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Comparison of in vitro antibacterial activities of two cationic peptides CM15 and CM11 against five pathogenic bacteria: *Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio cholerae, Acinetobacter baumannii,* and *Escherichia coli*

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Abstract In recent years, the widespread use of antibiotics has caused many bacterial pathogens resistance to conventional antibiotics. Therefore, generation of new antibiotics to control and reduce the effects of these pathogens is urgently needed. Antimicrobial peptides and proteins are important members of the host defense system in eukaryotes. These peptides are potent, broad-spectrum antibiotics that demonstrate potential as novel and alternative therapeutic agents for the treatment of drug-resistant infections. Accordingly, we evaluated two hybrid peptides CM11 (WKLFKKILKVL-NH2) and CM15 (KWKLFKK IGAVLKVL-NH2) on five important pathogenic bacteria. These peptides are short cecropin–melittin hybrid peptides obtained through a sequence combination approach, which are highly effective to inhibit the growth of important pathogenic bacteria. The activity of these two cationic peptides (CM11 and CM15) in different concentrations (2–64 mg/L) was investigated against standard and clinical isolates of important hospital infection bacteria by measuring MIC, MBC, and bactericidal assay. These peptides demonstrated the same ranges of inhibitory values: The organisms in early 24 h were more susceptible to polycationic peptides (MIC: 8 mg/L and MBC 32 mg/L), but after 48 h the MIC and MBC remained constant for the CM11 peptide. Bactericidal assay showed that all bacteria strains did not have any growth in agar plates after 40 min. The result showed that these two peptides are more effective than other peptides.

Keywords Hybrid peptide · Cecropin · Melittin · Antimicrobial peptides

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Introduction

Antimicrobial peptides (AMPs) virtually form an essential part of the innate immune system of all forms of life [1–5]. During the last decades, AMPs have been widely studied, as they may become an alternative to conventional antibiotics, especially for the treatment of drug-resistant infections [6–8]. Hundreds of antimicrobial peptides have been isolated and several thousands have been de novo designed and synthetically produced. They display a wide range of biological activities against bacteria, fungi, protozoa, enveloped viruses, and even tumor cells [9–16].

Many different peptide antibiotics have been identified, including defensins [17], insect cecropins [18], magainins, and melittin [19]. Cecropins and melittin belong to the group of antimicrobial peptides that exist in a random-coil

configuration in aqueous solutions but adopt a helix-turn-helix structure upon interaction with membranes [20, 21]. Cecropins, first isolated from the hemolymph of the giant silk moth *Hyalophora cecropia*, are some of the best studied antimicrobial peptides. These peptides are composed of 31–39 amino acids with antibacterial activity against both Gram-negative and Gram-positive bacteria but Gram-negative bacteria are generally more sensitive. Cecropins do not exhibit cytotoxic effects against human erythrocytes and other eukaryotic cells, but are susceptible to protease degradation [22, 23]. Also melittin, which is a linear 26-residue noncell-selective antimicrobial peptide, is isolated from the venom of European honey bee, *Apis mellifera*. Melittin displays strong lytic activity against bacteria and human red blood cells. Both of these peptides have one amphipathic α-helix and one hydrophobic α-helix, but the order of these helices in the two peptides is inverted.

To overcome the high production costs of such long peptides and to improve their biological properties and reduce toxicity, short peptide analogs have been designed and synthesized. Studies in this field led to the identification of nontoxic and more stable peptide sequences displaying a broader and higher activity than their natural counterparts including cecropin–melittin hybrid peptides that possess the amphipathic N-terminal α-helix of cecropin followed by the hydrophobic N-terminal α-helix of melittin [24, 25]. These peptides have been shown to have a broad range of antibacterial activity against both Gram-negative and Gram-positive bacteria with lower cytotoxic activity for mammalian cells [25].

A hybrid cecropin–melittin is one of them consisting of seven residues from 1 to 7 residues in the first segment of cecropin A and 8 residues from 2 to 9 of melittin, which was identified as the minimal sequence that has an antimicrobial effect. This recombinant peptide was named CM15 and like the native cecropin has two parts that consists of a highly basic N-terminal domain from cecropin A and relatively hydrophobic C-terminal domain from melittin [24].

In similar to CM15, WKLFKKILKVL-NH2 (pep3), a hybrid peptide derived from 2 to 8 cecropin A residues and from 6 to 9 residues of melittin with two parts that consists of a highly basic N-terminal domain from cecropin A and relatively hydrophobic C-terminal domain from melittin has been found to be sufficient for antifungal and antibacterial activities, while displaying low cytotoxicity [24, 26].

