved within 4 h (Figure 2E) using 8 μg CAMP113/mL but within 6 h using the same concentration of CAMP207 (Figure 2F). However, even at 4 mg /mL, CAMP207 completely failed in SaS18 eradication. Similar outcomes were obtained with the remainder of the tested strains.

Figure 2 also shows representative growth curves. With inocula up to 2 × 10^6 ~~colony forming units~~ (CFU)/mL, growth was fully prevented irrespective of the CAMP and the concentration tested (4, 8 or 16 mg/L). ~~By contrast, with~~ Higher inocula (up to 2 × 10^8 CFU/mL) gave initial growth ~~of the strain was~~ delay~~ed~~ at 4 mg/L, and a longer delay ~~effect that was much more apparent~~ at 8 mg/L. Finally, both CAMPs were able to fully inhibit growth at concentrations of 16 mg/L, ~~thus~~ demonstrating the concentration-dependence~~. of high-density inocula.~~

S. aureus~~, like other bacteria causing infection,~~ have evolved mechanisms to evade innate host ~~defences~~ defenses~~. These mechanisms~~ includinge proteolysis ~~against~~ of natural peptides, secretion of lytic enzymes, modulation of the electrical charge of the cell surface to enhance repulsion, and biofilm formation[18]. ~~As the main virulence factor of *S. aureus* and many other bacterial species [19],~~ Biofilms have been the focus of considerable research attention and their destruction the main goal of current antibacterial therapy. ~~The ability of a compound to reduce the survival of bacteria within biofilms can be quantitatively evaluated by determining the minimal biofilm eradication concentration (MBEC), as shown for the CAMPs in Figure 2~~. The MBECs of both CAMPs indicated the ability of these compounds to fully eradicate *S. aureus* biofilms at relatively low concentrations, in contrast to colistin, which had no effect. Confocal imaging (Figure 2) revealed the effects of the CAMPs (at 128 mg/L) on *S. aureus* biofilm during short exposure periods (4 h). No significant death occurred in either the controls or colistin-treated biofilms, whereas CAMP113 (5C) and CAMP207 (5D) were able to kill roughly one half the bacteria within the biofilm (47.27% and 50.46%, respectively). Given the current interest in CAMPs in the search for new approaches in anti-biofilm treatment [20], our study demonstrates the potency of these ~~two cyclic~~ peptides ~~analogues of polymyxin~~ in targeting biofilms harboring several clinical isolates of *S. aureus*. Under conditions in which a colistin dose of 2,048 mg/L was required, CAMP113 and CAMP207 eradicated the biofilms of all strains tested at much lower concentrations (Figure 2).

The CAMPs were also tested for their cytotoxicity towards the human hepatocellular cell line HepG2 (ATCC, American Type Culture Collection HB8065). As shown in Figure 2d, colistin had a relative low cytotoxicity *in vitro* since its mechanism of toxicity involves the accumulation of molecule in organs such as the kidney. At 5 mg/L, a slightly higher concentration than the MICs of the CAMPs, none of the tested compounds had detectable toxicity. A concentration of 50 mg/L yielded 89.49% HepG2 cell viability for colistin and 67.03% and 62.62% for CAMP113 and CAMP207, respectively. At 100 mg/L, colistin resulted in 68.75% HepG2 viability, CAMP113 in 53.2% and CAMP207 in 57.3%. At an antimicrobial concentration of 250 mg/L, HepG2 viability was 51.05%, 16.01% and 8.29%, respectively. At 500 mg/L, while 38.39% of the cells survived in the presence of colistin both CAMP113 and CAMP207 were highly toxic (7.74% and 7.4%, respectively) (Figure 3). Nonetheless, the selectivity index (the relationship between the IC50 and the MIC) in *S. aureus* strains [21] was inordinately high for the peptides (21.25 for CAMP113 and 23.75 for CAMP207 vs. 0.36 for colistin); that is, CAMP113 and CAMP207 are much more selective than colistin because the concentrations needed to kill the bacteria are much lower. Moreover, colistin is nephrotoxic, as it increases tubular epithelial cell membrane permeability to cations, anions and water, leading to cell swelling and cell lysis [22]. The presence in the newly synthesized CAMPs of a disulfide bond facilitates their biodegradation and thus avoids their accumulation in the kidney. This is in agreement with previous studies, in which the lethal dose (LD50) for peptides of this family in CD-1 mice was 283 mg/kg, compared to the 59.5 mg/kg determined for polymyxin.

