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Research Article

Study the activity of conjugated antimicrobial peptide WW-185 against clinically important bacteria

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Abstract

Multidrug-resistance bacteria are a serious problem for health specialists and all the people in the world. The main reasons for this problem are the misuse of antibiotics and the limited number of antibiotics as compared to the different human diseases. Important antibiotic-resistant bacteria include Methicillin-resistant Staphylococcus aureus (MRSA) and Extended-Spectrum β-Lactamases *E. coli* (ESBL *E. coli*), These two types of bacteria can cause life-threatening diseases and poses a big problem in choosing suitable antibiotics for infections caused by them.

Antimicrobial peptides (AMPs) are considered promising antimicrobial agents that meet the required criteria for novel antimicrobial drugs. This study aims to design novel and safe AMP to be used as antimicrobial agents. In this study, an unique modified AMPs called WW-158 was designed to have a hydrophilic and hydrophobic balance using arginine to represent the cationic part and tryptophan to show the hydrophobic part. It showed good activity against MRSA with a MIC value of 35 μM. These effective concentrations were associated with negligible toxicity toward human red blood cells. Furthermore, our results showed that most of the combined groups of peptides with eight conventional antibiotics displayed synergistic modes of action or additive effects.

Keywords

antimicrobial peptides, hemolytic activity, MRSA, antibiotics resistance and peptides

Introduction

The growing threat of antibiotic resistance has become a significant concern worldwide. The Interagency Task Force on Antimicrobial Resistance released an action plan in 2001 to combat the issue in the United States, which contained 84 action elements, 13 of which were designated as "top priorities" and fell into 4 overarching activity areas: surveillance, prevention and control, research, and product development (Ahn et al. 2017). However, the plan was never fully funded, and no additional measures have been proposed by the US government to stimulate research and development (R&D) of new diagnostics, vaccines, or (most critically) antibiotics. Pharmaceutical companies have been abandoning the development of anti-infectives, leading to a decline in the research and development of new antibiotics (Sanchez and Gustot 2019). Insufficient federal funding and surveillance are some of the causes of the growing threat of antibiotic resistance. The rate at which microbial adaptation to antimicrobials occurs is amenable to moderation, but the depth of microbial history and their adaptability suggest that resistance to future antimicrobial strategies is likely (Matzov et al. 2017). The current AMR crisis will not be resolved by a single new

product or therapy. Moderating the use of both current and forthcoming antimicrobials can extend the useful lives of existing and prospective therapies. Multidrug-resistant microbes are considered a substantial threat to US public health and national security by several organizations, including the National Academy of Science's Institute of Medicine, the federal Interagency Task Force on Antimicrobial Resistance, and the Infectious Diseases Society of America. Alternative therapies need to be explored, and the future focus of medical therapeutics and research is to look beyond antibiotics. The development of immune therapeutics and immune prophylactics has tremendous potential to reduce the overall burden of infection and infection-related deaths, and it should be a major focus of both government and industrial R&D. New antibiotics and immunological strategies complement one another, and both are needed to address the growing threat of antibiotic resistance. Identifying potential solutions is crucial in addressing this problem, and the drivers and levers of antimicrobial resistance need to be analyzed to understand the causes of this threat (Chokshi et al. 2019).

Antimicrobial peptides (AMPs) are natural antibiotics that are obtained from various living organisms such as plants, frogs, insects, fungi, bacteria, and other organisms. The increasing bacterial drug resistance has made AMPs a critical solution to combat bacterial infection (Kang et al. 2014) . AMPs are promising compounds that can fight microbial infections and contamination. They have potent antimicrobial activity and unique antimicrobial mechanisms, which give them advantages over traditional antibiotics. Additionally, they have broad-spectrum activity against a variety of microorganisms, including bacteria, fungi, and viruses (Mookherjee et al. 2020; Mylonakis et al. 2016) . This makes them a good alternative to current antimicrobials in fighting against drug-resistant bacterial infections. Furthermore, AMPs offer a potential alternative to traditional antibiotics, which is important due to the urgent need to obtain new antimicrobials as a result of the emergence of drug-resistant bacteria (Rajchakit and Sarojini 2017). Researchers have conducted clinical investigations on AMPs for drug-resistant bacterial infections and are integrating new technologies into their development. Synthetic antimicrobial peptides (SAMPs) are considered new weapons to fight infections caused by multidrug – resistant pathogens . Synthetic peptides are a promising new molecules to safeguard human and animal health. In this study, we designed anovel conjugated antimicrobial peptide called WW-158 and studied its effect against clinically important bacteria alone and in combination with traditional antibiotics (Ahmed et al. 2019).