The mechanism of bactericidal activity by these peptides has not been firmly identified, but it has been suggested that the bactericidal process is occurred. Studies have shown that cationic peptides cross the outer membrane by the self-promoted uptake pathway. In this pathway, cationic compounds displace the divalent cations that form stabilizing cross bridges between adjacent lipopolysaccharide (LPS) molecules. This results in a localized outer membrane perturbation through which the cationic compound is taken up, following this process, disruption of the cytoplasmic membrane occurs that leads to bacterial cell death. This process do not have a specific receptor, but the distortion of bilayer is occurred by disruption or pore formation via direct interaction with cell membrane [27]. In addition, the antimicrobial peptides have anti-endotoxic properties that comes from their ability to contact with the anionic and amphipathic nature of lipid A in LPS structure of Gram-negative bacteria [28]. During the last decades, resistance to most of the clinically available antimicrobial agents has emerged among several pathogens. Today antibiotic-resistant *Staphylococcus aureus, Pseudomonas aeruginosa, Vibrio cholerae, Acinetobacter baumannii,* and *Escherichia coli* are important hospital infection problem as an emerging cause of antimicrobial treatments failure and an increasing problem in community-acquired infections. In the hospital environment, antibiotic resistance to a variety of agents classes other than lactams is responsible for many life-threatening infections [29]. The species belonging to the *A. baumannii* and *P. aeruginosa* complexes act as causative agents for a wide variety of clinical conditions [30, 31]. It is particularly insidious in intensive care units, where the use of invasive devices, broad-spectrum antibiotics, and prolonged stay patient are associated with high morbidity and mortality rates [32–34].

Acinetobacter species and *P. aeruginosa* have shown an outstanding capacity to develop resistance against common antibiotics such as carbapenems and other broad-spectrum.

β-lactams, tetracyclines, fluoroquinolones, and aminoglycosides through a wide variety of mechanisms. This has led to a practical exhaustion in the repertoire of active antibiotics, including imipenem, which until recently was considered the gold standard for Acinetobacter treatment [26].

Methicillin-resistant *S. aureus* (MRSA) is responsible for a large proportion of nosocomial infections that makes treatment difficult due to the increasing resistance to multiple antibiotics [35]. Also *E. coli* is an important nosocomial pathogen that causes both community and nosocomial urinary tract infection (UTI) [36]. *Vibrio cholerae* is a water- and foodborne organism that can cause acute watery diarrhea, vomiting, severe dehydration and death. Similar to other pathogenic bacteria that resist to various antibiotics, *V. cholerae* strains have been isolated from both clinical and environmental place [37].

As mentioned previously, the resistance of pathogenic bacteria to various antibiotics are expanded, thus it appears that utilizing alternative agents are necessary to eliminate resistant pathogenic bacteria.

In this work, we determined the activity of selected cecropin–melittin CM11 (WKLFKKILKVL-NH2) and CM15 (KWKLFKKIGAVLKVL-NH2) hybrid peptides against five selected clinical strains of hospital infection

with the different degrees of antibiotic resistance and compared CM11 and CM15 with each other. This work is the first step toward the in vitro assay of these peptides as an alternative to antibiotics.

Materials and Methods

Peptide Synthesis

The CM15 and CM11 hybrid peptides were synthesized as a C-terminal carboxamide on a Rink p-methylbenzhydrylamine resin by the solid-phase synthesis method using standard method [38]. The peptides were purified by reversed-phase semipreparative HPLC on C18 Tracer column using a linear gradient from 10 to 60% acetonitrile in water with 0.1% trifluoroacetic acid over 50 min. The peptides were obtained with >95% HPLC purity. Electrospray ionization mass spectrometry was used to confirm peptide identity**.**

The in vitro activity of two cationic peptides, CM15 (KWKLFKKIGAVLKVL-NH2) and CM11 (WKLFKKILKVL-NH2) was separately investigated in different concentrations (2 to 128 mg/L) against five standard strains and clinical isolates by the standard method of macro dilution. Antimicrobial activities were measured by MIC, MBC, and bactericidal assay.