The emergence of MDR strains of several ~~bacterial~~ species has posed a tremendous challenge in clinical practice and ~~has~~ renewed interest in polymyxins, whose activity against gram-negatives ~~bacteria~~ is well established. Colistin is considered the drug of last-resort in the treatment of gram-negative-MDR bacteria such as *P. aeruginosa*, *A. baumanii* and *K. pneumoniae*[23,24]. Nonetheless polymyxins are intrinsically inactive against gram-positive bacteria because they lack lipid A, the primary binding site of polymyxins[16,23,25]

Novel peptides ~~have been~~ synthesized in an attempt to evade the increasing polymyxin resistance of gram-negative bacteria~~. Some of these peptides~~ have low MIC values in gram-positives ~~species~~ [8,26]. Similarly, our peptides showed activity against ~~gram positive~~ *S. aureus*. Teichoic acids have a chemical structure similar to that of the LPS regions to which polymyxins bind[15]. To determine whether teichoic acids were the targets of CAMP113 and CAMP 207, we performed isothermal titration calorimetry experiments. The results showed a three-step reaction between the peptides and teichoic acid, two of which were exergonic and the third endergonic (Figure 4).

To examine the spectrum of action of the CAMPs studied, we explored their action against gram-positive *Streptococcus pneumoniae*. Our results showed that in this species the MIC of colistin was consistently > 32 mg/L, whereas the MICs of both peptides were between 2 and 4 mg/L depending on the isolate. ~~Together with the above described results in the *S. aureus* isolate SasVR, a confirmed superbug resistant to vancomycin and linezolid (two new-generation antibiotics of choice in the treatment of MDR staphylococci).~~

~~Conclusions~~

Polymyxins, ~~are~~ regarded as inactive against gram-positives ~~bacteria. In this study, we showed that~~ after some small changes ~~in polymyxin molecule confers~~ have activity against *S. aureus* and *S. pneumoniae*~~. Our study demonstrates that~~ Peptides of this type, despite a more detailed determination of *in vitro* and *in vivo* toxicity as well as the development issues have to be addressed, may open up new frontiers in the treatment of "untreatable infections."

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Transparency declarations

None to declare

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Introduction

Among the world's most dangerous superbugs are multidrug resistant (MDR) bacteria of the so-called ESKAPE group[1,2]. The proportion of these bacteria in common infections has increased recently[3]. Moreover, patients with vascular prostheses, indwelling catheters, dialysis shunts, etc., are particularly vulnerable to the virtually untreatable infections resulting from ESKAPE colonization and subsequent biofilm formation.

The indiscriminate use of penicillin has increased the percentage of *S. aureus* penicillin-resistant strains to nearly 100 %[4]. The same is true for several other antibiotics.

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The synthesis of new molecules less toxic and with greater activity than conventional antibiotics is challenging. One strategy is to make small changes in known molecules, such as polymyxin, to generate derivatives effective against gram-positive bacteria. The antimicrobial activity of

two novel synthetic cyclolipopeptide analogues of polymyxin (CAMP113 and CAMP207) against *S. aureus* and other gram-positive bacteria is reported.

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MBEC Determination. A modification of the procedure described by Moskowitz et al. was used to determine MBECs as previously described[11].

Toxicity of peptides on human hepatocytes. Colistin, CAMP113 and CAMP207 were assayed for *in vitro* toxicity against human hepatocytes (HepG2) as previously described[12].

Confocal laser scanning microscopy imaging. Biofilms were washed with Ringer ¼ and treated with antimicrobials. After 4 h incubation (37°C), biofilms were rinsed with Ringer ¼ and stained using the Live/Dead BacLight kit (TermoFisher, Eugene, Oregon, USA). Fluorescence was observed using a Leica TCS-SL filter-free spectral confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with a 488-nm argon laser, 543-nm and 633-nm He/Ne lasers. All experiments were performed in duplicate. The images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The percentages of live and dead bacterial cells were calculated from the total cell number.

