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Synergistic microbicidal effect of cationic antimicrobial peptides and teicoplanin against planktonic and biofilm-encased *Staphylococcus aureus*

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ABSTRACT

Antibiotic resistance and biofilm formation are the main reasons for failure in treatment of bacterial infections. This study aimed to identify synergistic combinations of conventional antibiotics and novel synthetic antimicrobial and antibiofilm peptides (SAAPs) inspired by the structures of the natural human cationic peptides LL-37 and thrombocidin-1 (TC-1). The LL-37-inspired lead peptide SAAP-148 was combined with antibiotics of different classes against *Staphylococcus aureus*, and showed synergy with teicoplanin. Synergy with teicoplanin was also observed with LL-37, the LL-37-inspired SAAP-276 and the TC-1-inspired TC84. Interestingly, no synergy was observed against *Staphylococcus epidermidis*. Furthermore, teicoplanin combined with SAAP-148 or SAAP-276 showed strong interaction against *S. aureus* biofilms. The *dltABCD* operon and the *mprF* gene in *S. aureus* conferred resistance to LL-37, but SAAP-148 proved to be indifferently potent against wild-type, Δ*dltA* and Δ*mprF S. aureus* strains. When used alone, relatively high concentrations of both LL-37 and teicoplanin (30–120 μM and 4–32 mg/L, respectively) were required to kill *S. aureus*. Resistance to LL-37 in *S. aureus* was overcome by combined use of teicoplanin and LL-37. Thus, teicoplanin potentiates peptide LL-37, enhancing the efficacy of the innate defence, and combining the novel peptides with teicoplanin offers potential for enhanced efficacy of treatment of *S. aureus* infections, including biofilms.

1. Introduction

The development of antibiotic resistance and biofilm formation are major worldwide problems. If current clinical practice continues unchanged, it is predicted that antibiotic resistance will be the major cause of death by 2050, with 10 million deaths annually [1]. Novel antimicrobial agents are actively being developed, among which are cationic antimicrobial peptides (CAMPs) [2]. Natural CAMPs are a major immunological defence line in microorganisms, plants and animals [3]. Most CAMPs are amphipathic [4], and commonly interact with the negatively charged outer surface of bacteria, causing depolarization, permeabilization and disruption of the cytoplasmic membrane [5].

Two examples of natural CAMPs are the α-helical peptide LL-37, produced by human neutrophils, monocytes, dendritic cells, epithelial cells and a subset of macrophages [6]; and thrombocidin-1 (TC-1), residing in the α-granules of human blood platelets [7]. The authors recently designed highly potent novel antimicrobial peptides inspired by the structures of these native CAMPs. The LL-37-inspired synthetic antimicrobial and antibiofilm peptides (SAAP)-148 [8], SAAP-276 [9] and TC-1-inspired TC84 [10] (Table 1) are active in 50% human plasma at low micromolar concentrations against a wide range of multi-drug-resistant bacteria, have antibiofilm activity, and prevent and treat infection in different animal infection models [8–10].

However promising CAMPs may be as antimicrobials, intrinsic resistance against particular CAMPs exists [11]. For instance, *Staphylococcus aureus* and several other Gram-positive bacteria are able to add a protonated D-alanyl group to their cell wall teichoic acids through the enzymes encoded by the *dltABCD* (D-alanyl-lipoteichoic acid) operon [5]. These protonated D-alanyl residues, being positively charged, partially neutralize the negative charge of

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Table 1
Cationic antimicrobial peptide sequences.

Peptide	Sequence
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
SAAP-148	LKRVWKRVFKLLKRYWRQLKKPVR
SAAP-276	LKRVWKAVFKLLKRYWRQLKKPVR
TC84	LRAMCIKWWSGKHPKB

the bacterial outer surface, resulting in lower affinity for the positively charged CAMPs and reduced susceptibility to these CAMPs [12]. In addition, the *mprF* gene codes for MprF (multiple peptide resistance factor), which regulates the addition of positively charged L-lysine residues to phosphatidylglycerol, a major phospholipid of the staphylococcal cell membrane, also leading to repulsion of CAMPs [13].

In order to avoid the development of resistance to single antimicrobials and to benefit from synergy, antibiotics are often given in combination [14]. Synergy between antibiotics and CAMPs has also been reported. For example, LL-37 synergizes with several commonly used antibiotics against *Clostridium difficile* [15], and with amoxicillin/clavulanic acid against *S. aureus* [16]. However, to the authors' knowledge, a comprehensive screening among different classes of antibiotics for synergistic antimicrobial effects with innate and synthetic CAMPs has not been reported. Therefore, this study aimed to assess interactions of LL-37 and the novel CAMPs SAAP-148, SAAP-276 and TC84 with antibiotics acting against different targets in the bacterial cell. *S. aureus* was selected as the primary target bacterium because of its clinical importance [17], its *dlt*- and *mprF*-based CAMP resistance mechanisms [11], and its biofilm-forming properties [18].