Bacterial Strains

The control strains *P. aeruginosa* ATCC 27853*, V. cholerae* ATCC 11623, *A. baumannii* ATCC 17978, *S. aureus* subsp. *aureus* ATCC 33592, *E. coli* ATCC 43890 and 40 clinical isolates of *P. aeruginosa*, 30 clinical isolates of *V. cholerae*, 30 clinical isolates of *A. baumannii*, 40 clinical isolates of *S. aureus*, 40 clinical isolates of *E. coli* were tested. All clinical isolates used in this experiment have received from clinical microbiology laboratories and were confirmed by criteria laboratory control tests in these laboratories.

Antibiotic Resistance Assay

The agar disk diffusion test was used for investigating antibiotic resistance. The tests were carried out in Mueller–Hinton agar using 0.2 mL of inoculums (10^8 cells/mL) and special antibiotic disks were selected according to the National Committee for Clinical Laboratory Standards (NCCLS 2010, Table 1) [39]. The rate of antimicrobial resistance was determined by measuring the diameter of inhibition zone disk.

Peptide Soluble Preparation

The peptides were solubilized in phosphate-buffered saline (pH 7.2) to yield 1 mg/mL solution.

MIC and MBC Determinations

To measure antibacterial activity of CM15 and CM11 peptides, minimal inhibitory concentrations (MICs) were determined using a broth macro dilution method with Mueller–Hinton broth and an initial inoculum of $\sim 5 \times 10^5$ CFU/mL according to the procedures outlined by the NCCLS. Bacterial cultures with different peptide

Table 1 Antibiotic resistance patterns of five strains, *S. aureus*, *P. aeruginosa, V. cholerae, A. baumannii,* and *E. coli*

Antibiotic disks	Resistance (%)				
	S. aureus	*P. aeruginosa*	*V. cholerae*	*A. baumannii*	*E. coli*
Kanamycin	>80	–	>70	–	>60
Ceftazidime	–	–	–	>85	>80
Penicillin	>90	–	–	–	–
Rifampin	>70	–	–	–	–
Gentamicin	>80	>90	>75	>85	>75
Amikacin	–	>65	–	>60	>50
Ciprofloxacin	>75	>80	>80	>85	>90
Imipenem	–	>75	–	>90	>80
Norfloxacin	>80	>80	>80	–	>85
Cefotaxime	–	>60	–	>90	>85
Chloramphenicol	–	–	>85	–	–
Ampicillin	–	–	>70	–	–

Antibiotic resistance patterns for clinical isolates

Antibiotics selected according NCCLS-2010 for each bacterium

The data in each column was a representative of three independent experiments ($p < 0.05$)

concentrations from 2 to 128 mg/L were incubated in a shaking bath for 18 h at 37 °C. The lowest peptides concentration that inhibited bacterial growth was considered minimal inhibitory concentration (MIC). The minimal bactericidal concentration (MBC) was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction in the initial inoculums. Experiments were performed in triplicate.

Bacterial Killing Assay

Tubes containing freshly prepared Mueller–Hinton broth supplemented with minimal inhibitory concentration of CM15 and CM11 peptides were inoculated with standard and clinical strains to a density of $\sim 5 \times 10^5$ CFU/mL and incubated in a shaking bath at 37 °C. Aliquots were removed after 0, 5, 10, 15, 20, 30, 40, 50, 60 min. Samples were diluted serially and plated on Mueller–Hinton agar plates to obtain viable colonies.

Statistical Analysis

Statistical analysis was done by SPSS 15.0 (SPSS Inc, Chicago,IL). The data in each figure was a representative of three independent experiments expressed as the mean ± standard deviation (SD). The level of significance was determined at $p < 0.05$.

Results

Antibiotic Resistance Assay

To evaluate the antibiotic resistance of bacteria strains, we investigated the growth inhibitory effect of selected antibiotics on bacteria by measuring the diameter of inhibition zone around each antibiotic disk. Results were analyzed by NCCLS standards for each strain and relate antibiotics. Selection of the bacterial strains with highest resistance pattern was used after antibiogram test; results are summarized in Table 1.

MIC and MBC Determination

The peptides demonstrated same ranges of inhibitory values: the organisms in early 24 h were more susceptible to polycationic peptides (MIC: 4 mg/L and MBC 16 mg/L), but after 48 h, the MIC and MBC remained constant for the shorter peptide (CM11), the other peptide (CM15) was increased to two times. The MIC and MBC results are summarized in the Table 2.

Bacterial Killing Assay

Viable counts of *S. aureus, P. aeruginosa, V. cholerae, A. baumannii,* and *E. coli* treated with two CM11 and CM15 peptides are shown in Fig. 1a and b.