Results and Discussion

Peptides CAMP113 and CAMP207 have a disulfide bond between two cysteine residues, contained a dodecanoyl chain instead of (S)-6-methyloctanoyl, have a Cys residue at position 4, Norleucine (NLe) at position 7 and (D)-Cys at position 11, instead 2,4-Diaminobutyric Acid (Dab), Leu and Thr of polymyxin. CAMP113 was further modified with (D)-Phe and CAMP207 with (D)-(4F)-Phe instead of Leu at position 6. In addition, an Arg was substituted at position 8 of CAMP113 whereas colistin contains Dab (Figure 1).

Polymyxin action involves interaction with lipid A and the displacement of Mg^{2+} and Ca^{2+} bridging adjacent lipopolysaccharide (LPS) molecules[13]. MIC of both peptides is 4 mg/L. Tested strains were resistant to colistin (> 512 mg/L) and susceptible to vancomycin (1–0.5 mg/L), but

SasVR (supplementary data). The activity of the two CAMPs was high (Table 1) being MICs within the clinical use range. MIC values represent only the cumulative effect at 18 h, rather than the time course of activity, while time-kill kinetics or growth curves describe the antimicrobial action providing insights into the events during its first hours depicting the biological behaviour over time. Despite their chemical similarities both peptides killed the bacteria in a concentration-dependent manner (Figure 2.) being their behaviour very similar irrespective of the strain tested.

Two types of responses were observed in the presence of CAMPs: either the bacterium was completely inhibited at all concentrations tested or inhibition was achieved only at high concentrations (Figure 2). Neither peptide was able to fully eradicate the bacteria solely as a function of its concentration; rather, the activities of the two CAMPs strongly depended on the bacterial strain. For example, CAMP113 fully eradicated strain SaS06 (2A) in 1 h at 16 mg/L, in 2 h at 8 mg/L, but a few individuals survived at 4 mg/L whereas strain SaS16 was completely eradicated at this latter concentration (Figure 2B). At a concentration of 8 mg/L or higher, CAMP113 produced the rapid death of strain SaS16 (2C), during the first hour whereas at an equal concentration of CAMP207 (Figure 2D) 2 h were required. At 4 mg/L, both peptides required 4 h to fully kill strain SaS16. In tests of strain SaS18, complete eradication was achieved within 4 h (Figure 2E) using 8 μg CAMP113/mL but within 6 h using the same concentration of CAMP207 (Figure 2F). However, even at 4 mg /mL, CAMP207 completely failed in SaS18 eradication. Similar outcomes were obtained with the remainder of the tested strains.

Figure 2 also shows representative growth curves. With inocula up to 2×10^6 (CFU)/mL, growth was fully prevented irrespective of the CAMP and the concentration tested (4, 8 or 16 mg/L). Higher inocula (up to 2×10^8 CFU/mL) gave initial growth delay at 4 mg/L, and a longer delay at 8 mg/L. Finally, both CAMPs were able to fully inhibit growth at concentrations of 16 mg/L, demonstrating the concentration-dependence.

S. aureus have evolved mechanisms to evade innate host defenses including proteolysis of natural peptides, secretion of lytic enzymes, modulation of the electrical charge of the cell surface to enhance repulsion, and biofilm formation[14]. Biofilms have been the focus of considerable research attention and their destruction the main goal of current antibacterial therapy. The MBECs of both CAMPs indicated the ability of these compounds to fully eradicate *S. aureus* biofilms at relatively low concentrations, in contrast to colistin, which had no effect. Confocal imaging (Figure 2) revealed the effects of the CAMPs (at 128 mg/L) on *S. aureus* biofilm during short exposure periods (4 h). No significant death occurred in either the controls or colistin-treated biofilms, whereas CAMP113 (5C) and CAMP207 (5D) were able to kill roughly one half the bacteria within the biofilm (47.27% and 50.46%, respectively). Given the current interest in CAMPs in the search for new approaches in anti-biofilm treatment [15], our study demonstrates the potency of these peptides in targeting biofilms harboring several clinical isolates of *S. aureus*. Under conditions in which a colistin dose of 2,048 mg/L was required, CAMP113 and CAMP207 eradicated the biofilms of all strains tested at much lower concentrations (Figure 2).