2. Materials and methods

2.1. Bacteria and chemicals

S. aureus JAR060131 [19], *S. aureus* ATCC 49230, *S. aureus* SA113 (ATCC 35556), meticillin-resistant *S. aureus* (MRSA) AMC201 [20], MRSA LUH15101 [21], *S. epidermidis* RP62a (ATCC 35984), *S. epidermidis* AMC5 [22] and *S. epidermidis* O-47 [23] were used. In addition, *S. aureus* SA113 Δ*dltA* [12] and Δ*mprF* [13] mutants, and respective complemented mutant strains carrying the plasmids pRB474-dltASx1 and pRB474-mprF, were used. All bacterial strains were grown either in tryptic soy broth (TSB) or RPMI 1640 medium with 20 mM Hepes and L-glutamine and without sodium bicarbonate (referred to as RPMI; Gibco, ThermoFisher Scientific, Waltham (MA), USA). RPMI supports growth of the bacteria without affecting the bactericidal activity of CAMPs [24]. Antibiotics of different classes were used: fosfomycin (Bio Connect, Huissen, The Netherlands), cefuroxime (Enzo Life Sciences, Bruxelles, Belgium), vancomycin (Sigma, St. Louis (MO), USA) and teicoplanin (Sigma) that target cell wall synthesis; ciprofloxacin (Sigma) and moxifloxacin (Bayer, Leberkusen, Germany) which are DNA-gyrase inhibitors; the protein synthesis inhibitors gentamicin (Centrafarm, Etten-Leur, The Netherlands), tetracycline (Sigma) and doxycycline (Sigma); rifampicin (Sigma), an RNA-polymerase inhibitor; and daptomycin (Cubist Pharmaceuticals, Lexington (MA), USA) which disrupts the cell membrane. Stock solutions (2.56 mg/mL) of these antibiotics were made in RPMI. LL-37, SAAP-148, SAAP-276 and TC84 (Table 1) were synthesized by normal 9H-fluorenylmethyloxycarbonyl (Fmoc) chemistry [25]. Stock solutions (1.2 mM) of these CAMPs were made in RPMI.

2.2. Susceptibility testing of planktonic bacteria

Overnight bacterial cultures in TSB were diluted 100-fold in fresh TSB and cultured to mid-logarithmic growth phase at 37 °C on a rotary shaker (120 rpm). A culture was centrifuged (5000 × *g*, 5 min) and the pellet was washed with phosphate buffered saline (PBS) and resuspended in RPMI. Based on the optical density at 620 nm, the bacterial suspension was diluted to the desired inoculum concentration of 1×10^7 colony-forming units (cfu)/mL, and this was verified by culturing duplicate 10-µL aliquots from 10-fold serial dilutions of the suspension on blood agar (Biomérieux, Marcy l'Etoile, France) and determining the cfu/mL on the following day (quantitative culture). Stock solutions of CAMPs and antibiotics were two-fold serially diluted in RPMI, ranging from 120 to 0.06 µM for CAMPs and from 128 to 0.01 mg/L for antibiotics, in a 96-well polypropylene round-bottom microtitre plate (Corning Life Sciences, Corning (NY), USA) to a final volume of 90 µL. For the fosfomycin assays, 25 mg/L glucose-6-phosphate was added. Ten microlitres of the bacterial inoculum was added to each well, resulting in a final concentration of 1×10^6 cfu/mL. The plate was incubated at 37 °C on a rotary shaker (120 rpm) for 24 h in a moist environment. After quantitative culture of surviving bacteria, the 99.9% minimum bactericidal concentration (MBC_{RPMI}; the concentration that killed ≥99.9% of the bacteria in RPMI after 24 h) was determined. All assays were performed at least in duplicate.

2.3. Susceptibility testing of biofilms

An inoculum suspension containing 1×10^8 cfu/mL in TSB was prepared as described above. Two hundred microlitres of this inoculum was added to each well of a 96-well polystyrene flat-bottom microtitre plate (Greiner, Kremsmünster, AustriaElma) and to eight wells of an additional plate (for control biofilms). The plates were incubated at 37 °C without agitation overnight in a moist environment to allow biofilm formation. The biofilms were washed twice by carefully adding and removing 200 µL PBS to remove residual medium and planktonic bacteria. To assess the numbers of bacteria in the biofilms present before exposure to the antimicrobials, the control biofilms were sonicated (Elma Transonic T460, 35 kHz, Elma Schmidbauer GmbH, Singen, Germany) for 5 min to dislodge and resuspend the bacteria, and the sonicates were quantitatively cultured. One hundred microlitres of CAMP or antibiotic solution that had been two-fold serially diluted in RPMI to concentrations ranging from 120 to 1.875 µM for CAMPs and from 256 to 0.25 mg/L for antibiotics was added to the biofilms. After 24 h of incubation at 37 °C without agitation, the medium containing the antimicrobials was carefully removed from the well and 100 µL PBS was added. The plate was sonicated for 5 min and the bacteria were quantitatively cultured. The antibiofilm activity was expressed as the 99% minimal biofilm eradication concentration ($MBEC_{99}$), which was the concentration that eradicated ≥99% of the bacteria after 24 h of incubation compared with the numbers of bacteria cultured from the control biofilms. All assays were performed at least in duplicate.

2.4. Checkerboard assays with planktonic bacteria

Two-fold serial dilutions of an antibiotic and a CAMP across the columns and rows of a 96-well polypropylene microtitre plate, respectively, were prepared in a final volume of 90 µL. Concentration ranges, as established with susceptibility testing, were used for both the antibiotic and the CAMP. Ten microlitres of the bacterial inoculum was added to each well, resulting in a final concentration of 1×10^6 cfu/mL. The plate was incubated overnight at 37 °C on a rotary shaker (120 rpm) in a moist environment, and the MBC_{RPMI} values of the antimicrobials were determined after

Table 2
Activity of synthetic antimicrobial and antibiofilm peptide (SAAP)-148 combined with conventional antibiotics against *Staphylococcus aureus* JAR060131.