The time-kill curve was determined for the survival of five bacterial strains after challenge with the MIC of CM11 (Fig. 1a) and CM15 (Fig. 1b) peptides. The viable bacterial concentration decreased between 0 and 10 min and reached a plateau between 10 and 20 min, during the treatment with the MIC of peptides CM11 and CM15. There was a statistically significant difference between test and control groups ($p < 0.05$).

For each time, two peptides showed similar bactericidal effect, but reducing bacterial cell between times was more tangible for CM11 peptide. Also for *A. baumannii* and *E. coli,* bactericidal activity was completed after a 30 min, but it was completed for other bacteria after a 40 min.

Discussion

The activity of cationic antimicrobial peptides has been mainly connected to their interaction with membranes. The studies proved that for many of these peptides, membrane disruption is the primary mechanism of bactericidal activity [15, 40].

Antimicrobial cationic peptides play a significant role in host defenses and are now being considered for use as therapeutic agents, it is necessary to understand how these peptides work. Some studies have shown the ability of these peptides to form channels in lipid bilayer membranes [41]. In contrast, few studies have dealt with the issue of how these peptides interact with and cross the barrier of outer membrane in Gram-negative bacteria. The self-promoted uptake pathway that was originally proposed to be used by the cationic antibiotics polymyxin B and gentamicin was also suggested as the mechanism of uptake for the defensins macrophage cationic proteins 1 and 2 across the outer membrane of *P. aeruginosa* [42, 43]. Elucidating their mechanism of action and their specific membrane damaging properties are crucial for the rational design of novel antibiotic peptides with high antibacterial activity and low cytotoxicity [15].

In this study, we tested two small peptides (CM11 and CM15) against five important hospital infection strains of bacteria. Our results showed that these peptides are highly active against clinical isolates of *S. aureus, P. aeruginosa, V. cholerae, A. baumannii, and E. coli*. It seems that CM11 and CM15 similar to many cationic peptides might act by interaction with bacteria cell membrane and initiate the activity of bacterial lysis leading to the damage of cytoplasmic membrane structure [29].

Table 2 The MIC and MBC of CM11 and CM15 peptides against five strains *P. aeruginosa, V. cholerae, A. baumannii*, *S. aureus* and *E. coli*

	Peptide	MIC (mg/L)			MBC (mg/L)		
		Range	50%	90%	Range	50%	90%
Standard strains							
P. aeruginosa ATCC 27853	CM11	4	–	–	16	–	–
	CM15	4	–	–	16	–	–
V. cholerae ATCC 11623	CM11	4	–	–	16	–	–
	CM15	4	–	–	16	–	–
A. baumannii ATCC 17978	CM11	4	–	–	16	–	–
	CM15	4	–	–	16	–	–
Staphylococcus subsp.*aureus* ATCC 33592	CM11	4	–	–	16	–	–
	CM15	4	–	–	16	–	–
E. coli ATCC 43890	CM11	4	–	–	16	–	–
	CM15	4	–	–	16	–	–
Clinical isolates							
P. aeruginosa (40 isolates)	CM11	2–32	4	8	8–64	16	32
	CM15	4–32	4	8	16–128	16	32
V. cholerae (30 isolates)	CM11	2–16	4	8	16–64	16	32
	CM15	4–32	4	8	16–128	16	32
A. baumannii (30 isolates)	CM11	2–32	4	8	8–64	16	32
	CM15	4–64	4	8	16–128	16	32
S. aureus (40 isolates)	CM11	2–32	4	8	16–64	16	32
	CM15	4–32	4	8	16–64	16	32
E. coli (40 isolates)	CM11	4–32	4	8	16–64	16	32
	CM15	4–32	4	8	16–64	16	32

MIC minimal inhibitory concentration, *MBC* minimal bactericidal concentration

To obtain MIC and MBC, peptides were used at low to high concentration to find the concentration that inhibits bacterial growth in broth and agar cultures, respectively. Results showed the MIC and MBC of CM15 and CM11 peptides against the clinical isolates were significantly increased in comparison with standard controls. According to the bacteriostatic effect of antibacterial agents in MIC, we found that in the minimal inhibitory concentration of these peptides, the bacteria challenged with CM11 between 24 and 48 h have not grown, but the bacteria challenged with CM15 have grown at the same time. However, CM11 peptide is more effective than CM15 peptide in first 48 h; moreover, minimum bacterial growth inhibition in both peptides was the same. Time-killing curves were used to evaluate the antibacterial activities of CM15 and CM11 peptide.

