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Synergy of short antimicrobial peptides with β-lactam antibiotics against MRSA resides in the degradation of peptidoglycan barrier

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Introduction

The emerging resistance of bacteria to currently used antibiotics is becoming a significant global problem that requires searching for alternative antimicrobial agents. One of the most frequently reported pathogens, methicillin-resistant *Staphylococcus aureus* (MRSA), which causes infections of wounds, bones, implants, bloodstream, skin, pneumonia, etc., is a typical example of Gram-positive bacterium that possess resistance to several antibiotics, including β-lactams. Therapeutic options are becoming limited, and vancomycin remains as the last resort for treating MRSA, but not for long.

Antimicrobial peptides (AMPs) have long been considered as a possible new class of anti-infective agents that could be used as a supplement to, or substitute for, conventional antibiotics in the fight against multi-drug-resistant bacteria. Their potential advantage resides in a unique mechanism of action that involves interacting with the negatively charged phospholipid bilayer of bacterial cell membrane causing its disruption *via* pore formation or detergent-like disintegration, thereby leading to cell death. However, in Gram-positive bacteria, AMPs have to first pass through the cell wall that consists prevalently of a peptidoglycan layer, before reaching its target - the cytoplasmic membrane.

Several reports have shown a significant synergistic antimicrobial effect when AMPs were applied in combination with common antibiotics. In this work, we examined a combination of short linear AMPs, previously invented in our laboratory, with β-lactam antibiotics against a reference strain of MRSA (ATCC 43300) in order to shed light on the mechanism of the synergy between AMPs and β-lactam antibiotics.

Results and Discussion

In this study we used four AMPs (I: GKWMKLLKKILK-NH_2, VI: GKWVKLLKKILK-NH_2, VIII: GKWMKLLKKILK-NH_2, and IX: KWMKLLKKILK-NH_2) which were derived from the natural AMP halictine-2 [1] and were characterized recently [2]. These peptides showed potent anti-staphylococcal activity in concentrations ranging from 8 to 20 μM (Table 1). MIC values for both peptides and antibiotics were noticeably higher when they were measured in hypertonic LB 20 medium (LB medium supplemented with 20% [wt/vol] sucrose). All tested peptides acted synergistically in combination with β-lactam antibiotics - amoxicillin, ceftazidime, cefuroxime, and meropenem (FIC < 0.5, Table 2). On the other hand, the peptides showed only an additive effect with vancomycin (0.5 < FIC ≤2). Synergy between short α-helical peptides and β-lactam was previously described [3]. Some authors proposed a mechanism hypothesizing that the cell wall breakdown caused by β-lactams resulted in the enhanced access of AMPs to the cytoplasmic membrane [3, 4].

Table 1: Minimum inhibitory concentrations (MIC) of peptides and antibiotics against MRSA ATCC 43300 and its protoplasts measured in two different media.

Peptide Antibiotic	MIC (μM)		
	MRSA[a)]	Intact MRSA[b)]	MRSA protoplasts[b, c)]
Peptide I	8.1 ± 0.3	14.8 ± 1.7	3.8 ± 0.6
Peptide VI	15.5 ± 2.0	24.0 ± 2.0	6.0 ± 0.5
Peptide VIII	20.0 ± 1.1	48.0 ± 4.4	6.2 ± 1.0
Peptide IX	15.8 ± 0.6	27.0 ± 3.0	6.6 ± 0.6
Amoxicillin	20.7 ± 1.4	106.6 ± 8.0	>500
Ceftazidime	57.3 ± 5.0	466.7 ± 27.2	>500
Cefuroxime	9.4 ± 0.6	>500	>500
Meropenem	6.2 ± 0.6	26.9 ± 5.8	>500
Vancomycin	0.9 ± 0.1	0.8 ± 0.1	4.0 ± 2.1

a) Determined in LB medium; b) determined in LB20 medium; c) protoplast were prepared by digesting the peptidoglycan layer of bacteria with lysostaphin

To validate this theory, we measured MIC values for all AMPs against MRSA cells which were previously transformed into their protoplasts – cells that lack a cell wall. Interestingly, they exhibited a significant four-to eight-fold reduction. This was in contrast to all the antibiotics which had either higher, or at least same, MIC values against the protoplast compared to intact MRSA (Table 1). The protoplasts became resistant to the antibiotics since β-lactams and vancomycin are known to inhibit cell wall synthesis but do not act on the bacterial cell membrane.

Moreover, AMPs, as cationic molecules, can interact not only with the phospholipids of bacterial membranes but also with peptidoglycan, which contains components carrying a negative charge. In fact, such a study describing the direct interaction of AMPs with peptidoglycan was recently published [5].

Assuming that the peptide may bind to the peptidoglycan barrier before reaching the cytoplasmic membrane, we studied its interaction with peptidoglycan isolated from *Staphylococcus aureus* (InvivoGen, France). The study, based on RP-HPLC methodology, showed that when 10 μM of a peptide was mixed with peptidoglycan (0.02 mg in 1 mL), up to 25% of the peptide was almost immediately trapped into peptidoglycan. Under the same conditions, approximately the same amount of peptide was also absorbed into MRSA cells without killing them immediately, but the cells were killed during next two hours as determined by CFU counting.

Table 2: Fractional inhibitory concentration (FIC) indexes for the combination of studied peptides with selected antibiotics against MRSA ATCC 43300.

Peptides	Antibiotics				
	amoxicillin	ceftazidime	cefuroxime	meropenem	vancomycin
Peptide I	**0.30 ± 0.01**	**0.42 ± 0.03**	**0.33 ± 0.03**	**0.24 ± 0.04**	0.67 ± 0.07
Peptide VI	**0.25 ± 0.00**	**0.44 ± 0.05**	**0.35 ± 0.02**	**0.25 ± 0.00**	0.83 ± 0.14
Peptide VIII	**0.31 ± 0.03**	**0.38 ± 0.06**	**0.33 ± 0.07**	**0.21 ± 0.02**	0.86 ± 0.15
Peptide IX	**0.25 ± 0.00**	**0.38 ± 0.00**	**0.35 ± 0.06**	**0.20 ± 0.02**	1.07 ± 0.02

The FIC indexes were interpreted as follows: FIC $\leq$ 0.5 is synergistic (in bold), 0.5 < FIC $\leq$ 2 is additive, and FIC > 2 is antagonistic effect.

Our results indicate that the binding of AMP to peptidoglycan (and thus the bacterial cell wall) causes considerable interference during AMP-mediated killing of the bacteria due to a decreased AMP concentration that is available for the disruption of the cytoplasmic membrane. We hypothesize that in the course of the combination treatment, β-lactams cause the degradation of the peptidoglycan layer, resulting in a weakening of the cell wall, thus allowing AMP easier access to the cytoplasmic membrane and its subsequent disruption. Consequently,

lower concentrations of both antibiotics and AMPs are required to kill the bacteria that finally results in a synergistic effect. This would also explain the different MIC values of AMPs against intact MRSA and the protoplasts. Our conclusion is in accordance with previously published data showing that treatment of *S. aureus* with β-lactam antibiotics can result in its conversion to protoplasts [6]. In addition, vancomycin, which is known to inhibit cell wall synthesis, did not show any synergy with the peptides. The increase in its MIC against the protoplast, compared to intact MRSA, was only four-fold, thus, indicating that vancomycin probably possesses other direct effects on the bacterial cytoplasmic membrane other than cell wall inhibition, as was proposed by Hancock and Fitz-James [7]. This could explain the additive effect of combining vancomycin with AMPs, as they also disintegrate the membrane.

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