Agent	MBC_{RPMI} in μM or mg/L[a]				Fold decrease of MBC_{RPMI} in combination		$FBCI_{opt}$		Outcome
	Alone		In combination						
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	
SAAP-148	0.47	3.8	0.23	0.94	2	4	1.0	0.5	No interaction
Rifampicin	2.0	0.13	1.0	0.031	2	4			
SAAP-148	0.47	0.47	0.059	0.12	8	4	0.63	0.75	No interaction
Ciprofloxacin	1.0	0.50	0.50	0.25	2	2			
SAAP-148	0.94	0.94	0.47	0.47	2	2	0.53	0.63	No interaction
Moxifloxacin	0.13	0.031	0.0039	0.0039	32	8			
SAAP-148	0.23	0.23	0.23	0.23	–	–	1.1	1.1	No interaction
Daptomycin	2.0	2.0	0.13	0.25	16	8			
SAAP-148	0.47	1.9	0.12	0.23	4	8	0.38	0.25	Synergy
Teicoplanin	16	16	2.0	2.0	8	8			
SAAP-148	0.23	0.94	0.12	0.47	2	2	1.0	0.56	No interaction
Cefuroxime	4.0	2.0	2.0	0.13	2	16			
SAAP-148	0.47	0.47	0.23	0.23	2	2	0.75	0.75	No interaction
Fosfomycin	16	32	4.0	8.0	4	4			
SAAP-148	0.47	0.47	0.23	0.12	2	4	0.56	0.75	No interaction
Tetracycline	16	32	1.0	16	16	2			
SAAP-148	0.47	0.47	0.23	0.23	2	2	0.56	0.56	No interaction
Doxycycline	8.0	16	0.50	1.0	16	16			
SAAP-148	0.94	0.94	0.94	0.94	–	–	1.0	2.0	No interaction
Vancomycin	4.0	4.0	0.13	4.0	32	–			
SAAP-148	1.9	0.94	0.94	0.94	2	–	0.75	1.0	No interaction
Gentamicin	0.5	0.25	0.125	0.0019	4	128			

$FBCI_{opt}$, optimal fractional bactericidal concentration index; MBC_{RPMI}, concentration that killed ≥99.9% of the bacteria in RPMI after 24 h.
[a] Concentration of SAAP-148 in μM, concentration of antibiotic in mg/L.
Results of two independent experiments are shown. An $FBCI_{opt}$ value ≤0.5 indicates synergy, >0.5–4 indicates no interaction, and >4 indicates antagonism. A combination was considered synergistic or antagonistic if the two independent experimental results fell into the same category (synergy or antagonism); otherwise, the combination was considered non-interacting.

quantitative culture. To determine synergistic or antagonistic interactions of the antimicrobial agents, a fractional bactericidal concentration index (FBCI) [26] was calculated for each isoeffective combination (i.e. all of the wells corresponding to an MBC_{RPMI}) in the microtitre plate with the following formula:

$$FBCI = \frac{MBC_{RPMI}\text{ of antibiotic in combination}}{MBC_{RPMI}\text{ of antibiotic alone}} + \frac{MBC_{RPMI}\text{ of peptide in combination}}{MBC_{RPMI}\text{ of peptide alone}}$$

Of all isoeffective combinations, the combination with the lowest FBCI value was considered to be optimal ($FBCI_{opt}$). An $FBCI_{opt}$ value ≤0.5 indicates synergy, >0.5–4 indicates no interaction, and >4 indicates antagonism. The study protocol consisted of two independent experiments for all combinations of antibiotics and CAMPs tested. A combination was considered synergistic or antagonistic if the two independent experimental results fell into the same category (synergy or antagonism); otherwise, the combination was considered non-interacting.

2.5. Checkerboard assays with preformed biofilms

Overnight *S. aureus* biofilms were prepared as described above. The biofilms were washed twice by carefully adding and removing 200 μL PBS. Control biofilms were quantitatively cultured as described. Two-fold serial dilutions of an antibiotic and a CAMP across the columns and rows of a 96-well polypropylene microtitre plate, respectively, were prepared in a separate plate, and 100 μL of these mixtures was added to the biofilms. Concentration ranges, as established with susceptibility testing, were used for both the antibiotic and the CAMP. After 24 h of incubation at 37 °C without agitation, the medium with antimicrobials was removed from the wells and 100 μL PBS was added. The plate was sonicated for 5 min and the bacteria were quantitatively cultured. $MBEC_{99}$ values were calculated, and the effects of the antibacterial agents were assessed by determining the fold reduction of $MBEC_{99}$. Due to the high $MBEC_{99}$ values of the antimicrobial agents when used alone, it was not possible to calculate FBCI.

3. Results

3.1. Teicoplanin synergizes with CAMPs against S. aureus

Planktonic cultures of *S. aureus* JAR060131 were used in antimicrobial checkerboard assays. An initial screening for synergistic combinations was performed by combining the novel SAAP-148 with a panel of 11 antibiotics with different modes of action (Table 2). In combination with SAAP-148, MBC_{RPMI} of both moxifloxacin and doxycycline decreased eight-fold or more; however, synergy was only concluded for SAAP-148 with teicoplanin ($FBCI_{opt}$ of 0.25). Surprisingly, vancomycin, a glycopeptide antibiotic like teicoplanin, did not synergize with SAAP-148. Therefore, the interac-

Table 3
Activity of teicoplanin or vancomycin in combination with different cationic antimicrobial peptides (CAMPs) against *Staphylococcus aureus* JAR060131.