Giacometti et al. (2004) investigated the antibacterial activity of CM15 peptide on clinical isolates of *S. aureus* [29]. Their results showed that all isolates were inhibited by CM15 at concentrations of 1–16 mg/L, with MIC_{50} of 4 mg/L and MIC_{90} of 8 mg/L. For the control strain of *S. aureus*, peptide exhibited MIC and MBC of 2 and 4 mg/L, respectively, which are similar to our results. Also bacteria's killing by CM15 was completed after 20 min at a concentration of 8 mg/L, while our results showed that bactericidal activity by CM15 and CM11 peptides were completed after 30 min at a concentration of 4 mg/L for *S. aureus, P. aeruginosa,* and *V. cholerae* and for *A. baumannii* and *E. coli* after 40 min.

Rodriguez-Hernandez et al. [44] and Saugar et al. [26] reported the antibacterial activity of several peptides on clinical isolates of *A. baumannii*, among these peptides CM15 was used for antibacterial activity test [26, 43]. Their results showed that MIC for 3 standard strains of *A. baumannii* is 2 mg/L that is half of peptide concentration in our standard test, also in this research, results of clinical isolates showed a MIC range between 4 and 64 mg/L for CM15 peptide and 2–32 mg/L for CM11 peptide, which demonstrated that the clinical isolates are lower sensitive to CM15 peptide.

Also Ferre et al. [45] studied the antibacterial activity of CM11 (Pep3) peptide and 22 new analogs against the plant phytopathogenic bacteria *Erwinia amylovora*, *Xanthomonas vesicatoria* and *Pseudomonas syringae*. Their results showed that 10 to 14 mg/L concentration of CM11 was operative on three bacteria.

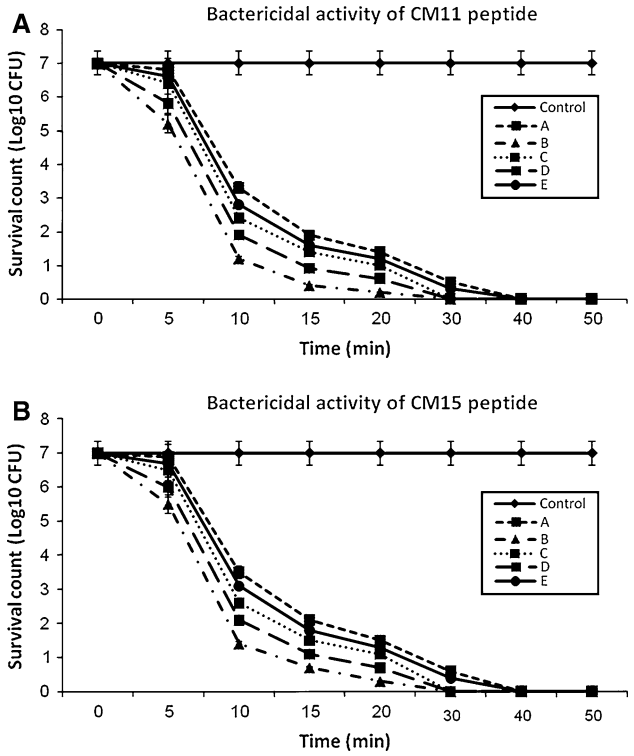

Fig. 1 Time-kill determinations for five strains, (*A*) *A. baumannii,* (*B*) *V. cholerae* (*C*) *P. aeruginosa,* (*D*) *S. aureus,* and (*E*) *E. coli* after treatment with two peptides CM11 (**a**) and CM15 (**b**). The *x*-axis represents the killing time, and the y-axis represents the logarithmic bacterial strains survival. The test of survival count was performed 3 times on different days, and the means and standard deviations are indicates a statistically significant difference survival rates ($p < 0.05$)

These different levels of bacterial susceptibility to antibacterial peptides with different amino acid sequences have been attributed to the variation in the plasma membranes components of target microorganism, for example, charge and lipid composition, which would influence the rates of binding of cationic peptides to the membranes.

In summary, the present study demonstrated that small peptides (CM11, CM15) have significant activity against clinical isolates of *S. aureus, P. aeruginosa, V. cholerae, A. baumannii, and E. coli* in vitro. We hope that these findings will lead to new treatment strategies for the eradication of resistance hospital infections, which is closely associated with persistent hospital environment.

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