The CAMPs were also tested for their cytotoxicity towards the human hepatocellular cell line HepG2 (ATCC, American Type Culture Collection HB8065). As shown in Figure 2d, colistin had a relative low cytotoxicity *in vitro* since its mechanism of toxicity involves the accumulation of molecule in organs such as the kidney. At 5 mg/L, a slightly higher concentration than the MICs of the CAMPs, none of the tested compounds had detectable toxicity. A concentration of 50 mg/L yielded 89.49% HepG2 cell viability for colistin and 67.03% and 62.62% for CAMP113 and CAMP207, respectively. At 100 mg/L, colistin resulted in 68.75% HepG2 viability, CAMP113 in 53.2% and CAMP207 in 57.3%. At an antimicrobial concentration of 250 mg/L, HepG2 viability was 51.05%, 16.01% and 8.29%, respectively. At 500 mg/L, while 38.39% of the cells survived in the presence of colistin both CAMP113 and CAMP207 were highly toxic (7.74% and 7.4%, respectively) (Figure 3). Nonetheless, the selectivity index (the relationship between

the IC50 and the MIC) in *S. aureus* strains [16] was inordinately high for the peptides (21.25 for CAMP113 and 23.75 for CAMP207 vs. 0.36 for colistin); that is, CAMP113 and CAMP207 are much more selective than colistin because the concentrations needed to kill the bacteria are much lower. Moreover, colistin is nephrotoxic, as it increases tubular epithelial cell membrane permeability to cations, anions and water, leading to cell swelling and cell lysis[17]. The presence in the newly synthesized CAMPs of a disulfide bond facilitates their biodegradation and thus avoids their accumulation in the kidney. This is in agreement with previous studies, in which the lethal dose (LD50) for peptides of this family in CD-1 mice was 283 mg/kg, compared to the 59.5 mg/kg determined for polymyxin[10].

The emergence of MDR strains of several species has posed a tremendous challenge in clinical practice and renewed interest in polymyxins, whose activity against gram-negatives is well established. Colistin is considered the drug of last-resort in the treatment of gram-negative-MDR bacteria such as *P. aeruginosa*, *A. baumanii* and *K. pneumoniae*[18,19]. Nonetheless polymyxins are intrinsically inactive against gram-positive bacteria because they lack lipid A, the primary binding site of polymyxins[18,20]

Novel peptides synthesized in an attempt to evade the increasing polymyxin resistance of gram-negative bacteria have low MIC values in gram-positive[8]. Similarly, our peptides showed activity against *S. aureus*. Teichoic acids have a chemical structure similar to that of the LPS regions to which polymyxins bind[15]. To determine whether teichoic acids were the targets of CAMP113 and CAMP 207, we performed isothermal titration calorimetry experiments. The results showed a three-step reaction between the peptides and teichoic acid, two of which were exergonic and the third endergonic (supplementary data).

To examine the spectrum of action of the CAMPs studied, we explored their action against gram-positive *Streptococcus pneumoniae*. Our results showed that in this species the MIC of

colistin was consistently > 32 mg/L, whereas the MICs of both peptides were between 2 and 4 mg/L depending on the isolate.

Polymyxins, regarded as inactive against gram-positives after some small changes have activity against *S. aureus* and *S. pneumonia;* peptides of this type, despite a more detailed determination of *in vitro* and *in vivo* toxicity as well as the development issues have to be addressed, may open up new frontiers in the treatment of "untreatable infections."

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Transparency declarations

None to declare

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Figure 1. Chemical structure of the cyclopolypeptides CAMP113 and CAMP207 (below) and polymyxin B (above). Common structural and chemical features that were modified are highlighted in gray. Differences in the structural features of the two synthetic peptides are indicated in the boxes R and Y.