Materials and method

Designing of AMPs

In silico designing of a series of novel, improved, and conjugated antimicrobial peptide of tripeptides was performed taking into account the balance between the hydrophobic and charged moieties

Peptide analysis

The synthesized peptide were analyzed using RP-HPLC for purification using an acetonitrile / H_2O gradient. The identification of the synthesized peptides was confirmed by ESI-MS.

Minimum Inhibitory Concentrations (MICs), and Minimum Bactericidal Concentrations (MBCs) determination of the peptide

Using sterile 96-well polypropylene microtiter plates, the micro broth dilution method outlined by the Clinical and Laboratory Standards Institute (CLSI) guidelines was adopted to determine the MIC and MBC of the peptide (Sahl et al. 2005). In brief, the MHB was used as the growth medium for organisms after removing it from the stock media of frozen glycerol. Bacterial cells were grown overnight in Muller Hinton Broth and diluted to 10^6 CFU/ml in the same medium before use. For peptide, different dilutions with concentrations in the range of 0.5–100 μM as final concentrations were prepared. Then, in 96-well microtiter plates, 50 μl of each peptide concentration and 50 μl of diluted bacterial suspension were added into each well. Each plate included six replicates of each peptide concentration divided into six wells. The plate was incubated for 18 h at 37 °C. After that, the growth of bacteria was determined by measuring OD at λ = 570 nm by an ELISA plate reader, MIC was determined accordingly (as the lowest concentration of antimicrobial drugs which is needed to inhibit the growth of the bacteria). Each plate included a positive control column (50 μl of bacterial suspension plus 50 μl MHB without any antimicrobial agents) and a negative control column (100 μl of MHB in each well) to ensure the activity of bacteria and the sterility of MHB respectively and repeated three times.

MBC was determined by taking 10 μL was taken from clear negative wells, and turbid positive control wells and they were streaked on sterile labeled nutrient media agar and incubated for 24 hours at 37 °C. The lowest concentration that led to having < 0.1% viable cells (killing 99.9%) was referred to as the MBC value (Fjell et al. 2012).

MIC and MBC determination of antibiotics alone

MICs and MBCs determined against stander bacterial strains of *S. aureus* and *E. coli* and resistance ESBL *E. coli* and MRSA via preparing different concentrations of each antibiotic (the concentration range was from 0.25 to 250 μM). Every antibiotic solution was prepared by dissolving it in water then diluted in the sterile broth (Lau et al. 2015).

MIC determination of peptides-antibiotics combinations

According to the broth microdilution checkerboard technique (Sueke et al. 2010), MICs of peptide-antibiotics combinations against stander bacterial strains of *S. aureus* and *E. coli* and resistance ESBL *E. coli* and MRSA were tested and determined as described. However, in this assay, each microtiter well contained a mixture of one peptide and one antibiotic in different concentrations. 25 µl of each peptide concentration and 25 µl of each antibiotic concentration (from 0.25 to 200 µM) were added to six wells of a sterile flat–bottomed 96 well-plate that contained 50 µl of the diluted bacterial suspension. MICs determination made in triplicate (Mishra et al. 2017).

Determination of synergism using fractional inhibitory concentration

The fractional inhibitory concentration (FIC) is the summation of the inhibitory concentration values of each component resulted in the antimicrobial combination divided by the inhibitory concentration alone (Meletiadis et al. 2010).

The FIC indices were interpreted as follows:

≤ 0.5: synergistic activity, 0.5–1: additive activity, 1–4 indifferent, >4: antagonistic. Interpretation and assessment of the FIC index and antimicrobial activity of peptides-antibiotics combinations were conducted according to the broth microdilution checkerboard technique (Brochado et al. 2018).

Erythrocyte hemolytic assay

Determination of the ability of the designed peptide to cause hemolysis to human erythrocyte, hemolytic assays was performed. Two ml of human blood was placed into a 50-ml centrifuge tube, centrifuged at 3000× g for 5 min. The supernatant was discarded and the cell pellet was suspended in 48 ml of PBS and centrifuged at 3000× g for 5 min; this step was repeated three times. Finally, the cell pellet was re-suspended in a sterile tube containing 50 ml PBS to reach a final concentration of 4% RBC and PBS containing different concentrations of the peptides. Then 1 ml of each concentration was added to 1 ml of erythrocyte suspension.

