REPORT

Antibacterial activity of a peptide derived from HIV-1 MN strain gp41 envelope glycoprotein against methicillin-resistant *Staphylococcus aureus*

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Abstract: Peptides derived from HIV-1 transmembrane proteins have been extensively studied for antimicrobial activities, and they are known as antimicrobial peptides (AMPs). These AMPs have also been reported to potently combat the drug-resistant microbes. In this study, we demonstrated that peptide #6383 originated from HIV-1 MN strain membrane-spanning domain of gp41 was active (2-log reductions) at 100µg/mL (56.5µM) against methicillin-resistant *Staphylococcus aureus* (MRSA) in 10% and 50% human plasma-supplemented phosphate buffered saline (PBS). The activity was further enhanced (3-log reductions) in the presence of 5% human serum albumin (HSA) alone. All bactericidal activities were achieved within 6 hours. At 100µg/mL, the peptide showed only 13% toxicity against human erythrocytes. This peptide can serve as an attractive template for a design of a novel peptide antibiotic against drug-resistant bacteria. By sequence-specific engineering or modifications, we anticipated that the bactericidal activity and the reduced toxicity against human erythrocytes will be improved.

Keywords: HIV-1 glycoprotein; antimicrobial peptide; MRSA; hemolysis; plasma; serum albumin.

INTRODUCTION

Emergence of multidrug-resistant bacteria in a striking manner has accelerated the search for novel microbicidal agents. AMPs that play important roles in first-line defense system for most organisms against invasion of microbes, are attractive sources of these agents. Due to several important features, they are ideal candidates to be used to combat multidrug-resistant bacteria (Marr *et al.*, 2006; Joanne, 2009; Huang *et al.*, 2010; Splith and Neundorf, 2011; Kang *et al.*, 2012). AMPs are microbicidal against a broad spectrum of bacteria (Pereira, 2006) at low doses (micromolar) (Splith and Neundorf, 2011) and with rapid activity (within minutes) (Boman, 2003). Unlike antibiotic treatments, the emergence of AMPs-resistant phenotype via multiple passages of bacterial strains is uncommon (Yeaman and Yount, 2003). In addition, they can act synergistically with conventional antibiotics to comprehensively kill the bacteria (Giacometti *et al.*, 2000).

The bactericidal activities of AMPs have been associated with their interactions with the anionic targets on microbial surfaces (Kang *et al.*, 2012). This electrostatic binding subsequently results in membrane disruption and cell damage that eventually lead to cell death (Otvos, 2005; Bechinger and Lohner, 2006; Huang, 2006; Kang *et al.*, 2012). Despite many AMPs exert potent activities *in vitro*, their activities are generally limited in physiological conditions (Tencza *et al.*, 1997; Zasloff, 2002). Moreover, toxicity to mammalian cells and sensitivity towards proteolytic degradation are the major obstacles (Van 'T Hof *et al.*, 2001; Marr *et al.*, 2006). To overcome these challenges, many of the highly potential AMPs that serve as lead compounds, have been subjected to amino acid sequence and structural modifications (Tencza *et al.*, 1999; Phadke *et al.*, 2003; Deslouches *et al.*, 2005).

In this study, we selected a peptide (#6383) derived from HIV-1 MN strain envelope glycoproteins, gp41 for evaluation of the antimicrobial activity against MRSA. As a comparison, its antibacterial activity against methicilin-sensitive *Staphylococcus aureus* (MSSA) was also evaluated. The antibacterial activity of this peptide has been previously reported against *Escherichia coli* and *Listeria monocytogenes* (Cole *et al.*, 2003). As this group of peptides share many characteristics, including size, structure and charge in common with defensins (Monelland Strand, 1994), which is one of the most studied AMPs (Brown and Hancock, 2006; Mookherjee and Hancock, 2007), we proposed that this peptide can potently kill *S. aureus*. In the current investigation, we examined the antibacterial activity of peptide against MRSA in different percentages of plasma isolated from healthy donors and its toxicity against human erythrocytes.

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MATERIALS AND METHODS

Reagents

Peptide derived from HIV-1 MN strain gp41was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, MD, USA (Catalog #6451). The peptide was dissolved in acidified water (0.01% acetic acid) at 1mg/mL for subsequent assays as described (Cole *et al.*, 2003). The sequence and other information of peptide used in this study are listed in table 1. HSA was purchased from CSL, Australia whereas plasma was isolated from blood samples taken from healthy donors.