Agent	MBC_{RPMI} in μM or mg/L[a]				Fold decrease of MBC_{RPMI} in combination		$FBCI_{opt}$		Outcome
	Alone		In combination						
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	
LL-37 Teicoplanin	120 32	120 32	1.9 0.50	3.8 1.0	64 64	32 32	0.031	0.063	Synergy
LL-37 Vancomycin	60 1.0	30 1.0	60 1.0	30 0.06	– –	– 16	2.0	1.1	No interaction
SAAP-276 Teicoplanin	1.9 32	0.94 32	0.47 4.0	0.12 2.0	4 8	8 16	0.38	0.19	Synergy
SAAP-276 Vancomycin	1.9 1.0	1.9 2.0	0.94 0.5	0.94 1.0	2 2	2 2	1.0	1.0	No interaction
TC84 Teicoplanin	15 16	30 16	3.8 0.50	7.5 1.0	4 32	4 16	0.28	0.31	Synergy
TC84 Vancomycin	60 8.0	15 4.0	30 2.0	15 1.0	2 4	– 4	0.75	1.3	No interaction

$FBCI_{opt}$, optimal fractional bactericidal concentration index; MBC_{RPMI}, concentration that killed ≥99.9% of the bacteria in RPMI after 24 h; SAAP, synthetic antimicrobial and antibiofilm peptide.
[a] Concentration of CAMPs in μM, concentration of teicoplanin in mg/L.
Results of two independent experiments are shown respectively. An $FBCI_{opt}$ value ≤0.5 indicates synergy, >0.5–4 indicates no interaction, and >4 indicates antagonism. A combination was considered synergistic or antagonistic if the two independent experimental results fell into the same category (synergy or antagonism); otherwise, the combination was considered non-interacting.

tion of both vancomycin and teicoplanin with LL-37, SAAP-276 or TC84 against *S. aureus* JAR060131 was investigated to assess the generality of the finding (Table 3). LL-37, SAAP-276 and TC84 all synergized with teicoplanin. The strongest synergy was found for LL-37 combined with teicoplanin ($FBCI_{opt}$ of 0.031), with isoeffective concentrations of 1.9 μM and 0.5 mg/L LL-37 and teicoplanin, respectively. This meant a 64-fold reduction in MBC_{RPMI} for LL-37 compared with LL-37 alone. The $FBCI_{opt}$ values for teicoplanin with SAAP-276 and teicoplanin with TC84 were 0.19 and 0.28, respectively. No synergy was observed between either LL-37, SAAP-276 or TC84 with vancomycin.

3.2. CAMP and teicoplanin combinations which synergistically eradicate S. aureus show no interaction against S. epidermidis

To investigate species specificity of the synergy between innate/synthetic CAMPs and teicoplanin, the authors chose to test LL-37 with teicoplanin and SAAP-148 with teicoplanin against different *S. aureus* and *S. epidermidis* strains in checkerboard experiments (Table 4). Alone, LL-37 appeared to be much more effective against the *S. epidermidis* strains (MBC_{RPMI} of 3.8–15 μM), than against the *S. aureus* strains (MBC_{RPMI} of 30–120 μM). Both LL-37 and SAAP-148 acted synergistically with teicoplanin against all of the *S. aureus* strains except MRSA LUH15101, for which the combination of SAAP-148 with teicoplanin did not meet the criteria for synergy ($FBCI_{opt}$ of 0.50–0.53). Interestingly, neither LL-37 nor SAAP-148 synergized with teicoplanin against any of the *S. epidermidis* strains tested.

3.3. Only SAAP-148 and SAAP-276 retain their synergy with teicoplanin against S. aureus biofilms

To determine the efficacy of the synergistic CAMP–teicoplanin combinations against biofilms of *S. aureus*, LL-37, SAAP-148, SAAP-276 or TC84 were combined with teicoplanin and tested against preformed biofilms of *S. aureus* JAR060131 (Table 5). When used alone, teicoplanin (≥256 mg/L), LL-37 (≥120 μM) and TC84 (≥120 μM) were only able to eradicate 99% of the biofilm bacteria at the highest concentrations tested. SAAP-148 and SAAP-276 did eradicate the biofilms, with $MBEC_{99}$ values ranging from 60 to 120 μM. Combining LL-37 with teicoplanin decreased $MBEC_{99}$ of both LL-37 and teicoplanin two- to four-fold. Strong antimicrobial enhancement was found for both SAAP-148 and SAAP-276 with teicoplanin (Table 5). When SAAP-148 was combined with teicoplanin, $MBEC_{99}$ of SAAP-148 and teicoplanin decreased two- to four-fold and 16- to 32-fold, respectively. Combining SAAP-276 with teicoplanin decreased the $MBEC_{99}$ values eight- to 16-fold and 64- to 128-fold, respectively. Combining TC84 with teicoplanin did not decrease the $MBEC_{99}$ values. Apparently, the observed synergy of the SAAPs with teicoplanin against planktonic *S. aureus* still takes effect against *S. aureus* in its biofilm-encased state.

3.4. The dltABCD operon and the mprF gene in S. aureus confer resistance against LL-37, but not against SAAP-148

S. aureus showed resistance to LL-37, both in planktonic culture (MBC_{RPMI} of 30–120 μM) and in biofilms ($MBEC_{99}$ ≥120 μM). To assess whether *S. aureus* resistance mechanisms based on neutralization of outer surface charge would influence susceptibility to LL-37 or SAAP-148, the susceptibilities for these CAMPs of wild-type (WT) *S. aureus* strain SA113, its *dltA* and *mprF* deletion mutants, and their respective complemented mutant strains SA113 pRB474-dltASx1 and SA113 pRB474-mprF were compared (Fig. 1). The SA113 WT strain was moderately resistant to LL-37 (MBC_{RPMI} of 50 μM); however, LL-37 was highly effective against the *dltA* (MBC_{RPMI} of 0.94 μM) and *mprF* (MBC_{RPMI} of 1.9 μM) deletion mutants. In comparison, the SA113 WT strain was highly susceptible to SAAP-148 (MBC_{RPMI} of 0.94 μM), and no increased susceptibility was observed for the mutants. Notably, the *dltA* complemented mutant showed an increase of resistance against LL-37 compared with the WT strain. This phenomenon has been observed in previous work, and is probably due to overexpression of the complementing gene [12].