Controls were prepared by the addition of 5 µl of Triton X-100 to 1 ml of RBC suspension (positive control). The blank (negative control) was prepared by adding 1 ml of RBC suspension with PBS. The suspension was incubated for 60 min at 37 °C. Tubes were gently vortexed and 1 ml of each sample was aspirated and placed into sterilized Eppendorf tubes and then centrifuged for 5 min at 3000× g. From each supernatant 100 µl were placed into the wells of a 96-well plate. Absorbance was measured at λ= 570 µM with the aid of an Absorbance Microplate Reader The percentage of hemolysis was calculated according to the following equation (Oddo and Hansen 2017):

$$\% \text{ Hemolysis} = \frac{(A - AO)}{(AX - AO)} \times 100$$

Where A: is OD 450 with the peptide solution,
A0: is OD 450 of the blank.
And A_X: is OD 450 of control (0.1% Triton X-100).

Results

Peptide design and synthesis

A novel improved tri- AMP contins two subunits of tryptophan and one ornithine amino acid. Ornithine was used to give the charge for the peptide; it has the advantage of being an unnatural amino acid and a non-coded amino acid, thus its stability against proteases is excellent. Tryptophan was integrated as hydrophobic moieties because of its membrane interface interaction. it exhibits a strong preference when compared to the other hydrophobic amino acids. our designed peptide was conjugated to Para-hydroxycinnamic acid (PHCA) which was used to increase the hydrophobic properties of the peptide. Also, PHCA has antimicrobial activity on its own. So it is expected to increase the activity of the peptide. The structure of the peptide is shown in Fig. 1.

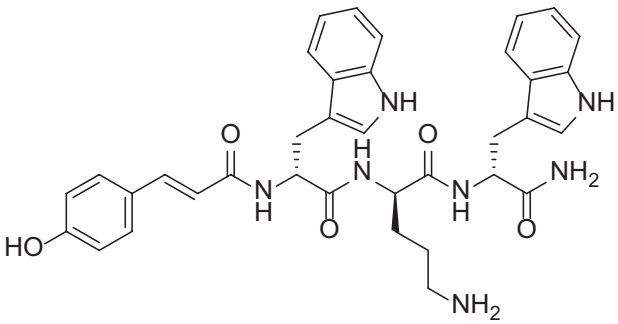

Figure 1. Structure of WW-158 peptide.

Bacterial susceptibility assay of the peptide

The peptide was tested against different strains of Gram-positive and Gram-negative bacteria, including resistant strains. The bacteria used were standard strains of *S. aureus* (ATCC 29215), MRSA (ATCC BAA-41) and standard strains of *E. coli* (ATCC 25922); in addition to ESBL – *E. coli*. To determine the MIC and MBC.

As shown in (Table 1) WW-158 displayed potent activity against the tested Gram-positive bacterial strains with a minimum inhibitory concentration (MIC) of 25 µM against the standard strain S. aureus and MIC of 35 µM against MRSA (ATCC BAA-41).As for the Gram-negative bacteria,

Table 1. MIC and MBC values of WW-158 against four bacterial strains.

Gram-positive strains	ATCC	MIC value (μM)	MBC value (μM)
S. aureus	6538	25	25
MRSA	BAA-41	35	35
Gram-negative strains	**ATCC**	**MIC value (μM)**	**MBC value (μM)**
E. coli	8739	30	30
ESBL *E. coli*	BAA-3054	50	50

the MIC was 30 μM against the standard strain of *E. coli* and 50 μM against ESBL *E. coli*. The MBC values were the same as the MIC values for the four bacterial types.

Hemolytic assay of the peptide

WW-158 was subjected to the hemolytic assay. the result is summarized in (Table 2).

Table 2. The hemolytic activity of the peptide against human erythrocytes after 60 minutes incubation.

Concentration (μM)	Hemolysis %
5	1
10	1
20	1
40	2
60	2
80	2
100	2

Determination of MIC, FIC Index of the Peptide and Antibiotics Combinations

According to the checkerboard dilution method, WW-158 was combined with the eight conventional antibiotics to determine the outcomes of these combinations on antibacterial potency. The shift in antibacterial potency is reported by calculating the MIC values of the ultrashort peptides and the antibiotic combinations against standard types of bacterial strains of Gram-positive and Gram-negative bacteria. Also, it is out righted by calculating the FIC indices, so these combinations can be classified as synergistic (FIC ≤ 0.5), additive (FIC 0.5< FIC≤ 1), indifferent (1<FIC≤4), or antagonist (FIC>4).