Bacterial strains

The MSSA and MRSA strains were purchased from American Type Culture Collection (ATCC). ATCC 25923 (MSSA) and ATCC 43300 (MRSA) were maintained as glycerol stocks at -80°C and cultured on Mueller Hinton Agar (MHA) (105437, Merck, Germany). For antibacterial assays, Muller-Hinton Broth (MHB) (M391, Hi Media Laboratories, India) was used. For agar plating, Luria-Bertani (LB) agar (244520, BD, USA) was used.

Kinetics of bacterial killing

The bacterial killing rate was determined as previously described (Tencza *et al.*, 1999; Deslouches *et al.*, 2005). Bacterial suspensions were cultured in MHB to mid-log phase and washed three times by centrifugation (1,400xg for 10 minutes) and resuspension in an equal volume of PBS (P3813, Sigma-Aldrich, USA), pH7.4. The A_{600} of the suspension determined by µ Quant ELISA Reader (Bio-Tek Instruments, USA), was adjusted with PBS to 0.5×10^6 to 1×10^6 CFU/mL for assays. Various test media were used to compare the influence of complexity of media on antibacterial effect of peptide. The basal medium used was PBS and it was supplemented with 5% HSA, 10%, 50%, and 98% pooled plasma to evaluate the activity of peptide. The test strains were treated with 0 to 100µg/mL peptide and incubated at 37°C for various time points to determine the rate of bacterial killings. Aliquots of 10µL of the peptide-treated suspension were collected from 0 to 6 hours, serially diluted with PBS, plated on LB agar plate and incubated overnight. The colonies were enumerated the next day and the data were expressed as log CFU/mL plotted against time-exposure.

Erythrocyte lysis assay

Peptide was screened for hemolytic activity as previously described (Deslouches *et al.*, 2005; Myhrman *et al.*, 2013), by treating a 10% suspension of freshly isolated human red blood cells (RBCs) with various concentrations of peptide (0, 25, 50, 100 and 150µg/mL). Briefly, the samples were centrifuged to pellet intact cells, and the amount of hemoglobin in the supernatant was measured to determine the extent of RBC lysis. The peptide concentrations of 0 to 150µg/mL were tested and the reaction mixtures were incubated at 37°C for 6 hours with gentle shaking. The suspensions were centrifuged at 20, 000 xg for 4 minutes using Beckman Coulter Microfuge 22R, and 50µL of the supernatant was diluted in 450µL of distilled water. A standard curve of RBC lysis was plotted by treating various amounts of cells (0, 10, 20, 40, 60, 80 and 100% of the volume of RBC used in test samples) with deionized water to a final volume of 500µL that completely lysed the cells. The supernatant of all samples (200µL) were measured in 96-well micro plate (92096, TPP, Switzerland) at 570nm. The standard curve was used to calculate the percent lysis at each peptide concentration.

STATISTICAL ANALYSIS

All experiments were performed independently in duplicate on three separated occasions. All values are expressed as the mean ± standard error of mean (SEM). Statistical comparisons were performed using a Student's *t*-test by IBM SPSS Statistics Version 21 software. A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Antibacterial activity of peptide

To evaluate the antibacterial activity, various concentrations of peptide were incubated with adjusted amount of test bacteria. Variable test media were selected to examine the activity of peptides in the presence of physiologically relevant conditions. As previously reported (Tencza *et al.*, 1997; Tencza *et al.*, 1999; Cole *et al.*, 2003; Joanne *et al.*, 2009), AMPs derived from HIV-1 envelope demonstrated potent activities against a wide range of bacteria at relatively short time. Degradation of peptides occurred with in hours as a results of the defensive mechanism exerted by bacteria. The peptide rapidly lost its activity and overcome by bacterial growth, which masked the antimicrobial activity of the agent if it has not been assayed within an appropriate time period. A preliminary screening of peptide at various concentrations and various time points against test bacteria was performed in 10% plasma. The antibacterial activity of

Table 1: Details of HIV-1 MN strain gp-41 peptide used in this study.

Protein	Peptide[a]	Structural motif	Isoelectric point	Amino acid sequence
HIV-1 gp41	6383	Membrane-spanning domain	12.30	RIVFAVLSIVNRVRQ

[a]Peptide identification corresponds to reagent numbers in the NIH AIDS Research and Reference Reagent Program.

100µg/mL peptide was maximized at 6 hours (about 2-log reductions, $p<0.05$) (fig. 1C) in following hours, the activity was overcome by bacterial growth.