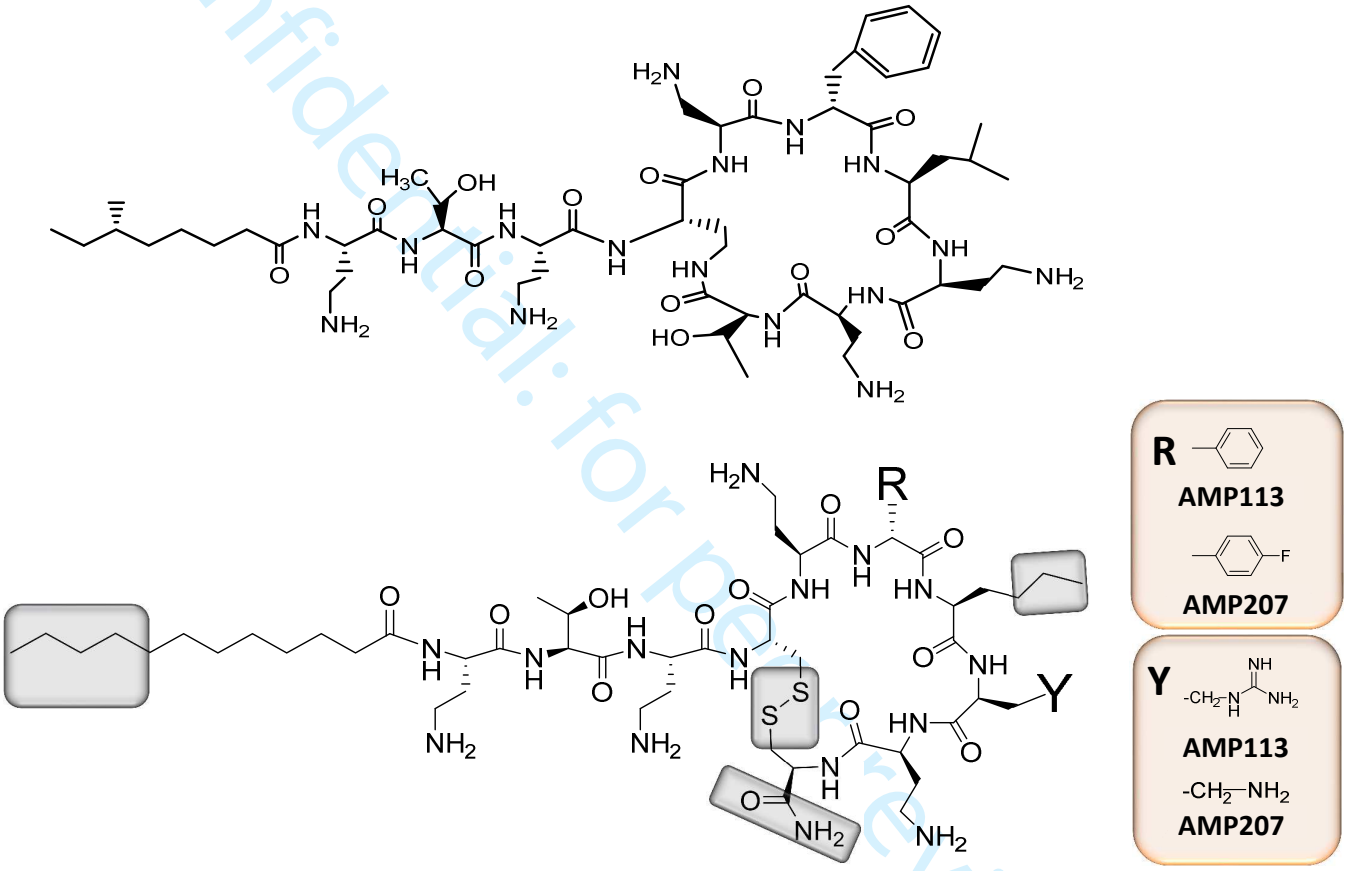
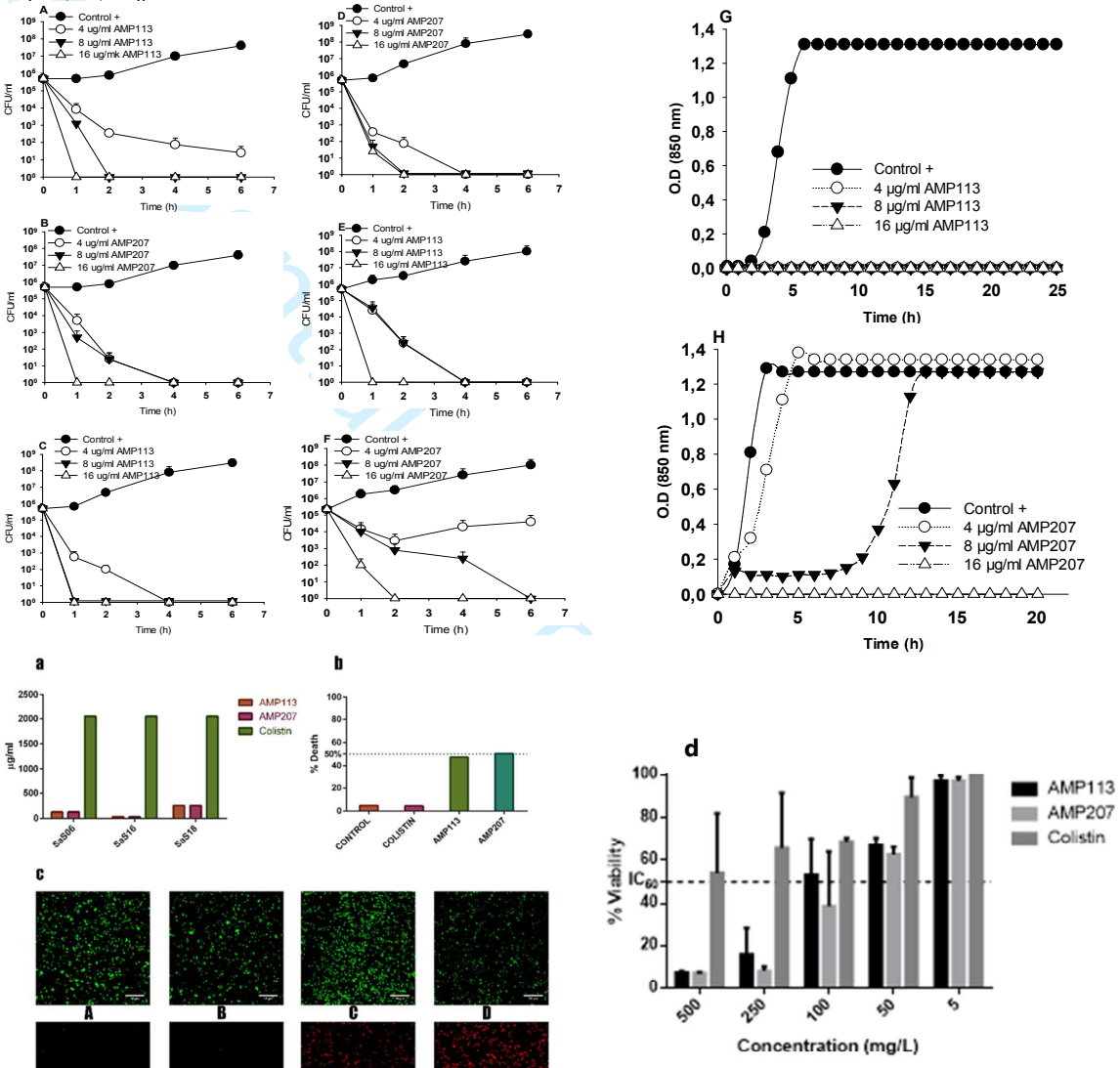