The only combination that showed a synergistic effect against Gram-positive strains was that with vancomycin against *S. aureus* and MRSA. with FIC values of 0.21 and 0.25 respectively, On the other hand, combinations of WW-158 with rifampicin showed a synergistic effect with a FIC index less than 0.5 against the control strain *E. coli*, while no combination showed synergistic effect against the resistance gram-negative strain ESBL *E. coli* isolated strain (BAA-3054). The results are summarized in Table 3.

Discussion

In the present study, novel antimicrobial peptide (WW-158) was designed based on rational design. The mode of antimicrobial activity of AMPs was proposed to occur as a result of targeting the bacterial cell membranes. The proposed mechanism of action for AMPs indicates that peptide entry and membrane targeting are facilitated by the hydrophilic positively charged moieties of AMPs, which are driven electrostatically towards bacterial negatively charged surfaces, leading to pore formation and later cell membrane lysis and death. Previous work on AMPs has shown that only those peptides that carried a cationic net charge (≥ +3) managed to possess antibacterial activities and inflict damage on bacterial membranes ; thus, our strategy relied on designing novel peptides displaying a net cationic charge around +3 (Porto et al. 2018). While balancing other physicochemical parameters within the primary sequence, such as hydrophobicity, the design hypothesis is in complete alignment with the proposed mechanism of action of AMPs. Our in-house designed peptide will be adequately able to create sufficient electrostatic attraction with the negative head groups of phospholipids in the bacterial cell membrane and consequently cause membrane perforation and cell death (Xia et al. 2018). The cationic charge of AMPs designed in our study is mainly attributed to the presence of arginine amino acids within the primary structure. Since the side chain of the amino acids interacts through the formation of hydrogen bonds and also through electrostatic interaction with the negatively charged surface of bacteria (Rodriguez et al. 2016). Our results are consistent with previous studies , which demonstrated that the membrane disruption is cationic residue-specific and hence the positive charges must be dependent on arginine and not lysine or ornithine, especially when the peptide is less than three residues (Torres et al. 2019). This phenomenon explains the role of arginine's guanidine moiety , which is responsible for increasing the binding of the cationic moiety in AMPs to the membrane surface via forming a complex with the phosphate groups belonging to the membrane phospholipid bilayer (Yadav et al. 2018). The hydrophobicity of AMPs has been well shown to play a major role in defining AMPs' activity. The hydrophobic moieties of the active peptides in our study are represented mainly by tryptophan. Tryptophan, when compared to other hydrophobic amino acids, plays a significant role in enhancing peptide-membrane interaction in comparison with other amino acids (Porto et al. 2018). It has been reported that a high propensity for membrane insertion happens as a consequence of the specific affinity between the indole group of tryptophan and the carbonyl moieties of phospholipids and hence membrane disruption (Cole and Keenan 1987). WW-158 displayed antimicrobial activity against Gram-positive bacteria only. This difference in the activity of WW-158 between the Gram-positive and Gram-negative can be explained due to the structural differences in their cell wall composition. Gram-negative bacteria contain a thin peptidoglycan layer surrounded by an outer membrane composed of a lipopolysaccharide layer while Gram-positive bacteria possess a thick peptidoglycan lipid but lack an outer layer of lipopolysaccha-

Table 3. Minimal inhibitory concentrations (MIC) and the FIC index of combinations of WW-158 & the antibiotics against all the tested bacterial strains.