In time-course study, various concentrations of peptide were tested against MRSA. As depicted in fig. 1, the peptide was not active in PBS ($p>0.05$), but at 100µg/mL, significant antibacterial effects were clearly seen in 10% (2-log reductions, $p<0.05$), 50% (2-log reductions, $p<0.05$) and 98% (1-log reduction, $p<0.05$) plasma at 6-hour incubation. This suggests that the activity of peptide is facilitated by some components in plasma. In the absence of these components, the activity is inhibited by one of the defensive mechanisms of MRSA such as enzymatic protein degradation, causing the peptide to be inactive in PBS. To validate this result, we supplemented normal PBS with 5% of HAS, which accounts for 55% - 60% of total plasma protein. Interestingly, 3-log reductions ($p<0.05$) of MRSA growth are observed at peptide concentration of 100µg/mL after 3 hours of incubation (fig. 1B). This might be caused by the protective role of HSA from proteolytic degradation of peptide, thereby enhancing the bacterial killing activity.

As a comparison, we repeated the experiments against non-resistant *S. aureus* MSSA. Agreeable results are seen as shown in fig. 2. At 100µg/mL, about 2-log reductions ($p<0.05$) were observed both in PBS supplemented with 5% HSA and 10% plasma, with more prominent activity in former ones. Activity of peptide was not examined against MSSA in other test media as our main interest in this report is MRSA, which possesses a distinct property of antibiotic resistances. The consistent antibacterial effect seen in both MSSA and MRSA suggests that the mechanism of this peptide towards *S. aureus* does not correspond with the mechanism of antibiotic resistance.

Selective toxicity in human red blood cells

Selectivity, or bacterial killing at concentrations that is not harmful to normal eukaryotic cells, is highly desirable for an antibacterial agent. To determine the selectivity, peptide was evaluated for its toxicity to RBC relative to its antibacterial activity. The test concentration of peptides in hemolysis was within the range of those in bacterial killing assay, with identical condition of incubation (at 37°C for 6 hours). 10% RBC suspension was prepared by diluting human whole blood from healthy donor 10-fold with PBS. No hemolysis was observed in RBC treated with peptides up to 50µg/mL (fig. 3). Exceeding this concentration, dose-dependent hemolytic activity was seen. In RBC treated with 100µg/mL peptide at which 2-log reductions were shown against MRSA in 50% plasma, 13% of hemolysis ($p<0.05$) was seen compared to the untreated controls. About 28% of RBC lysis ($p<0.05$) took place in the RBC suspension treated with 150µg/mL peptide, higher level of hemolysis is anticipated in the increasing concentration of peptide.

DISCUSSION

AMPSs have been extensively investigated for their potentials as peptide antibiotics (Vaara, 2009). Currently, there are 15 different peptide-based therapeutic agents undergoing different clinical phases that are only limited to topical administration (Fjell *et al.,* 2012; Yount and Yeaman, 2012), more potent peptides must be identified for systemic uses as other conventional antibiotics. Interaction of cationic AMPs and anionic bacterial membrane is known as a critical step for microbicidal action. In addition to rapid activity and pronounced effect at low concentration, AMPs can target the bacterial cell membrane without specific receptors, thus diminishing the occurrence of resistance due to bacterial mutations (Huang *et al.*, 2010). Generally, cationic AMPs do not bind to mammalian cell membranes enriched in the neutral zwitterionic phospholipids (Kang *et al*., 2012) and this result in the highly selective activity of peptides.

In the past ten years, several AMPs that are efficacious against *S. aureus* have been reported (Vaara, 2009). For instance, Omignan, a tryptophan-rich AMP is highly active (MIC_{90} of 16µg/mL) against *S. aureus* (Sader *et al.*, 2004). $(RW)_3$-NH_2, a derivative of Omiganan is inhibitory against *S. aureus* at 8µg/mL (Liu *et.al*., 2007). Other active AMPs include PMX30063, LL-37 and so on (Choi *et al.,* 2009; Noore *et al.,* 2013). Despite many AMPs studies have used *S. aureus* as model, little is known about their efficacies against the drug-resistant strains such as MRSA. Here, we describe a peptide (#6383) originated from the viral membrane-spanning domain of HIV-1MN strain gp41, which demonstrated a potent antibacterial activity against MRSA. The peptide is cationic, having p*I* of12.30, nearly 50% hydrophobic, valine- and arginine-rich (RIVFAVLSIVNRVRQ), and small size with only15 amino acid sequences. The physiochemical properties of this peptide are similar to general AMPs with known antimicrobial activity, which make it an ideal candidate for therapeutic agent (Kang *et.al*., 2012). The antibacterial effect of this peptide against *E. coli* and *L. monocytogenes* at low concentrations (2.73 and 3.10µg/mL respectively) in the presence of high salt concentration (100mM NaCI) has been previously reported (Cole *et al.,* 2003), and it is active at 100µg/mL against MRSA in this report. The active concentration against MRSA is 50 folds higher than that used against *E. coli* and *L. monocytogenes*. This is more likely due to the decreased susceptibility of bacteria towards the peptide in the presence of physiological conditions, but not the antibiotic resistance since we have shown that the active doses against MRSA and MSSA are similar. Taken together, this peptide shows the broad-spectrum microbicidal action against both gram-positive and gram-negative bacteria.