Table 4
Combined activity of teicoplanin with LL-37 or synthetic antimicrobial and antibiofilm peptide (SAAP)-148 against different *Staphylococcus aureus* and *Staphylococcus epidermidis* strains.

Organism	Agent	MBC_{RPMI} in μM or mg/L[a]				Fold decrease of MBC_{RPMI} in combination		$FBCI_{opt}$		Outcome
		Alone		In combination						
		Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	
S. aureus ATCC 49230	LL-37	120	120	1.9	1.9	64	64	0.031	0.047	Synergy
	Teicoplanin	64	32	1.0	1.0	64	32			
	SAAP-148	1.9	1.9	0.47	0.47	4	4	0.38	0.28	Synergy
	Teicoplanin	16	32	2.0	1.0	8	32			
MRSA AMC201	LL-37	60	120	3.8	1.9	16	64	0.094	0.078	Synergy
	Teicoplanin	3	16	1.0	1.0	32	16			
	SAAP-148	1.9	0.94	0.47	0.23	4	4	0.31	0.50	Synergy
	Teicoplanin	32	32	2.0	8.0	16	4			
MRSA LUH15101	LL-37	60	30	1.9	1.9	32	16	0.094	0.125	Synergy
	Teicoplanin	16	16	1.0	1.0	16	16			
	SAAP-148	1.9	1.9	0.94	0.94	2	2	0.50	0.53	No interaction
	Teicoplanin	32	16	0.063	0.50	512	32			
S. epidermidis O-47	LL-37	3.8	3.8	1.9	0.23	2	16	1.0	0.56	No interaction
	Teicoplanin	2.0	2.0	1.0	1.0	2	2			
	SAAP-148	0.94	0.94	0.47	0.47	2	2	0.75	1.0	No interaction
	Teicoplanin	32	32	8.0	16	4	2			
S. epidermidis RP62A	LL-37	30	7.5	30	3.8	–	2	1.5	1.0	No interaction
	Teicoplanin	4.0	2.0	2.0	1.0	2	2			
	SAAP-148	0.94	0.94	0.059	0.94	16	–	0.56	1.0	No interaction
	Teicoplanin	8.0	2.0	4.0	0.063	2	32			
S. epidermidis AMC5	LL-37	3.8	15	0.23	0.47	16	32	1.1	1.0	No interaction
	Teicoplanin	20	2.0	2.0	2.0	–	–			
	SAAP-148	0.94	0.94	0.94	0.94	–	–	1.0	1.0	No interaction
	Teicoplanin	2.0	2.0	0.016	0.016	128	128			

$FBCI_{opt}$, optimal fractional bactericidal concentration index; MBC_{RPMI}, concentration that killed ≥99.9% of the bacteria in RPMI after 24 h; MRSA, meticillin-resistant *Staphylococcus aureus*.

[a] Concentration of LL-37 and SAAP-148 in μM, concentration of teicoplanin in mg/L.

Results of two independent experiments are shown. An $FBCI_{opt}$ value ≤0.5 indicates synergy, >0.5–4 indicates no interaction, and >4 indicates antagonism. A combination was considered synergistic or antagonistic if the two independent experimental results fell into the same category (synergy or antagonism); otherwise, the combination was considered non-interacting.

Table 5
Activity of teicoplanin in combination with LL-37, synthetic antimicrobial and antibiofilm peptide (SAAP)-148, SAAP-276 or TC84 against preformed *Staphylococcus aureus* JAR060131 biofilms

Agent	$MBEC_{99}$ in μM or mg/L[a]				Fold decrease of $MBEC_{99}$ in combination	
	Alone		In combination			
	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
LL-37	120	>120	60	60	2	>2
Teicoplanin	256	>256	64	128	4	>2
SAAP-148	60	120	15	60	4	2
Teicoplanin	256	>256	16	8.0	16	>32
SAAP-276	120	60	7.5	7.5	16	8
Teicoplanin	256	>256	2.0	4.0	128	>64
TC84	120	>120	120	>120	–	–
Teicoplanin	>256	>256	256	>256	≥2	–

$MBEC_{99}$, concentration that eradicated ≥99% of the bacteria after 24 h of incubation; CAMPs, cationic antimicrobial peptides.

[a] Concentration of CAMPs in μM, concentration of teicoplanin in mg/L.

Results of two independent experiments are shown. The highest concentration tested was 120 μM for the CAMPs and 256 mg/L for teicoplanin. When the highest concentration was not able to eradicate 99% of the bacteria in the biofilm, '>120' (μM) for the CAMPs or '>256' (mg/L) for teicoplanin was given in the table. The fold decrease of the concentrations in combinations as compared with the agents when used alone is given.