Figure 2. Biological activity of CAMP113 and CAMP207. Control (•), 4 mg/L (○), 8 mg/L (▼), 16 mg/L (△). (**A**) Strain SaS06 incubated with CAMP113. (**B**) Strain SaS06 incubated with CAMP207. (**C**) Strain SaS16 incubated with CAMP113. (**D**) Strain Sas16 incubated with CAMP207. (**E**) Strain Sas18 incubated with CAMP113. (**F**) Strain Sas18 incubated with CAMP207. **G)** growth curve of strain SaS16 (inoculum of 10^6 cfu/mL) in the presence of /CAMP113. **H)** Growth curve of strain SaS06 (inoculum of 10^8 CFU/mL) in the presence of /CAMP207**.** a) Minimal biofilm eradication concentration assays. Strain SaS06 (CAMP113, 128 mg/L; CAMP207, 128 mg/L; colistin >2048 mg/L). Strain SaS16 (CAMP113, 32 mg/L; CAMP207, 32 mg/L; colistin >2048 mg/L). Strain SaS18 (CAMP113, 256 mg/L; CAMP207, 256 mg/L; colistin, >2048 mg/L); b) Percentage of biofilm death after 4 h of exposure. c) Confocal laser scanning microscopy assays. The images (1024 × 1024 pixels) were taken after 4 h of exposure to: control (A), colistin (B), CAMP113 (C), or CAMP207 (D). d) Toxicity of CAMP113 and CAMP207 towards HepG2 human