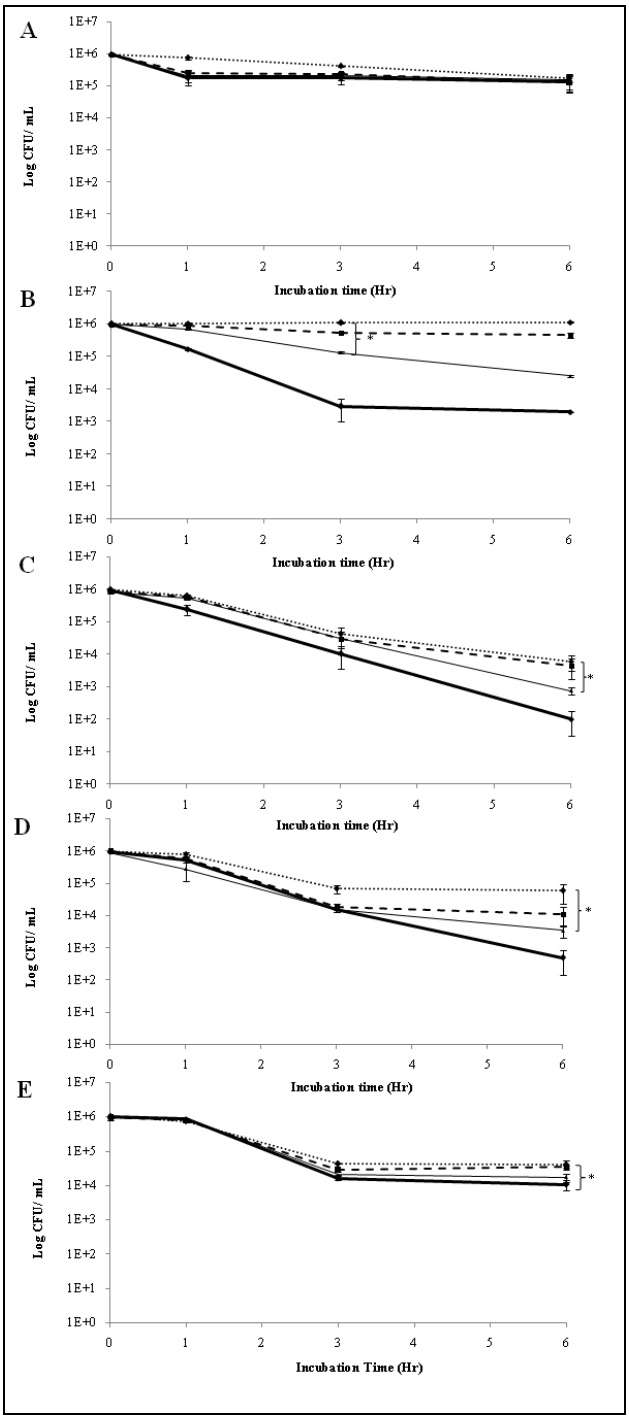

Fig. 1: Kinetics of bacterial killing of MRSA in various media. MRSA was treated with 0 to 100µg/mL of peptides in PBS (A), PBS supplemented with 5% HSA (B), 10% plasma (C), 50% plasma (D), and 98% plasma (E). The results showed that the peptide was inactive in PBS and 98% plasma, but activities were shown in other media. Data plotted are representative average values of three independent experiments. 0µg/mL; 25µg/mL; 50µg/mL; 100µg/mL; *p<0.05.

Many AMPs have shown reduced activity when tested in physiological conditions (Tencza *et al*., 1997; Zasloff, 2002). In this report, the peptide demonstrates its potent activity against MRSA in diluted plasma (up to 50%), at concentration 100µg/mL. This suggests the high resistance of peptide towards proteolysis or degradation in plasma. In the present study, serum albumin was also proven to enhance the antimicrobial action of peptide. Albeit exact mechanism is not known, we believed that a complex protein interaction has taken place, and the net release of the protein resulted in the enhanced microbicidal activity. On top of the antimicrobial activity, we attempted to evaluate the selective toxicity of peptide. At 100µg/mL, only mild hemolytic activity (~13%) was seen.