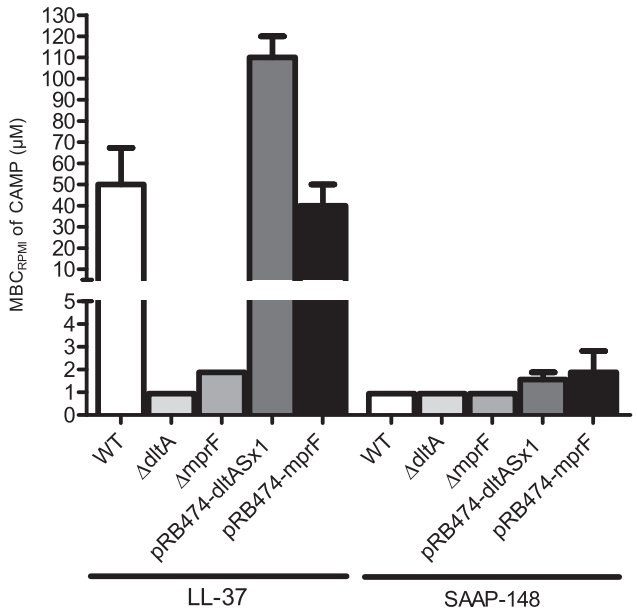

Fig. 1. Microbicidal activity of LL-37 and synthetic antimicrobial and antibiofilm peptide (SAAP)-148 against the *Staphylococcus aureus* SA113 wild-type (WT) strain, Δ*dlt*A mutant, Δ*mprF* mutant, Δ*dlt*A pRB474-dltASx1 complemented mutant and Δ*mprF* pRB474-mprF complemented mutant. All microbial susceptibility assays were performed three times independently. The mean and standard deviation are given for each peptide–strain combination. Of note, the graph is segmented to clearly visualize the low MBC$_{RPMI}$ values (concentration that killed ≥99.9% of the bacteria in RPMI after 24 h) of SAAP-148. CAMP, cationic antimicrobial peptide.

3.5. *Synergy of LL-37 with teicoplanin overcomes* dltA*BCD- and* mprF*-dependent LL-37 resistance in* S. aureus

Next, the study aimed to determine if the strong synergistic interaction between teicoplanin and LL-37 is dependent on *dlt*- and/or *mprF*-based resistance to LL-37. Teicoplanin and LL-37 were used in combination against the *S. aureus* SA113 WT strain, the *dltA* and *mprF* deletion mutants, and the complemented mutant strains (Fig. 2). The WT SA113 strain showed results similar to those of the other *S. aureus* strains tested previously (Table 4). LL-37 used in combination with teicoplanin had an FBCI$_{opt}$ of 0.13 against the WT SA113 strain, indicating synergy. The *dltA* deletion mutant was highly susceptible to LL-37 (MBC$_{RPMI}$ of 0.94 μM), and susceptibility was not further increased in combination with teicoplanin, so no synergy occurred. The optimal isoeffective MBC$_{RPMI}$ values of LL-37 and teicoplanin used in combination against the *dltA* complemented mutant were 3.8 μM and 1 mg/L, respectively, showing that the combination overcomes the *dlt*-dependent LL-37 resistance. The *mprF* deletion mutant was also highly susceptible to LL-37 (MBC$_{RPMI}$ of 1.9 μM), but despite this lack of resistance, addition of teicoplanin still increased susceptibility and synergy was apparent for the LL-37–teicoplanin combination. As with the *dltA* complemented mutant, the moderate LL-37 resistance of the *mprF* complemented mutant (MBC$_{RPMI}$ of 30 μM) was overcome by the combination of LL-37 with teicoplanin. In the optimal isoeffective combination, the MBC$_{RPMI}$ of LL-37 was ≤1.9 μM (i.e. ≥16-fold lower than the MBC$_{RPMI}$ of LL-37 when used alone).

4. Discussion

Screening for synergy between SAAP-148 and antibiotics with diverse modes of action against *S. aureus* revealed a synergistic effect of teicoplanin combined with SAAP-148. Interestingly, this combination did not synergize against *S. epidermidis* strains, and no interaction was found between CAMPs and vancomycin (analogous to teicoplanin) against *S. aureus*. Combining SAAP-148 or SAAP-276 with teicoplanin against notoriously difficult to treat *S. aureus* biofilms resulted in strong enhancement of the efficacy of teicoplanin, as judged from the reduction in MBEC$_{99}$. Although conferring resistance to LL-37, the *dltABCD* operon and the *mprF* gene did not provide resistance to SAAP-148. The addition of teicoplanin to LL-37 overcame *dlt*- and *mprF*-based resistance mechanisms through synergistic interaction.

Interaction of CAMPs with bacterial membranes can facilitate antimicrobial action of cell-wall-targeting antibiotics, and vice versa. The observation of synergy between teicoplanin and CAMPs is in line with earlier reports on synergy between glycopeptide antibiotics and CAMPs [27–33]. In several of these studies, certain CAMPs synergized with vancomycin against Gram-positive bacteria, including *S. aureus*; however, CAMPs used in this study showed no interaction with vancomycin. In previous work, Breidenstein et al. observed synergy between the CAMP plectasin NZ2114 and teicoplanin, and similar to the present findings, plectasin NZ2114 did not synergize with vancomycin [33]. Apparently, synergy requires a 'match' between the CAMP and the glycopeptide. Teicoplanin and vancomycin both bind the C-terminal $_{D}$-Ala-$_{D}$-Ala of the UDP-MurNAc-pentapeptide to subsequently inhibit transglycosylation and transpeptidation in the peptidoglycan synthesis [34]. However, the charge difference at physiological pH between vancomycin and teicoplanin (isoelectric points of 8.0 and 5.2, respectively) [35] and the lipid tail of teicoplanin which anchors it into the cytoplasmic membrane [36,37], and which is absent in vancomycin, could be of influence. The cytoplasmic membrane is likely to become more negatively charged by binding of teicoplanin, while vancomycin does not contribute to negative charge near the membrane. It is hypothesized that CAMPs, particularly those with a high positive net charge, may have a higher affinity to the bacterial membrane when the negatively charged teicoplanin is inserted, resulting in a synergistic effect (Fig. 3). Another possible mechanism that could drive the observed synergy is binding of teicoplanin to the $_{D}$-alanyl residues on the teichoic acids in the staphylococcal cell wall. The masking of the protonated $_{D}$-alanyl residues would, in theory, reduce the repulsion of cationic peptides (Fig. 3).