MIC in combination with bacterial strains						FIC* Index
MIC in combination and MIC alone						
Bacterial strains	Antibiotics	Antibiotic MIC before combination	Antibiotic MIC after combination	WW-158 MIC before combination	WW-158 MIC after combination	
***S. aureus* (ATCC 6538)**	**Levofloxacin**	.5	.25	25	10	.9
	Chloramphenicol	30	15	25	15	1.1
	Rifampicin	.025	.0125	25	10	0.9
	Amoxicillin	5	.5	25	15	0.7
	Clarithromycin	1.5	.5	25	20	1.13
	Doxycycline	10	2	25	8	0.52
	Vancomycin	.5	0.00125	25	5	0.21
	Cefixime	4	2	25	15	1.1
MRSA (*ATCC* BAA-41)	**Levofloxacin**	10	6	35	20	1.2
	Chloramphenicol	40	15	35	15	0.8
	Rifampicin	.005	.0025	35	20	1.1
	Amoxicillin	40	40	35	30	1.9
	Clarithromycin	100	100	35	25	1.7
	Doxycycline	20	10	35	15	0.9
	Vancomycin	2	.05	35	8	0.25
	Cefixime	30	15	35	20	1.1
***E. coli* (ATCC 8739)**	**Levofloxacin**	2	.0025	100	60	0.6
	Chloramphenicol	100	20	100	80	1
	Rifampicin	15	1	100	40	0.47
	Amoxicillin	25	10	100	80	1.2
	Clarithromycin	150	100	100	60	1.3
	Doxycycline	15	2	100	40	0.53
	Vancomycin	150	60	100	50	0.9
	Cefixime	6	1	100	40	0.57
ESBL *E. coli* (BAA-3054)	**Levofloxacin**	12	6	100	60	1.1
	Chloramphenicol	200	40	100	80	1
	Rifampicin	50	15	100	40	.7
	Amoxicillin	250	150	100	80	1.4
	Clarithromycin	200	100	100	40	0.9
	Doxycycline	25	6	100	60	0.84
	Vancomycin	200	175	100	50	1.4
	Cefixime	80	40	100	60	1.1

*the synergistic FIC values are highlighted

rides. Therefore, the electrostatic interaction between the cationic residues of the WW-158 and the negative charges of the bacterial cell membrane, the initial step required for antibacterial activity, decreases significantly in Gram-negative bacteria due to the existence of the outer lipopolysaccharide layer (Pasupuleti et al. 2012). Evaluating the hemolytic toxicity of our active peptide towards human red blood cells revealed negligible hemolytic activity. Increasing the cationic charge to around +3 contributed to minimizing the hemolytic toxicity towards red blood cells. Accordingly, mass charge plays a major role in creating a sufficient electrostatic attraction and hence targeting the negative head groups of the bacterial cell membrane with very negligible toxic effects toward human erythrocytes (Floch et al. 2000).

MIC values of eight conventional antibiotics (levofloxacin, chloramphenicol, rifampicin, amoxicillin, clarithromycin, vancomycin, cefixime, and doxycycline) against various strains of Gram-positive (*S. aureus* (ATCC **6538**) MRSA (ATCC BAA-41)) and Gram-negative (*E. coli* (ATCC **8739**) and ESBL *E. coli* isolated strain (BAA-3054)) demonstrated that rifampicin is the most potent antibiotic against Gram-positive bacteria namely, *S. aureus* (ATCC: **6538** and BAA-41) with MICs of 0.025 and 0.005 μM, respectively. Regarding the Gram-negative bacterial strains *E. coli* (ATCC: **8739** and BAA-3054), levofloxacin exhibited the highest antimicrobial activity with a MIC value equal to 2 and 12 μM. The combination between WW-158 and rifampicin shows a synergistic effect. The mechanism of the synergistic effects of the peptide- rifampicin combinations is unclear yet, but one hypothesis for the synergistic effect proposes that the destruction and pore formation effects of AMPs in the bacterial membranes enhance the intracellular entry of antibiotics, which allows them to reach their targets and accomplish their function rapidly (Spänig and Heider 2019). Vancomycin does not work intracellularly but functions by inhibiting cell wall synthesis by binding to the D-Ala-D-Ala terminal of the growing peptide chain during cell wall synthesis, resulting in inhibition of the trans peptidase, which prevents further elongation and cross-linking of the peptidoglycan matrix (Ruzin et al. 2004). It displayed a synergistic effect

against Gram-positive bacteria. This might be explained based on vancomycin's function against the cell wall thus facilitating the ease of entry of the peptides to their target sites in the cell membrane, which finally causes rapid cell lysis and decreases the effective concentrations needed to inhibit bacterial growth displayed by peptides and vancomycin (Deslouches and Di 2017).

Conclusion

In conclusion, we report the design and antibacterial properties of a novel conjugated ultrashort antimicrobial peptide with potent activity against clinically resistant strains of Gram-positive and Gram-negative bacteria with negligible hemolytic activity. When used in combination with traditional antibiotics, the peptide showed multiple synergistic effects and may prove to be an important candidate for further development of antibacterial drugs.

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