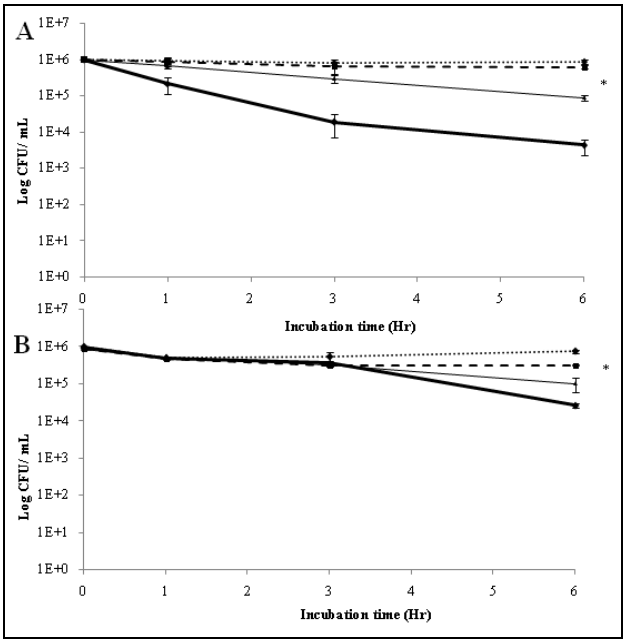

Fig. 2: Kinetics of bacterial killing of MSSA in various media. MSSA was treated with 0 to 100µg/mL of peptides in PBS supplemented with 5% HSA (A), and 10% plasma (B). Similar to MRSA, peptide was active against MSSA in test media above. Data plotted are representative average values of three independent experiments. 0µg/mL; 25µg/mL; 50µg/mL; 100µg/mL; *p<0.05.

Notably, the native peptide described here has not been subjected to any engineering work. Based on the current understanding of peptide engineering, this peptide can be potentially developed into peptide antibiotics (Vaara, 2009). For instance, minimizing the size of peptide (length of the amino acid sequence) and systematically substituting each residue with other amino acids for structure-activity relationship studies (Fjell *et al.*, 2012) can be considered. These will not only result in reduced proteolysis in physiological fluids that enhances microbicidal action, but minimizing hemolysis and toxicity to other eukaryotic cells. Although sequence

engineering and other peptide modification works are common to maximize the desired activity of peptides, several crucial considerations must be taken during the process. Following the peptide modifications, immune-modulation and immunogenicity may be potentially triggered after the administration into a biological system (Mader and Hoskin, 2006; Yeung *et al.*, 2011). Despite being uncommon, bacterial resistance has been reported in response to certain antimicrobial peptides (Dawson and Liu, 2008).

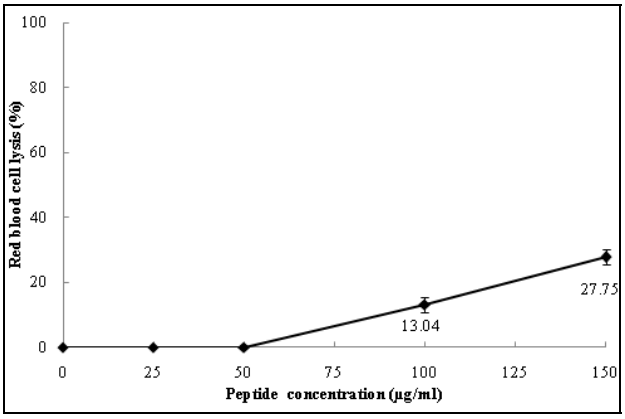

Fig. 3: RBC lysis by peptide in the absence of bacteria. RBCs were incubated for 6 hours in PBS containing various concentrations (0, 25, 50, 100 and 150μg/mL) of peptides. RBC lysis was measured compared with standard curve (RBCs in deionized water) as described in Materials and Methods. Data plotted are the average of three independent experiments of RBC lysis at each peptide concentration. *. $p<0.05$.

CONCLUSIONS

We have demonstrated that the HIV-1 envelope glycoprotein-derived antibacterial peptide was active against MRSA at concentration 100μg/mL. This glycopeptide was able to partially overcome the challenges of physiological conditions in 50% plasma within 6 hours meanwhile showing only mild hemolytic activity. Overall, this peptide serves as a good template to be developed into a therapeutic antimicrobial agent. Further investigations must be carried out to reveal the mechanism involved. The cytotoxicity caused by the interactions of peptide with cell membrane must be examined in depth to evaluate the safety profile of peptide.

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