Biofilms associated with persistent infections pose major healthcare challenges. Therefore, the promising results of synergy between teicoplanin and CAMPs encouraged the authors to investigate the activity of teicoplanin in combination with LL-37, TC84, SAAP-148 and SAAP-276 against *S. aureus* biofilms. When used alone, teicoplanin, LL-37 and TC84 were only able to eradicate preformed biofilms of *S. aureus* JAR060131 at the highest concentrations tested. SAAP-148 and SAAP-276, however, did eradicate the biofilms at lower concentrations and were even more effective when combined with teicoplanin. Apparently, there are constraints to the structure, charge and/or size of CAMPs for synergy with teicoplanin. LL-37, which is larger than SAAP-148 and SAAP-276, might be less able to pass through *S. aureus* biofilm matrices. Single use of either SAAP-148 or SAAP-276 required relatively high concentrations to eliminate these biofilms; however, both SAAP-148 and SAAP-276 show potential in biofilm eradication when used in combination with teicoplanin.

The *S. aureus* WT strains used in this study were resistant to LL-37, but highly susceptible to SAAP-148. It is known from previous work that deletions in *dltABCD* and *mprF* render *S. aureus* more susceptible to a wide variety of CAMPs [12,13]. In this study, both the *dltA* and *mprF* deletion mutants were significantly more susceptible to LL-37 than the WT strain. However, the WT strain and the mutants were all killed by SAAP-148 at 0.94 μM, indicating that *dltABCD* and *mprF* confer resistance in *S. aureus* to LL-37, but not to SAAP-148. Importantly, addition of teicoplanin to LL-37 overcomes *S. aureus* resistance associated with the presence of *dltABCD* and *mprF*. The present results suggest that the electrostatic repulsion of CAMPs by the *S. aureus* WT cell envelope, caused by

A — *S. aureus* SA113 WT

LL-37 (μM) \ Teicoplanin (μg/mL)	64	32	16	**8**	4	2	1	0.5	0.25	0.13	0.06	0
240												
120												
60												
30										0.52	0.51	
15									0.28			
7.5								0.19				
3.8					0.56	0.31	0.19	0.13				
0												

B — *S. aureus* SA113 Δ*dltA*

LL-37 (μM) \ Teicoplanin (μg/mL)	64	32	16	8	4	**2**	1	0.5	0.25	0.13	0.06	0
3.75												
1.88												
0.94								1.3	1.1	1.1	1.0	
0.47							1.0					
0.23							0.75					
0.12						1.1						
0.06						1.1						
0												

C — *S. aureus* SA113 Δ*dltA* + pRB474-dltASx1

LL-37 (μM) \ Teicoplanin (μg/mL)	64	32	16	**8**	4	2	1	0.5	0.25	0.13	0.06	0
240												
120										1.0		
60									0.53		0.51	
30								0.31				
15							0.25					
7.5							0.19					
3.75					0.53	0.28	0.16					
0												

D — *S. aureus* SA113 Δ*mprF*

LL-37 (μM) \ Teicoplanin (μg/mL)	64	32	16	8	**4**	2	1	0.5	0.25	0.13	0.06	0
3.75												
1.88									1.1	1.0	1.0	
0.94								0.63				
0.47								0.38				
0.23							0.38					
0.12							0.31					
0.06						0.53						
0												

E — *S. aureus* SA113 Δ*mprF* + pRB474-mprF

LL-37 (μM) \ Teicoplanin (μg/mL)	64	32	16	8	**4**	2	1	0.5	0.25	0.13	0.06	0
120												
60												
30											1.0	
15									0.56	0.53		
7.5								0.38				
3.75							0.38					
1.88						0.56	0.31					
0												

Fig. 2. Checkerboard assays with LL-37 and teicoplanin in combination against the *Staphylococcus aureus* SA113 wild-type (WT) strain, Δ*dltA* mutant, Δ*mprF* mutant, Δ*dltA* pRB474-dltASx1 complemented mutant and Δ*mprF* pRB474-mprF complemented mutant. The white boxes in the block diagrams represent the wells in the microtitre plate where ≥99.9% of the bacteria were killed; the grey boxes represent the wells where >0.1% of the bacteria survived. The MBC_{RPMI} values (concentration that killed ≥99.9% of the bacteria in RPMI after 24 h) of the single agents are boxed and given in bold. Fractional bactericidal concentration index (FBCI) values are given in the white boxes that represent the isoeffective combinations. An $FBCI_{opt}$ value (i.e. the lowest FBCI of a checkerboard) ≤0.5 indicates synergy, >0.5–4 indicates no interaction, and >4 indicates antagonism. Experiments were performed in duplicate, and one representative result is shown. (A) SA113 WT strain, (B) SA113 Δ*dltA* mutant, (C) SA113 Δ*dltA* complemented mutant, (D) SA113 Δ*mprF*, (E) SA113 Δ*mprF* complemented mutant. Of note, the scales for the LL-37 concentrations in (B) and (D) are smaller than in (A), (C) and (E).