Antimicrobial susceptibility (minimum inhibitory concentration, MIC; mg/L).

	Origin	CAMP113	CAMP207	COLISTIN	VANCOMYCIN	LINEZOLID	ERYTHROMYCIN	TETRACYCLINE	CIPROFLOXACIN
ATCC 700698*	Sputum	4	4	>512	1(S)	4(S)	>32(R)	>32(R)	>32(R)
ATCC 29213	Wound Smear	4	4	>512	1(S)	4(S)	1-2(S)	1(S)	1(S)
SaSVR	*[1]*Conj. exd*	2-4	4-8	>512	>32(R)	>32(R)	>32(R)	1-2	4(R)
SaSVI	*[2]*Spleen nec.*	16	4-8	>512	2-4	4(S)	>32(R)	>32(R)	0,25-0,5(S)
SaS01	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	1(S)	2-4(R)
SaS02	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	1(S)	1(S)
SaS03	Wound Smear	4	4	>512	1(S)	4(S)	>32(R)	0,5-1(S)	1(S)

R: resistant; S: susceptible.

*SaS04**	Wound Smear	4	4	>512	0,5(S)	4(S)	>32(R)	0,125(S)	>32(R)
*SaS05**	Wound Smear	4	4	>512	0,5(S)	2(S)	>32(R)	>32(R)	>32(R)
*SaS06**	Sputum	4	4	>512	1(S)	4(S)	0,5(S)	0,125(S)	>32(R)
*SaS07**	Nasal Smear	4	4	>512	0,5	8(R)	0,25(S)	4(R)	>32(R)
*SaS08**	Wound Smear	4	4	>512	1(S)	4(S)	>32(R)	0,5 - 1(S)	>32(R)
SaS09	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	1(S)	>32(R)
SaS10	Urine	4	4	>512	1(S)	4(S)	1(S)	1(S)	0,5-1(S)
*SaS11**	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	2(R)	>32(R)
*SaS12**	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	2(R)	>32(R)
*SaS13**	Total Blood	2	2-4	>512	0,5(S)	4(S)	1(S)	8(R)	0,5(S)
*SaS14**	Wound Smear	4	4	>512	0,5(S)	4(S)	>32(R)	0,5(S)	>32(R)
*SaS15**	Total Blood	4	4	>512	1(S)	4(S)	1(S)	>32(R)	>32(R)
SaS16	Skin Smear	4	4	>512	1(S)	4(S)	1(S)	0,5(S)	>32(R)
SaS17	Wound Smear	4	4	>512	1(S)	4(S)	1(S)	8(R)	4(R)
*SaS18**	Wound Smear	4	4	>512	1(S)	4(S)	>32(R)	4(R)	4(R)

* MRSA. *[1]Conjunctival exudate. *[2]Sample from a spleen necropsy.

Isothermal titration calorimetry curve for CAMP207 (5290 μM) vs. teichoic acids (500 μM)