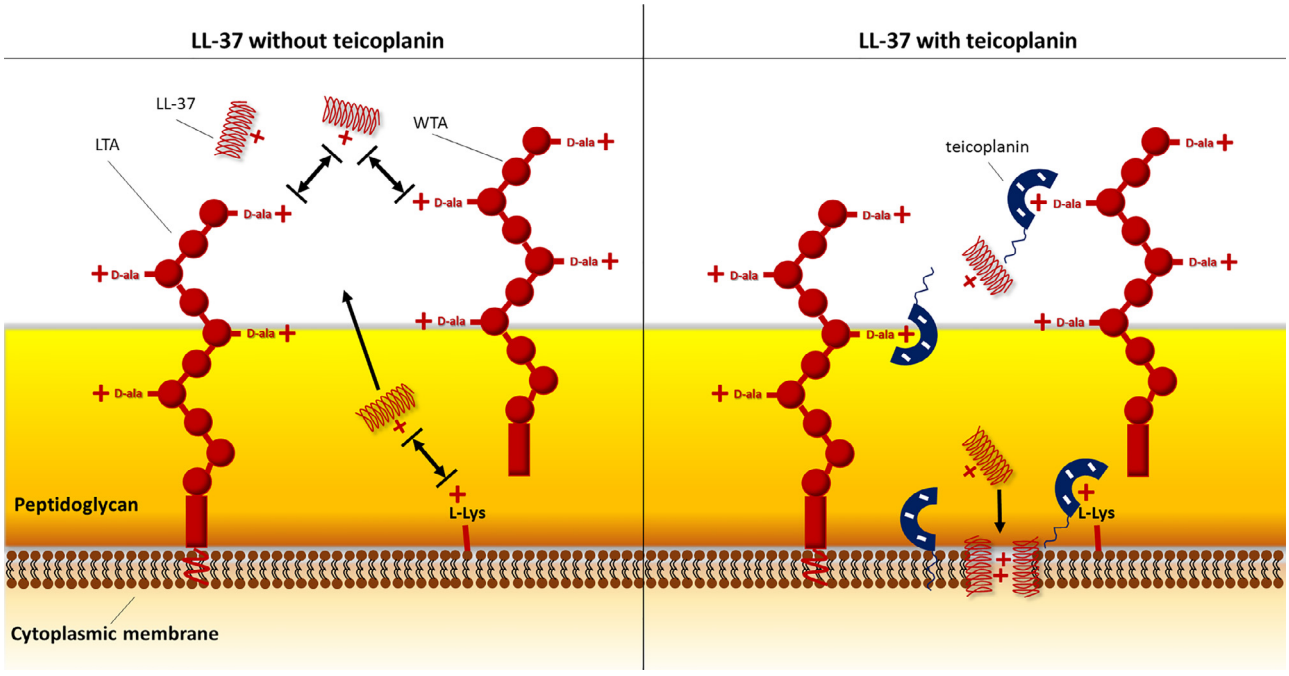

Fig. 3. Model of synergy between LL-37 and teicoplanin against *Staphylococcus aureus*. When LL-37 is used alone (left panel), electrostatic repulsion by the $_D$-alanyl ($_D$-ala) teichoic acids (LTA/WTA) and lysinylated ($_L$-lys) phosphatidylglycerol prevents LL-37 molecules from accumulating at the cytoplasmic membrane. Addition of teicoplanin (right panel) could: (i) contribute to negative charge near the membrane via membrane insertion, (ii) shield the positive charges of the $_D$-alanyl substituted teichoic acids, and (iii) mask the positively charged $_L$-lysyl residues coupled to phosphatidylglycerol. These mechanisms could abrogate electrostatic repulsion and thereby increase the accessibility of the bacterial membrane for LL-37 molecules.

$_D$-alanine substitution of teichoic acids and addition of $_L$-lysine to phosphatidylglycerol, is cancelled out by the negatively charged teicoplanin molecule (Fig. 3). *S. epidermidis* strains were more susceptible to LL-37 than WT *S. aureus*, and no synergy of CAMPs with teicoplanin against *S. epidermidis* was observed. It is important to note that *S. aureus* contains comparatively high amounts of lysylphosphatidylglycerol, whereas *S. epidermidis* produces only trace amounts of this substituted phospholipid [38]. Indeed, the susceptibility of *S. epidermidis* for CAMPs was similar to the susceptibility of the *S. aureus mprF* mutant, which is not capable of phosphatidylglycerol lysinylation.

The need for more antimicrobial treatment options approved for clinical use has never been more pressing. SAAP-148 and SAAP-276 show higher staphylocidal potency than other synthetic CAMPs, even those in clinical development [8]. Furthermore, these CAMPs show no resistance invocation [8,9], and seem to be promising for clinical applications such as implant coatings to prevent biomaterial-associated infections [9], or topical creams and other locally applied products for patients suffering from bacterial skin infections [8]. Currently, implant-associated infections often result in replacement of the implant combined with the local and systemic delivery of conventional antibiotics, which are infamous inducers of antimicrobial resistance and are often ineffective in preventing biofilm formation [39]. In light of this study, it is suggested that implant coatings containing teicoplanin in combination with CAMPs like SAAP-148 or SAAP-276 should be designed in order to benefit from synergy, avoid resistance development, prevent biofilm formation and ensure a broad antimicrobial spectrum.

5. Conclusions

Teicoplanin synergized with the human innate CAMP LL-37 and with the synthetic CAMPs SAAP-148, SAAP-276 and TC84 against the opportunistic human pathogen *S. aureus*. Combined use of SAAP-148 or SAAP-276 with teicoplanin was effective against teicoplanin-tolerant biofilms of *S. aureus*. Furthermore, the combination of teicoplanin and LL-37 overcomes *dltABCD*- and *mprF*-based resistance of *S. aureus* for LL-37, suggesting an enhancing effect of this antibiotic on the innate LL-37 activity during infection. Thus, these results show the importance of selecting antibiotics for their synergy with endogenous antimicrobial molecules to enhance innate immune effectivity, and of clearly defining the spectrum of CAMPs that act synergistically with antibiotics for optimal clinical application development.

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Competing interests

The Amsterdam UMC and the Leiden University Medical Centre are holders of Patent No. WO2015088344, relating to the synthetic peptides SAAP-148 and SAAP-276 used in this paper.

Ethical approval

None sought.

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